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New soy protein ingredients production and characterization

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New soy protein ingredients production and characterization

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ABSTRACT

This body of research focuses on three major areas related to soy protein ingredients. The first area is the use of genetically modified high-sucrose/low-stachyose soybeans (HS/LS) in a new simplified procedure to prepare soy protein concentrate; secondly, fractionating soy protein into ingredients enriched in either glycinin or β-conglycinin; and lastly, processing effects on soy protein isolate functionality. It was hypothesized that the physicochemical properties of soy proteins affect compositions, solubilities, and recoveries of soy protein fractions, soy protein isolate, and soy protein concentrate, and these changes in protein profile and structure affect functionalities of these ingredients.

Soy protein fractionation was significantly improved by increasing protein yields and by reducing processing costs. In the three-step or Wu fractionation procedure, significant advances were made by identifying the optimum SO_2 concentration to be 5 mM, the optimum NaCl concentration to be 250 mM, and the optimum dilution factor to be 1-fold. Furthermore, this procedure was modified by using mM amounts of $CaCl_2$ at pH 6.4, eliminating salting-in and salting-out steps, and improving both yield and purity of the β-conglycinin-rich fraction.

A new two-step fractionation procedure was developed based on the differential calcium reactivity of glycinin and β-conglycinin by using mM amounts of sulfite and calcium ions. The use of 5 mM SO_2 in combination with 5 mM $CaCl_2$ in the two-step fractionation procedure yielded the improved purities in the glycinin-rich (85.2%) and β-conglycinin-rich (80.9%) fractions. This procedure yielded fractions with improved solids, protein, and isoflavone yields, and similar purities to the three-step fractionation procedure. In addition, the ingredients produced by this method had unique and improved functional properties. Phytic acid was proposed as playing an important role in fractionating soybean storage proteins because of its ability to complex with calcium ions and soy protein.

HS/LS soybeans were used to produce a new soy protein concentrate that was low in fiber, high in isoflavones and soluble sugars, and had unique functional properties, which were, in most cases, similar to or better than those found in traditional soy protein isolates. HS/LS soybeans were identified as good starting material for fractionating glycinin and β-

conglycinin because they solubilized proportionally more β-conglycinin in solution when compared to regular soybeans. In the Wu fractionation procedure, HS/LS soybeans yielded high amounts of the individual storage proteins with 100% electrophoretical purity.

The functionality of soy protein isolate was affected by extraction temperatures (25, 40, 60, and 80°C) and other processing variables and method of preservation. Spray-dried soy protein isolate (SPI) were more soluble, hydrophobic, and formed more stable emulsions than did freeze-dried SPI. The drying method, however, did not affect denaturation enthalpy of SPI.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Although soybeans produce the highest protein yield per unit area of land and have the highest protein content of all seed crops (Kitamura 1993), only a small portion of the annual soybean production is used for food consumption. About 3% of the protein available from soybeans was used for food in 1992 (Johnson and others 1992). This amount has increased over the last few years to 4.8% in 2002 (Golbitz 2003). There is also a strong incentive for using low-cost vegetable sources of protein in the world economy (Endres 2001). World soybean production has grown 400% in 30 years. Consumer acceptance of soybean protein products has been growing and the perception of soy as a healthy food is strong (Golbitz 2003).

There are, however, some restraints associated with soyfoods and soy protein consumption. Soybeans have antinutritional factors such as protease inhibitors, phytic acid, and flatus-producing oligosaccharides. Many soyfoods have low consumer acceptance due to undesirable flavors. In most food systems, proteins from animal origin are used because of their better functional properties and the use of soy proteins is also limited due to lack of desirable functional properties.

Soy proteins being an inexpensive source of high quality protein, are increasingly associated with health benefits. Recently, the Food and Drug Administration (FDA) approved in October 1999 a health claim for soy protein that can be used on labels of soy-based foods to promote their heart-healthy benefits. Food manufacturers are permitted to place a health claim (healthy heart logo) on the package labels of food products containing more than 6.25g of soy protein per serving (Henkel 2000). These properties are driving new developments in soy protein research. The ultimate goal of many research efforts is to develop soy protein foods and ingredients with proven health benefits and to overcome all the inherent restraints associated with them. The long-term goal of this body of work is to contribute to the knowledge base that will allow the food industry to reduce or eliminate some of the restraints associated to soy protein consumption to further advance soy protein

consumption. I will address new genetic traits to produce unique soy protein ingredients; process improvements and developments to produce ingredients of individual soy proteins; functional and compositional characteristics of these ingredients; and the effects of some processing variables on soy protein functionality.

The two main storage proteins in soybeans are glycinin and β-conglycinin. Considerable efforts have been made to fractionate these two proteins into relatively pure fractions in order to study their inherent properties and evaluate their potential food and industrial applications. Earlier fractionation studies have focused on isolating high purity fractions of the individual storage proteins to study their physicochemical characteristics and structure-function relationships. Recent research, however, has been focused on producing higher amounts of these individual fractions in order to study their potential health benefits thorough feeding and clinical studies. In addition, these fractions should have unique functional properties that are important to their applications in the food industry.

The central hypothesis for this body of work is that the physicochemical properties of soy proteins affect compositions, solubilities and recoveries of soy protein fractions, soy protein isolate and soy protein concentrate, and these changes in protein profile and structure affect functionalities of these ingredients. The specific research hypotheses addressed in the nine papers are: Paper 1 - The concentration of reducing agent during soy protein fractionation affects protein partitioning and alters protein functionality; Paper 2 - The concentrations of NaCl during the salting-in and salting-out steps affect soy protein fractionation and subunit distribution due to differences in behavior of the individual storage proteins, glycinin and β-conglycinin; Paper 3 – Differences in calcium sensitivity of glycinin and β-conglycinin in the presence of a reducing agent can be used to more effectively fractionate soy proteins; Paper 4 - Physicochemical and structural changes in the soy proteins fractionated with calcium in the presence of a reducing agent improves isoflavone recovery and functional properties of the fractions produced; Paper 5 - The use of high-sucrose/low-stachyose soybeans allows high yields of a new soy protein concentrate that is low in fiber and high in soluble sugars and isoflavones; Paper 6 - Intrinsic (chemical composition) and extrinsic (preparation variables) factors of the new soy protein concentrate prepared from high-sucrose/low-stachyose soybeans affect its functionality; Paper 7 - Differences in

fractionation behavior of high-sucrose/low-stachyose soybeans compared to normal soybeans affect functional properties of the glycinin-rich and β-conglycinin-rich fractions that are recovered; Paper 8 - Protein-phytate interaction and phytate content and partitioning are affected by the starting material and processing variables that account for differences in fractionating protein from high-sucrose/low-stachyose soybeans compared to regular soybeans; and Paper 9 - Extraction temperature and preservation method affect the functional properties of soy protein isolate by introducing physicochemical changes to the protein structure.

Dissertation Organization

This dissertation consists of a literature review, in the format of the *Journal of Food Science*, and nine papers, each paper presented as a chapter. The first paper, "Effects of Reducing Agent Concentration on Soy Protein Fractionation and Functionality," will be submitted to the *Journal of Food Science*. The second paper, "Effects of NaCl Concentration on Salting-in and Dilution during Salting-out on Soy Protein Fractionation," will be submitted to the *Journal of Food Science*. The third paper, "Fractionating Soybean Storage Proteins Using Calcium and $NaHSO_3$," will be submitted to the *Journal of Food Science*. The fourth paper, "Characterizing Fractionated Soy Protein Produced by a Simplified Procedure," will be submitted to the *Journal of the American Oil Chemists' Society*. The fifth paper, "Compositions of Soy Protein Ingredients Prepared from High-Sucrose/Low-Stachyose Soybeans," will be submitted to the *Journal of the American Oil Chemists' Society*. The sixth paper, "Functional Properties of Soy Protein Ingredients Prepared from High-Sucrose/Low-Stachyose Soybeans," will be submitted to the *Journal of the American Oil Chemists' Society*. The seventh paper, "Fractionation of Glycinin and β-conglycinin from High-Sucrose/Low-Stachyose Soybeans," will be submitted to the *Journal of Agricultural and Food Chemistry*. The eighth paper, "Fate of Phytic Acid during Preparation of Soy Protein Ingredients," will be submitted to the *Journal of Agricultural and Food Chemistry*. The ninth paper, "Effects of Extraction Temperature and Preservation Method on Functionality of Soy Protein Isolates," will be submitted to the *Journal of Food Science*.

A general conclusion section follows the ninth paper. Appendix A documents linear regression equations and plots to predict the effects of NaCl concentration on protein yields and purities during soy storage protein fractionation presented in Chapter 3. Appendix B documents correlation coefficients of compositional and functional properties of soy protein ingredients described in chapters 2, 5, 6, 7, 8, 9, and 10.

Literature Review

Soybeans

Soybeans (*Glycine max* [L.] Merr.) is a native crop of eastern Asia where they have served as an important part of the diet for centuries. Soybean production has expanded over the last 40 years from a regional minor crop to a major world commodity. Currently, global production is estimated at 150 million metric tons with the major producing counties being the United States, Brazil, China, and Argentina (Soya & Oil Bluebook 1999). This rapid growth can be attributed to five main reasons.

Soybeans have excellent agronomic properties. Soybeans have good adaptability to wide ranges of soils and climates. Soybean are a legume and have the ability to fix nitrogen, which makes them a good rotational crop for use with high nitrogen-consuming crops, such as corn and rice.

Soybeans have a unique chemical composition. They contain about 40% protein, 20% oil, and 35% carbohydrates on a dry matter basis (Perkins 1995), ranging from 13.9 to 23.2% in oil content and from 32.4 to 50.2% in protein content (Vaidehi and Kadam 1989). Soybeans rank first in terms of protein content among all food crops, and second with respect to oil content among all the food legumes (Liu 1999a).

Soybeans have good nutritional value. Soy oil contains high proportions of unsaturated fatty acids, and soy protein contains all essential amino acids in amounts that closely match those required by humans and animals. Based on the protein digestibility corrected amino acid score (PDCAAS), purified soy protein has a score very close to 1, which is the highest score possible, and similar to animal sourced proteins such as egg white and casein (FAO/WHO 1991).

Soybeans have functional health benefits. During recent years, there has been a great deal of interest and research carried out on the roles of soybeans and soyfoods in preventing and treating chronic diseases. The main focus of this research has been on isoflavones present in soy protein products and their interaction with soy proteins to prevent chronic diseases, such as cancer, blood cholesterol and lipid lowering effects, and inhibition of bone resorption (Messina 1999).

Soybeans have versatile end uses. They have been used as human foods, animal feed, and industrial materials. Traditional soy foods consumed mainly in Asia include soymilk, tofu, soy nuts, soy sauce, miso, natto, tempeh, among others. In western countries, on the other hand, soybeans are mainly crushed into oil and meal. Properly processed soybean oil can be used as major ingredient for making almost every commercial edible oil and fat product (Perkins 1995). Although soybean oil is almost used entirely for human consumption, soy meal is utilized primarily as animal feed. Only a small portion is destined for producing soy protein products (Johnson and others 1992), which are used as functional food ingredients. These products include soy flour and flakes, protein concentrates, protein isolates, and texturized soy proteins. These ingredients are widely used in a great range of food systems, such as bakery, dairy, meat, breakfast cereals, infant formulas, and dairy and meat analogs (Lusas and Rhee 1995).

Composition of soybeans

Lipids

Soybean lipids are deposited in spherosomes, which can be identified by electron microscopy. Spherosomes in soybeans are interspersed between protein bodies and are about 0.2 to 0.5 μm in diameter (Saio and Watanabe 1968). The oil contained in the soybean is protected against exogenous contaminations and oxidation processes due to its natural compartmentalization and to the presence of natural antioxidants, which ensures its preservation and maintenance of quality. However, once the oil is released from the spherosomes stability is compromised, and as a result, the bean must be protected from external agents such as moisture and high temperatures (Nakayama and Kito 1981). The total lipid content of soybeans ranges from 18 to 23% of which 88.1% is neutral lipids, 9.8%

is phospholipids, and 1.6% is glycolipids. Neutral lipids primarily consist of triglycerides and much smaller amounts of free fatty acids, sterols, and sterol esters. The main fatty acid components of neutral, phospho-, and glyco-lipids of regular soybeans are palmitic (10.7%), oleic (22.8%), linoleic (50.8%), and linolenic (6.8%) fatty acids, but these amounts may vary due to varietal and environmental factors (Vaidehi and Kadam 1989). Liu and others (1995) reported that among 10 normal soybean genotypes grown in Arkansas, oil content ranged 16.3 and 21.6%, fatty acid composition was also influenced by genotype. The high proportions of unsaturated and polyunsaturated fatty acids make refined soybean oil unstable (Liu 1999a).

Carbohydrates

Soybeans contain about 35% total carbohydrates and defatted soy flours contain about 17% soluble and 21% insoluble carbohydrates (Perkins 1995). In addition to sucrose, soybeans contain appreciable amounts α-linked oligosaccharides, mainly raffinose and stachyose (Fig. 1). Typical amounts of sucrose, raffinose, and stachyose are 4.1, 1.1, and 3.7 % of the whole seed, and 6.2, 1.4, and 5.2 of defatted soy flour, respectively, but these amounts may be affected by environment and genetics (Vaidehi and Kadam 1989). The oligosaccharides in soybeans are non-reducing sugars, containing fructose, glucose and galactose as one or more units, linked by β-fructosidic and α-galactosidic linkages. As shown in Figure 1, raffinose contains one galactose unit and stachyose contains two. The presence of these flatulence-causing oligosaccharides is one of the restraints for broader use of soybeans in human diets. Humans lack α-1,6-galactosidase in their intestinal mucosa. When ingested, these soluble sugars remain unabsorbed, pass into the lower intestinal tract where they are metabolized by intestinal microflora, which contain the enzyme, leading to production of gas (Liener 1994).

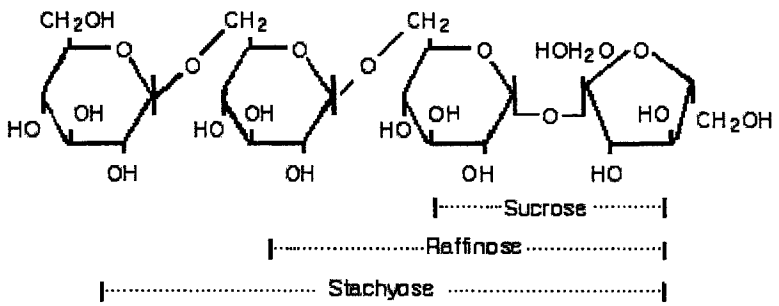


Figure 1 - Structure of soluble carbohydrates found in soybeans (Perkins 1995)

Protein

Soybean seeds contain between 35 and 46% protein at maturity (Nagano and others 1996). This protein is a heterogeneous group that may be classified in terms of their biological function as metabolic and storage proteins, their solubility profiles as albumin or globulins, or their molecular sizes, which are often qualified via sedimentation rate by fractional centrifugation (Thiering and others 2001). The two major storage proteins, the 7S globulins or β-conglycinin (37-39% of total protein) and the 11S globulins or glycinin (31-44% of total protein) have different intrinsic properties leading to different functional behaviors (Bazinet and others 2000).

Most soy proteins are insoluble in water at their isoelectric point (pH 4-5), but their solubilities increase at these pH ranges in the presence of salt (Nielsen 1985a). This behavior is typical of proteins that have been classified as globulins and is attributed to thermodynamic linkage between the free energy of salt binding and solubility of the soybean proteins (Yuan and others 2002). Studies of soy proteins by analytical ultracentrifugation in a phosphate buffer of pH 7.6 with an ionic strength of 0.5 containing 0.01 M mercaptoethanol have revealed the presence of four distinct peaks. These peaks have approximate Svedberg coefficients of 2S, 7S, 11S, and 15S with peak molecular weights of approximately 25, 160, 350, and 600 kDa, respectively (Wolf 1983). A typical soy extract yields approximately 22% 2S, 37% 7S, 31% 11S, and 11% 15S (Wolf 1983), but these amounts may vary significantly depending on variety (Zhang and others 2002, Fehr and others 2003), crop year (Yagasaki and others 1997, Fehr and others 2003), and handling and

thermal histories (Wu and Inglett 1974). Furthermore, these fractions are heterogeneous groups. They have been broadly studied and characterized.

2S ultracentrifugal fraction

The 2S fraction has been reported to contain from 8 to 22% of the extractable soybean protein and consists of a number of enzymes (Wolf 1983). This fraction has been assigned an average molecular weight of 25 kDa, but it is composed of a number of proteins with various molecular weights.

Probably the most studied proteins of this fraction are the trypsin inhibitors. Two different trypsin inhibitors have been identified in soy. The smaller of them, is known as the Bowman-Birk inhibitor. This protease inhibitor consists of 72 amino acid residues, has a molecular weight of 7.9 kDa and an isoelectric point of 4.2. This protein forms dimers and trimers in solution, which probably explains its association with the 2S fraction. The molecular configuration is stabilized by seven disulfide bonds, which give this small protein very rigid structure, and, as a consequence, makes it very resistant to heat denaturation (Liener and Kakade 1969).

The other main trypsin inhibitor of soybeans is the Kunitz inhibitor. This is a larger molecule composed of 197 amino acid residues with a molecular weight of 21.5 kDa and an isoelectric point of 4.5. This protein contains two disulfide bridges and is not as heat stable as the Bowman-Birk inhibitor (Vaidehi and Kadam 1989).

These proteins have biologically active functions based on the inhibition of proteolytic enzymes. The Kunitz trypsin inhibitor inhibits trypsin activity; but, the Bowman-Birk trypsin inhibitor is capable of inhibiting chymotrypsin as well (Liener 1994). This ability to inhibit proteolytic enzymes is the basis for trypsin activity assays. On the other hand, several studies have suggested that these trypsin inhibitors may help prevent cancer, especially the Bowman-Birk inhibitor. Whether commercially available soy products contain enough biologically active Bowman-Birk inhibitor is not clear (Kennedy 1998).

Other minor components of this fraction are cytochrome C, chalcone-flavone isomerase, alcohol dehydrogenase, and β-amylase with molecular weights of 12.5, 15.6, 53, and 57 kDa, respectively (Wolf 1983).

7S ultracentrifugal fraction

The 7S fraction of soy protein comprises about 35% of the soluble protein. It contains some enzymes, a number of hemagglutinins, and a protein known as the 7S globulin or β-conglycinin. The enzyme of greatest commercial significance in this fraction is lipoxygenase, having a molecular weight of about 105 kDa. This enzyme causes the formation of hydroperoxides by catalyzing the addition of oxygen to the double bonds of linoleic and linolenic fatty acids commonly present in soybean oil. Breakdown of these hydroperoxides leads to further lipid oxidation and creation of off-flavors in the soy protein. Much of the typically objectionable beany flavor associated with soy products is believed to come from the oxidative deterioration of lipids that are tightly complexed with protein molecules (Nielsen 1985a). Three isozymes of lipoxygenase, termed L1, L2, and L3, have been identified and each has unique characteristics. Their isoelectric points and optima pH activities are 5.68 and 9.5; 6.25 and 6.5; and 6.15 and 5.5-8.0 for L1, L2, and L3, respectively. While L2 activity is stimulated by the presence of Ca^{2+}, L3 is inhibited and L1 is not affected. L1 is heat stable and L2 is heat labile. L2 and L3 preferentially use esters as substrate and L1 preferentially acts on free fatty acids (Vaidehi and Kadam 1989).

The 7S fraction also contains a group of proteins referred to as lectins or hemagglutinins that have a molecular weight of about 122 kDa and an isoelectric point of 5.8. Defatted soy flour contains about 3% of hemagglutinins (Vaidehi and Kadam 1989). These glycoproteins, containing 4.5% mannose and 1% glycosamine, are composed of four different subunits designated A, B, C, and D with an average molecular weight of 30 kDa each (Nielsen 1985a). These proteins were named hemagglutinins because they have the capacity to agglutinate erythrocytes *in-vitro*. In some cases, hemagglutinins have been shown to lower the utilization of proteins *in-vitro*, but they seem to have no effect on the protein quality of soybeans (Vaidehi and Kadam 1989). In contrast, lectins are reported to inhibit growth in rats, lower insulin levels in the blood, induce degenerative changes in the liver and kidney, and interfere with iron absorption from the diet (Liener 1994).

The majority of the 7S protein fraction, however, consists of 7S globulins, also called β-conglycinin. This protein makes up about 85% of the 7S fraction and consists of four to

six different components and contains about 6% carbohydrate. The molecular weights of the various components of the 7S fraction range from 140 to 180 kDa (Thanh and Shibasaki 1977). β-Conglycinin consists of three subunits, α, α', and β, with molecular weights of 57, 57, and 42 kDa, respectively (Thanh and Shibasaki 1977, Shattuck-Eidens and Beachy 1985, Ogawa and others 1995). The isoelectric points for β-conglycinin is 4.9 (Koshiyama 1968) and for the individual subunits are 4.9, 5.18, and 5.66-6 for α, α', and β, respectively (Thanh and Shibasaki 1977). All of the β-conglycinin subunits are glycoproteins, mannose and glucosamine are present in a 3:1 ratio. There is no apparent difference in the carbohydrate contents of the α and α' subunits, but the β subunit contained one-half as much carbohydrate as the former two (Nielsen 1985a). Native β-conglycinin is composed of trimers of these subunits. Seven combinations of these three subunits have been identified in soybeans, ααα, ααα', ααβ, αα'β, αββ, and α'ββ (Thanh and Shibasaki 1978) and βββ (Yamauchi and others 1981). This heterogeneity in subunit combinations explains the range of molecular weights reported for this protein. β-Conglycinin undergoes complex associations and dissociations in response to changes in ionic strength and pH. At low ionic strengths ($I < 0.2$), the β-conglycinin subunits form dimers in the pH region 4.8-11.0 with sedimentation coefficients of 10S, although this protein starts to precipitate as the isoelectric point is approached. Increased ionic strength reduces the extent of precipitation, the protomeric form remaining stable and soluble. At neutral pH, β-conglycinin is found as a 7S protomer when the ionic strength is above 0.5 (Thanh and Shibasaki 1979). Other minor components, comprising less than 5% of the protein present in this ultracentrifugal fraction are malate dehydrogenase, γ-conglycinin, lactate dehydrogenase, α-D-galactosidase, α-D-mannosidase, and acid phosphatase giving an idea of the heterogeneity of the 7S ultracentrifugal fraction (Wolf 1983).

11S ultracentrifugal fraction

The 11S fraction comprises from 31 to 52% of the soluble soy protein with about 85% of the total protein being glycinin, also called 11S globulin, with a molecular weight range of 320 to 360 kDa. Glycinin does not contain sugar and does not undergo the association-dissociation behavior typical of β-conglycinin (Nielsen 1985b). Glycinin is the

major component of the protein fraction recovered from soybeans by cold precipitation. Glycinin is composed of six subunits with molecular weights of 58-62 kDa and when completely dissociated, two fractions with molecular weights of 31-45 and 18-20 kDa are recovered (Brooks and Morr 1985). The larger species have isoelectric points of 4.8 to 5.5 (Staswick and others1981) and are called acidic components (A), the smaller components have isoelectric points of from 6.5 to 8.5 (Lei and Reek 1987) and are called basic components (B). The isoelectric point of the glycinin molecule is reported to be 4.6 (Nielsen 1985a).

Acidic and basic components are linked by a single disulfide bond (A-SS-B). These fundamental units for assembly of glycinin are non-random gene products (Nielsen 1985b). Five different subunits have been purified and account for the majority of the glycinin subunits present in the seed, although other minor subunits may also be present in the genome (Nielsen and others 1989). The prevalent subunits originate from a family of homologous genes. Nielsen (1985b) reported two groups of subunits, group I with a molecular weight of 58 kDa was composed by $A_{1a}B_2$, $A_{1b}B_{1b}$, and A_2B_{1a} subunits, while group II was composed by the A_3B_4 subunit with molecular weight of 62 kDa and the $A_5A_4B_3$ subunit with a molecular weight of 69 kDa. All acidic polypeptide components except for A_4 are covalently linked to a basic polypeptide component (Nielsen 1985a).

In developing seeds, the constituent subunits are synthesized as a single polypeptide precursor, preprotein, the signal sequence of which is removed co-translationally. The resulting proproteins assemble into trimers of ~8S in the endoplasmatic reticulum. The protein trimers are transported to the protein storage vacuoles, where they are cleaved to form acidic and basic polypeptides. Finally, the mature proteins assemble into hexamers (Dickinson and others 1989). The amino acid sequences essential for trimer assembly are located in the basic chain (Dickinson and others 1990). Adachi and others (2003) further studied the crystal structure of glycinin A_3B_4 homohexamer and found that the hexamer has a 32-point group symmetry formed by face-to-face stacking of the two trimers. The interface contains the highly conserved interchain disulfide. As a consequence, during the hexamer assembly the basic polypeptides are buried in the interior of the glycinin molecule (Dickinson and others 1989). Further study of the interfaces suggested that the disulfide-

containing face has high positive potential at acidic pH, which induces dissociation of the hexamer into trimers (Adachi and others 2003). The glycinin molecule can also be dissociated in the presence of urea to form individual subunits of ~60 kDa, which can be further reduced with β-mercaptoethanol into the acidic and basic polypeptide components (Nielsen 1985b).

15S ultracentrifugal fraction

This fraction comprises about 5% of the total extractable soy protein. Wolf (1983) first reported this fraction to contain urease with a molecular weight of 480 kDa. In a later report (Wolf and Nelsen 1996), the 15S component of a soy protein extract was found to be a dimer of glycinin. Isolation of this dimer was difficult since it tended to dissociate into glycinin during sample processing. Gel electrophoresis and amino acid analysis confirmed the identity of the 15S fraction to be glycinin.

Non-protein components

Phytic acid

Phytic acid (myo-inositol 1,2,3,4,5,6, hexakis dihydrogen phosphate) is present in soybeans and soybean products in concentrations between 1.0 and 3.5% (Camire and Clydesdale 1982, Mohamed and others 1986, Liener 1994, Chitra and others 1995). Phytic acid content increases during seed development. Yao (1983) reported an increase from 0.84 to 1.26% (as is) during maturation stages of the soybean seed. Phytate is the principal source of phosphorous in soybeans. Selle and others (2003) analyzed 22 different soybean meals and found that 68.3% of the phosphorous present in their samples were associated with phytic acid. Phytate is known to be located in the protein bodies, principally as crystalline globoid inclusions (Prattley and Stanley 1982, Hegeman and others 2001).

At pH values normally encountered in food systems, phytic acid is strongly negatively charged, having considerable potential to complex or bind to positively charged molecules such as cations and proteins (Cheryan 1980). Phytate is also capable of forming complexes with negatively charged protein molecules at alkaline pH through calcium and

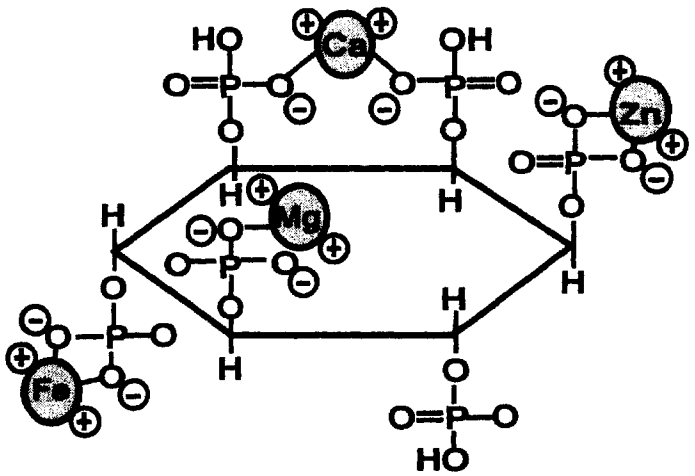


Figure 2 - Phytate binding cations (Cheryan 1980).

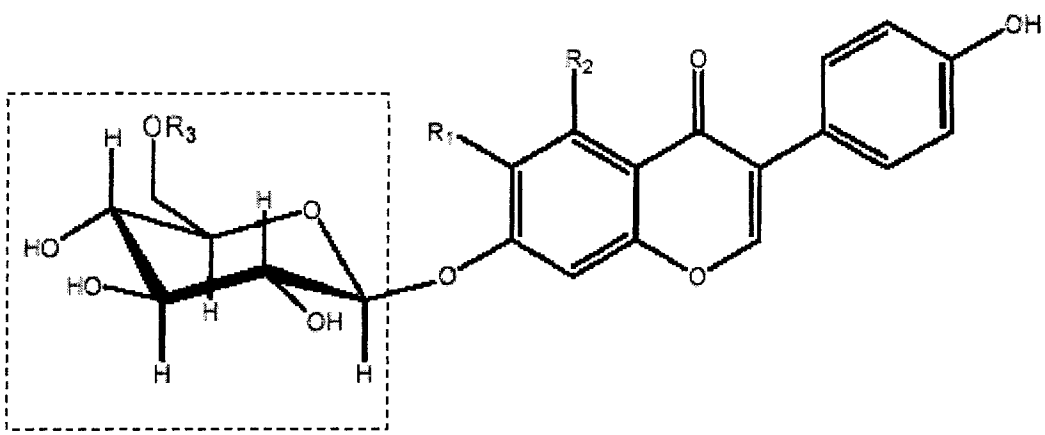
magnesium binding mechanisms (Liu 1999a). Figure 2 shows the structure of phytic acid binding to cations. Phytate binds minerals, such as iron, zinc, and calcium, and because it also binds to protein, the iron (Weaver and others 1984) and zinc (Prattley and others 1982) from soy foods are poorly absorbed. Direct experiments with human subjects have given mixed results and phytate may play minor roles in affecting the availability of minerals in humans (Liener 1994). In addition, phytate has been shown to inhibit enzymes important in digestion such as amylases (Knuckles and Betschart 1987) and pepsin (Vaintraub and Bulmaga 1991).

There is also evidence for protective functions of phytic acid such as the prevention of the formation of free radicals, the decrease of plasma cholesterol and triglycerides, and a change in the carryover of heavy metals (Pallauf and Rimbach 1997). In an animal study by Koba and others (2003), phytate was found to have only a limited role in the cholesterol-lowering effect of soy protein when Sprague-Dawley rats were fed a phytate-depleted soy-based diet and compared to a phytate-replenished soy-protein-based diet with and without added cholesterol. The basic mechanism by which phytic acid may exert these health beneficial effects is not clear.

Isoflavones

Isoflavones from soybeans are classified as phytoestrogens because they have weak estrogenic activity in mammalian systems (Murphy and others 1999). Isoflavones have limited distribution in nature although they have been found in other sources such as red clover (de Rijke and others 2001), alfalfa (Franke and others 1994), chickpeas, and lentils (Mazur and Adlercreutz 1998, Wieseman and others 2002). The majority of these diphenolic compounds in the human diet are the isoflavones from soy protein ingredients and soyfoods (Murphy and others 1999). These phytoestrogens are being extensively studied to determine their beneficial health role and therapeutic potential. Numerous biological activities have been attributed to isoflavones including antioxidant and antihemolytic (Naim and others 1976), antifungal and antibacterial (Hosny and Rosazza 1999), cancer chemoprotectant (Sanderson and others 2004, Allred and others 2004, Fischer and others 2004), cardiovascular health promoting by reduction of serum cholesterol (Anthony and others 1996), and estrogenic (Cassidy and others 1994).

Soybeans typically contain 1.9 to 9.5 mg/g of isoflavones, but these concentrations vary depending on crop year, variety and location (Lee and others 2003). Soyfoods on a wet weight basis contain from 1 μg/g in soy sauces to 540 μg/g in tempeh (Murphy and others 1999). Three isoflavones are found in soybean and soy products: genistein, daidzein, and glycitein (Fig. 3). There is typically more genistein than daidzein in soybeans, but isoflavone proportions vary greatly among different soy products (Wang and Murphy 1996). Each isoflavone can occur in four possible forms, the aglucon, the β-glucoside, the malonyl-β-glucoside, and the acetyl-β-glucoside. Typically, the β-glucoside and malonyl-β-glucoside forms predominate in soybeans (Wang and Murphy 1994), but the isoforms distribution varies depending on soy product and treatment (Wang and Murphy 1996).



Isoflavone	R_1	R_2	R_3
Daidzein	H	H	Aglucon
Genistein	H	OH	Aglucon
Glycitein	OCH_3	H	Aglucon
Genistin	H	OH	H
Glycitin	OCH_3	H	H
Daidzin	H	H	H
Malonylgenistin	H	OH	$COCH_2COOH$
Malonylglycitin	OCH_3	H	$COCH_2COOH$
Malonyldaidzin	H	H	$COCH_2COOH$
Acetylgenistin	H	OH	$COCH_3$
Acetylglycitin	OCH_3	H	$COCH_3$
Acetyldaidzin	H	H	$COCH_3$

Figure 3 – Isoflavone structures

Other minor components

Saponins are complex glycosides of triterpenoid alcohols and are present in soybeans at about 0.5% (Wolf 1983). Soyasaponins are composed of a lipid-soluble aglucon (sapogenol) consisting of either a sterol or a triterpenoid and water-soluble sugar residues differing in type and amount of sugars. When the sugars are added to the sapogenol, the resulting compounds are called glucosides. The most common sugars that are added to the sapogenols to form glucosides are glucose, arabinose, glucuronic acid, and xylose. The glucosides are highly surface-active due to their amphiphilic nature (Rao and others 1995). Because of their polarities, the saponins are insoluble in hexane and remain in defatted meal,

which contains about 0.6%. The saponins are not absorbed, but are hydrolyzed by bacterial enzymes in the cecum and colon (Hu and others 2004). The major dietary sources of saponins are legumes, especially soybeans. More than 20 saponins have been identified from soybean extract that vary in the structure of the sapogenin aglucon and their glucosides (Shiraiwa and others 1991). Hu and others (2002) developed a high-performance liquid chromatographic method for the isolation and quantitative determination of soysaponins. Hu and others reported (2002) saponin concentrations of soy ingredients and soy products, including tofu, tempeh, soy milk and soy protein isolate as 0.59, 1.53, 0.47, and 9.51 μmole total saponins /g product, respectively. Saponins are claimed to have hypocholesterolemic, immunostimulatory and anticarcinogenic properties (Kennedy 1995). Several *in-vitro* and *in-vivo* studies have recently been reported that support anticarcinogenic properties of saponins, more specifically soyasapogenol B (Hu and others 2004). In a review of Kennedy (1995), the proposed mechanisms of anticarcinogenic properties of saponins included antioxidant effect, direct and select cytotoxicity of cancer cells, immune-modulation, acid and neutral sterol metabolism, and regulation of cell proliferation. The saponins non-specifically inhibit several enzymes including cholinesterase (Ishaaya and Birk 1965) and chymotrypsin (Shingo and others 1996). The interaction of saponins with soybean proteins is still unknown.

Soybean seeds are a good source of water-soluble vitamins. Soybeans contain 0.2-0.4 μg/g β-carotene, 11.0-17.5 μg/g thiamin, 2.3 μg/g riboflavin, 20.0-25.9 μg/g niacin, 12 μg/g pantothenic acid, 6.4 μg/g pyridoxine, 0.6 μg/g biotin, 2.3 μg/g folic acid, 3.4 mg/g choline, and 0.2 mg/g ascorbic acid (Vaidehi and Kadam 1989). Although soybeans contain a fair amount of pyridoxine, most of it is in the form of pyridoxine glucoside that has been reported to have reduced bioavailability, as a consequence soy is not an important dietary source for vitamin B6 (Hansen and others 1996). Some anti-vitamin effects have been identified in soybeans for vitamins A, B_{12}, D, and E, but were related mainly to unheated soybean meal (Liener 1994).

The majority of inorganic compounds in soybeans are minerals and these concentrations vary on environmental and varietal factors. Grieshop and Fahey (2001) determined the ash contents of soybeans from three major soybean producing countries and

found that soybeans produced in Brazil had the lowest ash content (5.10%) followed by China (5.42%) and the United States (5.44%). Usually, the minerals present in soybeans, in order of importance, are potassium, sodium, phosphorous, magnesium, calcium, and iron. Their concentrations will also vary depending upon the product produced and the process utilized (Perkins 1995). Garcia and others (1998) analyzed commercial soybean products and found ash contents of 9.9, 6.8, 7.4, and 5.23% for soy protein isolate, soy flour, textured soy protein, and whole soybeans, respectively. They also analyzed the composition of the ash and found that it contained significant amounts of phosphorus (7.4-4.4 mg/g), calcium (7.4-1.2 mg/g), copper (2.0-0.3 mg/100g), iron (7.0-1.9 mg/100g), potassium (26.9-2.4 mg/g), and zinc (5.4-3.5 ng/100g). Araujo and others (1986) found that commercial soy protein isolates contained 0.92% calcium, 0.51% magnesium, 1.08% sodium, 0.19% potassium, and 0.64% phosphorus, but they did not measured phytate. Honig and Wolf (1991) analyzed soybean meal extracts by gel filtration and found that the highest concentrations of iron and calcium were recovered in the fraction that was richest in glycinin and β-conglycinin and also contained phytic acid. They concluded that there was apparently protein-mineral associations with phytic acid.

Soybeans and health

During the past two decades, the scientific community has firmly established a connection between diet and risk of chronic diseases such as cancer and heart disease. In addition, there have also been advances in recognizing the nutritive and nonnutritive components of soy foods and their roles in reducing risks of these chronic diseases. Several soyfoods components have been suggested to be related to health beneficial effects. These include macronutrients, such as protein, and micronutrients such as isoflavones, saponins and phytic acid. Soyfoods are gaining in popularity because of their specific health attributes and because they make for an easy transition from an animal-based diet to a plant-based diet (Messina 1999).

Components of soybeans that may contribute to cancer prevention are trypsin inhibitors, saponins, phytic acid, isoflavones, and hydrolyzed subunits of β-conglycinin. Kobayashi and others (2004) showed that dietary supplementation with soybean Kunitz

trypsin inhibitors was a useful means to reducing total tumor burden in a mouse model. Bowman-Birk trypsin inhibitor was shown to be an effective cell growth inhibitor when conjugated with concavalin A (Lin and Hu 1986). An extensive review by Kennedy (1998) deals with the anticarcinogenic properties of the Bowman-Birk trypsin inhibitor. Gurfinkel and Rao (2003) studied the effects of soyasaponins on colon cancer cells and they found that the most potent compounds, in suppression of cell growth, were the aglucon form soyasapogenol and further hypothesized that the increased bioactivity might be due to higher hydrophobicity of the aglucon forms. Hu and others (2004) showed that soyasaponins have low absorbability and are converted to soyasapogenol B by gut microflora. Phytic acid has also been identified as a possible cancer preventing agent. Tantivejkul and others (2003a, 2003b) showed that phytate inhibited metastasis in a human breast cancer cell model by reducing cancer cell adhesion, migration and invasion. Shamsuddin (2002) and later Vucenik and Shamsuddin (2003) published thorough reviews on the role of phytic acid in cancer prevention and strongly advocated the use of phytate in the diet, even suggesting phytic acid to be an essential nutrient.

Isoflavones are probably the soybean component most studied in relation to health beneficial effects and some have suggested isoflavones to be potential cancer preventing agents. Several new studies have been published in this area. Allerd and others (2004) studied the effects of different commercially available soy products in a breast cancer model system and suggested that consumption of products containing soy flour was more effective in cancer treatment for estrogen-dependent breast cancer, than were products with added purified forms of isoflavones. In contrast, Fischer and others (2004) found that high doses of purified soy isoflavones were effective for men with prostate neoplasia. Sharma and Sultana (2004) studied the effects of isoflavones in a mouse skin model and concluded that isoflavones were potentially protective against a well-known tumor-promoting agent. Tsuruki and others (2003) developed immuno-stimulating peptides derived from the α' subunit of soybean β-conglycinin and named them soymetides. These peptides promoted tumor necrosis factor production following oral administration in mice.

In spite of extensive research done on relating soy intake and cancer risk including *in-vitro*, animal, and epidemiologic studies, there are insufficient data to conclude that soy

components are protective against carcinogenesis. The inconsistencies in research results may be due to the complexity of soybean composition and the complexity of the cancer process (Birt 2001). More epidemiological studies are needed to elucidate the complexity of these relationships. Epidemiological studies are often contrasting and use traditional soyfoods, making it difficult to identify specific components and their relationships to disease prevention (Wu 2001). In considering the potential for cancer prevention by soybeans, it will be important to identify the optimal intake of all constituents that may interact in preventing cancer (Birt 2001). The development of soy protein ingredients with known compositions would help to answer the questions remaining on this subject.

More consistent results were found when relating the effects of consumption of soy foods and coronary heart diseases. In October 1999, the Food and Drug Administration approved a health claim that can be used on labels of soy-based foods to promote their heart-healthy benefits. The agency reviewed research from 27 studies that demonstrated the benefits of soy protein in lowering levels of total cholesterol and low-density lipoprotein (Henkel 2000). Although most studies examined the effects of substituting soy protein for animal protein, some researchers indicate that the simple addition of soy protein to the diet is effective (Friedman and Brandon 2001). Soy protein is primarily effective in persons with moderate to severe hypercholesterolemia. In addition, there is a dose-response relationship between soy protein and cholesterol reduction. Some data also suggest that the combination of soy protein and isoflavones in some situations or people not only lower LDL-cholesterol, but also raise HDL-cholesterol. The amount of soy protein needed to lower cholesterol could be achieved more easily by consuming soy protein products high in soy protein content (Messina 1999).

Recent research suggests that β-conglycinin is beneficial to heart health in populations consuming high amounts of soy protein. These benefits include reducing blood cholesterol (Adams and others 2004, Duranti and others 2004, Manzoni and others 2003) and plasma triglyceride levels (Aoyama and others 2001, Baba and others 2004, Moriyama and others 2004), which impact cardiovascular health. All these studies, with the exception of Baba and others (2004), used animal or cellular models and, as a consequence, there is a critical need for human-feeding studies to corroborate these findings. More specifically,

Baba and others (2004) studied the effects of β-conglycinin on the body fat ratio and serum lipid levels of healthy female volunteers and suggested that a continuous intake of 5 g of this protein per day was effective eliminating excessive lipids and keeping a normal body fat ratio and serum lipid level. Manzoni and others (2003) using a cellular model confirmed that the α' subunit from β-conglycinin plays a key role in the cell cholesterol homeostasis and it is involved in cell protection against oxidative stress. Duranti and others (2004) found that this same subunit reduced plasma lipids and upregulated liver β-VLDL receptors in rats fed a hypercholesterolemic diet. Moriyama and others (2004) found that β-conglycinin could be a potentially useful dietary protein source for prevention of atherosclerosis. The development of β-conglycinin-rich soy products and ingredients rich in the α'subunit would allow epidemiologic studies to confirm these animal studies and to understand the roles of specific proteins and subunits in human diets.

Other health benefits attributed to soybeans are related to bone health and kidney disease. The relationship between soy protein and preventing osteoporosis is attributed to a reduced calcium excretion when animal protein is replaced by soy protein in the diet. In addition, several animal-feeding studies have suggested that genistein increases bone density by stimulating bone formation (Messina 1997, 1999, Friedman and Brandon 2001).

Soy protein and reducing agents

Intermolecular disulfide bridges are important with respect to structural properties of food proteins. Cleavage of disulfide bonds is necessary to separate and characterize individual polypeptide chains. In general, the extent of disulfide bond cleavage depends upon the reducing agent used and its concentration (Kella and Kinsella 1985). The complete cleavage of intramolecular disulfide bridges usually requires total disruption of the protein structure.

Glycinin has 18-20 disulfide bonds of both inter- and intramolecular nature that contribute to the compact structure of this protein (Kella and others 1986). Glycinin has single intermolecular disulfide bridges that covalently bond acidic and basic polypeptides (Nielsen 1995b). The rapid separation of the basic chains from the acidic chains of glycinin during heating in the presence of reducing agents indicates that the disulfide bond holding the

two chains together is easily cleaved (Wang and Damodaran 1991). Wolf (1993) suggested that during weak reducing treatments, such as the addition of dilute reducing agents during protein fractionation, the intermolecular disulfide bridges are preferentially cleaved. Further reducing treatment would also cleave intramolecular disulfide bonds with consequential changes in protein structure. The cleavage of intramolecular disulfide bonds could expose many of the non-polar groups that were buried in the protein interior these groups might interact leading to aggregation and consequent precipitation (Kella and others 1986). There are three major areas where reducing agents are used to cleave disulfide bridges: 1. for analytical purposes, 2. to modify protein functionality, and 3. to fractionate soy protein. In analytical applications, the reducing agent β-mercaptoethanol is added to separate individual polypeptides for further analysis. In general, the research to improve protein functionality was carried out through partial reduction of disulfide bonds and has focused on the glycinin component and, in some cases, the reduction of disulfide bond was followed by oxidation (Kella and others 1986, Gonzalez and Damodaran 1991). Kim and Kinsella (1987) found that reducing soy glycinin improved its surface-active properties. Abtahi and Aminlari (1997) found that solubility increased when added reducing agents to a soy milk base and attributed this effect to disulfide bond cleavage. Boonvisut and Whitaker (1976) and Kella and others (1986) found that cleaving disulfide bonds also improved solubility, surface hydrophobicity and, as a consequence, *in-vitro* digestibility of soy proteins. Further studies were conducted by Petrucelli and Anon (1995) and Wagner and Gueguen (1995, 1999a, 1999b) in which they related altered surface activity to partial reduction of disulfide bonds. Most of these researchers agreed that cleaving the disulfide bonds between glycinin subunits introduced significant changes in surface hydrophobicity and, as a consequence, improved the surface-active properties.

Another interesting body of research was done to understand the effects of reducing agents during soy protein fractionation. Wolf (1993) concluded that adding a reducing agent to precipitate glycinin significantly improved this fraction's purity when using the fractionation method described by Thanh and Shibasaki (1976). Reducing agents are believed to prevent co-precipitation of other and unwanted species (Thiering and others 2001). Suitable reducing agents for use as protein fractionation aids have been reported to be any

sulfite compound that yields SO_2 in solution, glutathione, cysteine (Hirotsuka and others 1988), and β-mercaptoethanol (Thanh and Shibasaki 1976).

Soy protein and calcium interactions

Several researchers have focused on determining the nature of the interactions between calcium ions and soy proteins. Some reports deal with specific mechanisms of Ca^{2+} binding. Rao and Rao (1976) studied calcium binding to β-conglycinin and the binding of calcium to the protein appeared to occur at the imidazole groups of the histidine residues. This observation was based in that the maximum number of ions bound matched with the histidine content of the protein and the addition of calcium increased the heat of coagulation of β-conglycinin, but the addition of 0.5M NaCl suppressed precipitation. The interaction of glycinin with Ca^{2+} was reported by Sakakibara and Noguchi (1977) who found that calcium binding was pH dependent and, in contrast with later reports, glycinin bound calcium at pH 8 but not at pH 6 or 7.

Kroll (1984) studied the effects of pH on Ca^{2+} binding by soy proteins and concluded that pH strongly affects the extent of Ca^{2+} binding because hydrogen ions compete with calcium ions for the same binding sites on the protein molecule. These binding sites where identified as being the side-chain carboxyl groups of the aspartic and glutamic acid residues and with the imidazole group of the histidine residues. The affinity of the binding sites for calcium ions increased as pH increased over the pH range of 4 to 9, since the binding constant increased from $0.07x10^3$ (moles of $Ca^{2+}/10^5$g of protein) at pH 4 to $6.38x10^3$ (moles of $Ca^{2+}/10^5$g of protein) at pH 9 a small change in pH resulted in a large change in the amount of Ca^{2+} bound. At low pH (4-5), calcium ions are quite loosely bound because they are in direct competition with H^+ for the binding sites in the protein molecule. At high ph (8-9), calcium ions are tightly bound and the binding sites have high affinity for calcium ions; at this pH, the carboxyl and imidazole groups are completely deprotonated (Kroll 1984).

The effects of pH and Ca^{2+}-induced associations of individual soy proteins were reported by Yuan and others (2002). This later study concluded that the amount of Ca^{2+} necessary to precipitate a mole of β-conglycinin was much greater (164 number of calcium ions/mole of protein) than the amount required to precipitate the glycinin fraction (79 number

of calcium ions/mole of protein), and related these findings to the charge density per surface area of the proteins with -0.47 e^-/nm^2 for β-conglycinin and -0.17 e^-/nm^2 for glycinin. The amount of calcium ions required to precipitate these proteins increased to 1000 and 435 for β-conglycinin and glycinin, respectively, with the addition of 0.1M NaCl. This work suggested that differential precipitation and complete partitioning of these two storage proteins could be achieved by adjusting the addition of Ca^{2+} and changing the pH of the medium in the presence of these Ca^{2+} ions.

Soy protein and phytate interactions

Data interpretation of studies using mixed systems containing proteins, salts, and other components in addition to phytate are difficult to interpret because phytic acid can interact strongly with positively charged ions and functional groups. The solubility profile of phytic acid is quite different in the presence of proteins than in its absence (de Rahm and Jost 1979). The solubility of phytic acid parallels the solubility behavior of the proteins in soybean systems and between pH 7 to 11. Above pH 11.5, phytate is insoluble even in the presence of protein and remains in solution at the isoelectric point of soy proteins (de Rahm and Jost 1979). Ultrafiltration experiments reported by Okubo and others (1975) and Omosaiye and Cheryan (1979) have suggested that the interaction at the three pH regions (<pH5, pH 5-7, and pH>7) are sufficiently different from each other that they should be discussed separately.

At low pH, the protein possesses a net positive charge (Yuan and others 2002) and phytic acid is negatively charged (Okubo and others 1976). Consequently, the protein-phytic acid interaction at low pH is strong electrostatic interaction. This interaction involves the anionic phosphate groups of phytate and the cationic groups of proteins, and is shown in Figure 4 (Okubo and others 1975). This interaction was further studied by Grynspan and Cheryan (1989) who found that below the isoelectric point of soy protein, phytate and protein solubility profiles paralleled each other indicating protein-phytate interaction. Addition of phytate shifted the isoelectric point and minimum solubility of soy proteins to lower pHs. Similar observations were also reported by Chen and Morr (1985), where phytate-reduced soy protein extract exhibited minimum solubility at pH 4.8-5.0, whereas the control extract

(without phytate removal) had minimum solubility at pH 4.2-4.5. Phytate-reduced soy protein isolate was more soluble and functional at pH 3, whereas the control isolate was at pH 6 and 9.

At high pH, multivalent cations, such as calcium, seem to be essential for the protein-phytate complex formation (Okubo and others 1976). Furthermore, Saio and others (1968) found that a single protein molecule may bind many molecules of calcium and phytic acid. The behavior of phytate at alkaline pH appears to be strongly influenced by salt linkages or alkaline-earth ion bridges as shown in Figure 4 (Omosaiye and Cheryan 1979). This mechanism also explains why phytic acid appears to be soluble in the presence of protein above pH 6, even though phytate salts by themselves are insoluble at alkaline pH (Saio and others 1968). This mechanism was further supported by studies of Okubo and others (1976) where co-elution of calcium-free glycinin and phytate during gel filtration did not occur at pH >6. DeRahm and Jost (1979) observed that at pH 7.5, 40% of the phytate was nondialyzable, increasing the calcium concentration in the system (from 2.6 mol to 3.5 mol of calcium per mole of phytate) increased protein-bound phytate to ~80%. The addition of citric acid (Jovani and others 2000), EDTA (Okubo and others 1975), or NaCl (de Rahm and Jost 1979) improved the removal of phytate by dialysis, ultrafiltration, and precipitation, respectively.

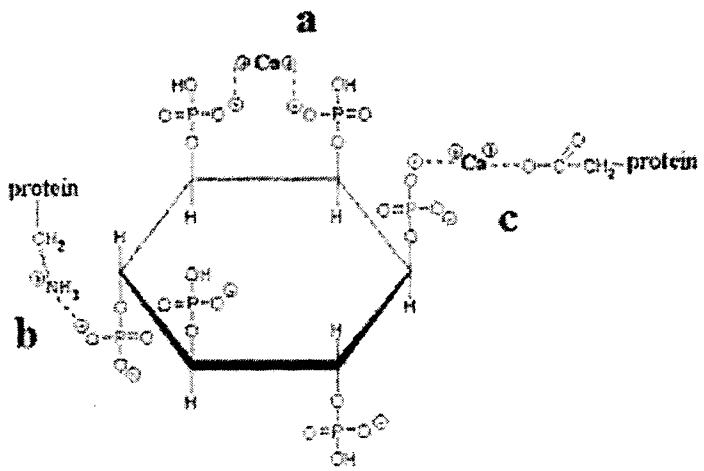


Figure 4 - Phytate binding to calcium (a) and proteins at low pH (b), and calcium-protein bridge formation at high pH (c) (Cheryan 1980).

At intermediate pH, both the proteins and phytic acid have net negative charge, however, data indicate some type of protein-phytate complex formation (de Rahm and Jost 1979). Also evidenced in the work of Omosaiye and Cheryan (1979), where at these intermediate pHs significant amounts of phytate remained with the protein fraction even after two ultrafiltration passes. It is possible that direct salt-like linkages occurs between phytic acid and the amino terminal groups and the amino groups of lysine, since these groups are still protonated at these pHs.

A considerable amount of research has focused on removing phytic acid from soy protein products. Addition of excess NaCl was used to disrupt alkaline-earth ion bridges to produce phytate-reduced soy protein products (DeRahm and Jost 1979). Ford and others (1978) used low pH in combination with added $CaCl_2$ to remove 90% of the phytate from soy protein concentrates. Omosaiye and others (1979) developed a method using ultrafiltration to eliminate phytic acid from soy protein isolates and full-fat concentrates. Brooks and Morr (1985a) compared two phytate removal treatments from defatted soy flakes and found that the ion exchange phytate removal was more effective, since removed 86% of the total phosphorus originally present without major alterations to the proteins molecular weight profile and subunit compositions. The control alkaline phytate removal treatment only removed 62% of the phosphorus present with major amounts of glycinin and β-conglycinin aggregation. Chen and Morr (1985) were able to remove 77% of the phytate by using a pilot-scale ion-exchange process. Kumagai et al. (2002) removed phytate by using ion-exchange resins. Saito et al. (2001) reported on a method for separating soybean glycinin and β-conglycinin using phytase and suggested that phytate may affect protein solubility and related functional properties.

Several patents have been filed in this area. Nardelli and others (1993) patented a method of separation of phytate from soy protein using anion-exchange resins. They claimed that more than 90% of the phytate is removed, while more than 90% of the protein is recovered. Westfall and others (1992) patented a method of preparation of flour proteins low in phytate and aluminum by ultrafiltration. They claimed a reduction of 60 and 88% for phytate and aluminum, respectively. Simell and others (1990) patented a process to produce

phytate-free or low-phytate soy protein isolate and concentrate by adding phytase enzymes during manufacture. They claimed 66% phytate reduction for both soy protein ingredients.

Genetically modified soybeans

Biotechnology can be defined broadly as a set of tools that allow scientists to genetically improve living organisms. Other emerging sciences, such as genomics and proteomics, are starting to impact plant improvement as well (Soper and others 2003). In spite of soybeans being an important world commodity, only a small portion of them is destined to food production. Some of the constraints for using soybeans in food systems are beany flavor, flatulence, oxidative and flavor instability, deficiency in sulfur-containing essential amino acids for some species, poor protein digestibility, lack of certain functional properties, and presence of antinutritional factors. In addition, soybeans are prone to attacks by diseases, insects, and weeds. To overcome some of these constraints advanced plant breeding and genetic engineering are being used. Although one of the first genetically modified soybeans was the Roundup Ready® variety, which has been widely adopted by growers (Soper and others 2003), increasing research efforts are focusing on improving soybeans quality for food, bioenergy and biomaterials. These improvements can offer products that provide healthier and more functional ingredients in our diet. Some target strategies for breeding higher value-added soybeans include increasing protein and oil contents, reducing unstable and saturated fatty acids, eliminating beany flavor, eliminating lipoxygenase activity, increasing levels of essential amino acids, modifying ratios of glycinin/β-conglycinin, and lowering levels of oligosaccharides.

Breeding to improve protein quality

Improving protein quality would add value to soybeans and resulting soy products. Two major areas have been identified regarding to protein quality, nutritional and functional properties. Lines with improved nutritional or functional properties include specific glycinin/β-conglycinin proportions (Ogawa and others 1989, Bringe 2001); high-cysteine, -methionine and high-lysine (George and de lumen 1991); low-lipoxygenase activity (Kitamura 1984, Hajika and others 1991); low activity or expression of trypsin inhibitors

(Stahlhut and Hymowitz 1983); and low-oligosaccharides and low-phytate (Hitz and others 2002, Crank and Kerr 1999). Some of these lines have already been developed and are commercially available.

High-sucrose/low-stachyose soybean lines

Raffinose and stachyose, the main components of the soluble sugar fraction in soybeans, are indigestible to humans and cause flatulence. There are a number of ways in which biotechnology could improve soy-based foods and help increase consumption. Removing indigestible and flatus-causing sugars is one obvious example and has been achieved (Hitz and others 2002). There is considerable variation in raffinose (0.1-0.9%) and stachyose (1.4-4.1%) contents among varieties of soybeans (Hymowitz and others 1972).

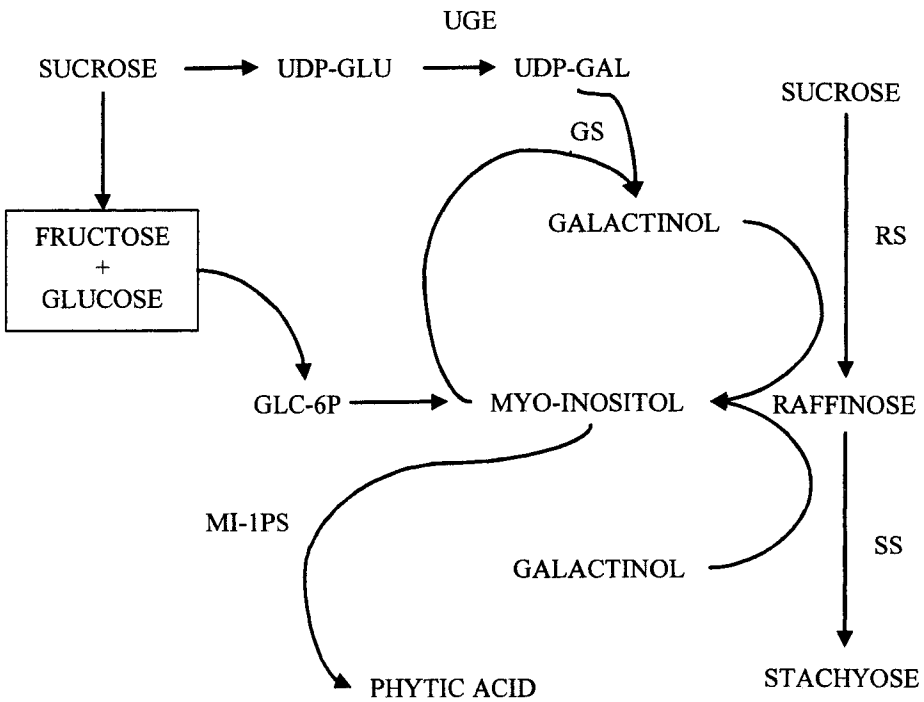


Figure 5 – Biosynthetic pathway of oligosaccharide synthesis in soybeans. Adapted from Wilson (2001). UGE denotes UDP-glucose-4'-epimerase; GS, galactinol synthase; RS, raffinose synthase; SS, stachyose synthase; and MI-1PS, myo-inositol phosphate synthase.

It is also possible to use molecular biology to produce genetically modified soybeans that are low in oligosaccharides, as described by Crank and Kerr (1999). These researchers describe the development of soybean lines with low raffinose and lines with high sucrose and low stachyose (HS/LS) contents by two separate methods of conventional breeding, germplasm screening and chemical mutagenesis. The activities of six enzymes, myo-inositol 1-phosphate synthase (MI1PS), myo-inositol 1-phosphatase (MI1P), UDP-glucose-4'-epimerase (UGE), galactinol synthase (GS), raffinose synthase (RS), and stachyose synthase (SS) could be reduced to decrease either raffinose or stachyose synthesis without decreasing sucrose content (Fig. 5). Three of these enzymes (GS, RS, and SS) are unique to raffinose and stachyose syntheses and could be reduced in activities without decreasing phytic acid content. Only MI1PS appears to be involved in the synthesis of all three, phytic acid, raffinose, and stachyose, and a reduction in its activity would change the amounts of all three end products (Hitz and others 2002). Utilizing defatted meal from these HS/LS soybean lines as starting soy flour opened the possibility for new products development (Johnson 1999, Crank and Kerr 1999).

Other high-quality protein lines

Genetic manipulation of the glycinin/β-conglycinin ratio could improve nutritional and functional quality of soy proteins. Kitamura (1995) reported finding two different low-β-conglycinin mutants. One lacked the α' subunit and the other lacked both α and β subunits. The glycinin contents of these lines were 15% higher than those of normal varieties and a negative correlation was observed between the two individual storage protein contents. The sulfur-containing amino acids content was 20% higher in these lines, compared to regular varieties. Kitamura also reported finding several low-glycinin lines, but no apparent increase in β-conglycinin content was detected in these lines. Bringe (2001) on the other hand, disclosed the use of a soybean line having a glycinin to β-conglycinin ratio of 1:4 to produce traditional soyfoods and ingredients with enhanced functional properties. These soybeans were also reported to have superior content of sulfur-containing amino acids (methionine + cysteine) compared to those of normal soybean lines. These new lines allow the

development of new food products and ingredients with unique functional and nutritional properties.

Methionine, a sulfur-containing amino acid, is the first limiting essential amino acid for livestock in soy protein. There have been two genetic approaches to increase the content of this and other amino acids. One approach is to transfer genes coding methionine-rich or lysine-rich proteins from other species. An example of such an approach is the successful transfer of methionine-rich protein from Brazil nut. Transgenic soybean lines accumulated up to 8% of this protein, equivalent to a gain of 26% in methionine (Liu 1999b). Unfortunately, the 2S albumin transferred to soybean is allergenic and, as a consequence, the transgenic soybeans were also allergenic (Nordlee an others 1996). Another approach is to modify amino-acid biosynthetic pathways to increase lysine, methionine and threonine.

Soy protein food ingredients

Typically, soy protein ingredients are classified by their protein contents and they are usually edible dry soybean products that are added to food systems for their nutritional and functional values. They can be divided in three groups: soy flakes and flours that may be full-fat, defatted, and refatted or lecithinated; soy protein concentrates that are produced by three different processes, aqueous-ethanol washing, acid leaching, and hot-water leaching; and soy protein isolates produced by neutral to alkali extraction and isoelectric precipitation, membrane filtration, and salt extraction. Each of these products may be modified physically, chemically or enzymatically to produce unique products for specific applications.

Soy flakes and flours

Three types of full-fat soy flours are produced, enzyme-active, toasted, and extruder-processed. Full-fat flours are used for the action of their lipoxygenases in bleaching wheat flours and conditioning doughs in breadmaking. In addition, soy β-amylases are heat-stable and remain active in early stages of baking contributing to improve texture. The cleaned soybeans are cracked and the hulls removed by aspirating. The dehulled pieces are ground into flours with different particle sizes. Commercial full-fat, enzyme-active soy flours contain about 42% protein (db, N x 6.25), 10% moisture, 21% fat, and 4.7% ash (Lusas and

Rhee 1995). Toasted full-fat soy flour and flakes are heat treated to minimize lipoxygenase activity, yielding products with a nutty flavor and tan color. The cleaned soybeans are steamed and then cooled, dried, cracked, dehulled, and milled into flours and flakes with different particle sizes. Usually these products have protein dispersibility indices (PDI) from 20 to 35. Extruder-prepared full-fat soy flours are texturized products that are processed by an extruder prior to processing them into flours.

The vast majority of soy protein ingredients are made from white flakes or flour (hexane-defatted flakes of dehulled soybeans graded for food use). White flakes are usually graded on their nitrogen solubility index (NSI) or protein dispersibility index (PDI). The production of high-PDI flakes typically utilizes a flash desolventizing system (FDS). A FDS with solvent vapor recirculation can be operated to produce flakes ranging from 10 to 85%, depending on how much steam is applied (Lusas and Rhee 1995). Defatted soy flours usually contain 56-59% protein (N x 6.25), 0.5-1.1% fat, 2.7-3.8% crude fiber, 2.1-2.2% soluble fiber, 17.0-17.6% insoluble fiber, 5.4-6.5% ash, and 32.0-34.0% carbohydrates, on moisture-free basis (Endres 2001). Defatted soy flours are finely ground flakes to pass through a No. 100 mesh US standard screen and steam injection is provided to render "white" (NSI 85-90), "cooked" (NSI 20-60), and "toasted" (NSI <20) grades (Endres 2001).

The uses for soy flour depend upon their PDI/NSI. High PDI (>90) flours are used as a white-bread bleaching agent, as fermentation aids, and as materials from which to produce soy protein isolates and spun protein fibers. Flours with 60-75 PDI are used in a variety of food systems to control fat and water absorption and to produce soy protein concentrates. Low PDI flours (10-45) are used as nutritional extenders in meat systems, bakery mixes, baby foods, sauces and gravies, and to produce hydrolyzed vegetable protein ingredients. Flakes or grits are used as nutritive meat extenders (Lusas and Rhee 1995).

Soy protein concentrates

Soy protein concentrates (SPC) contain at least 65% (N x 6.25) protein on moisture-free basis as defined by the US Department of Agriculture's Food and Nutrition Service in January 1983 (Lusas and Rhee 1995). SPCs usually contain 65.0-72.0% protein, 0.5-1.0% fat, 3.5-5.0% crude fiber, 2.1-5.9% soluble fiber, 13.5-20.2% insoluble fiber, 4.0-6.5% ash,

and 20-22.0% carbohydrates on moisture-free basis (Endres 2001). The objective in making SPCs is to immobilize the protein while leaching the indigestible soluble sugars (raffinose and stachyose). Three different processes, extracting soy flour with aqueous (20 to 80% water) ethyl alcohol or acid leaching, and denaturing the protein with moist heat and extracting with water, can achieve this objective (Johnson and others 1992). The yields of SPCs range from 60 to 70% based on the dry weight of the initial defatted flakes or flours (Vaidehi and Kadam 1989).

In the aqueous-alcohol process (Fig. 6), soy flour is typically extracted with 60% ethanol because soy protein solubility is minimum at this concentration (Campbel and others1985). The protein solubilities of SPCs made by using this process are low, but are not necessarily related to functionality because the mechanism of denaturation is different from heat denaturation. Typically, ethanol-washed soy protein concentrate (EWSPC) will contain 66.0% protein (N x 6.25), 6.7% moisture, 0.3% petroleum-ether-extractable fat, 3.5% crude fiber, 5.6% ash, and a NSI of 5 (Lusas and Rhee 1995).

In the acid-leaching process, defatted soy flour or flakes are leached with water at pH 4.5 (isoelectric point of soy protein) to remove soluble sugars. Then, the insoluble material is normally adjusted to neutrality and spray-dried. Some losses of soluble protein occur, but the resulting SPC has relatively high solubility. Acid-leached SPCs contain about 67.0% protein, 5.2% moisture, 0.3% petroleum ether extractable fat, 3.4% crude fiber, 4.8% ash, and a NSI of 69 (Lusas and Rhee 1995).

In the hot-water-leaching process, defatted soy flour is subjected to moist heat to denature the protein, followed by water leaching and drying. This process generally results in dark colored end-products due to the heat treatment (Ohren 1981). A typical moist-heat water-leached SPC contains 70% protein, 3.1% moisture, 1.2% petroleum-ether-extractable fat, 4.4% crude fiber, 3.7% ash, and a NSI of 3 (Lusas and Rhee 1995).

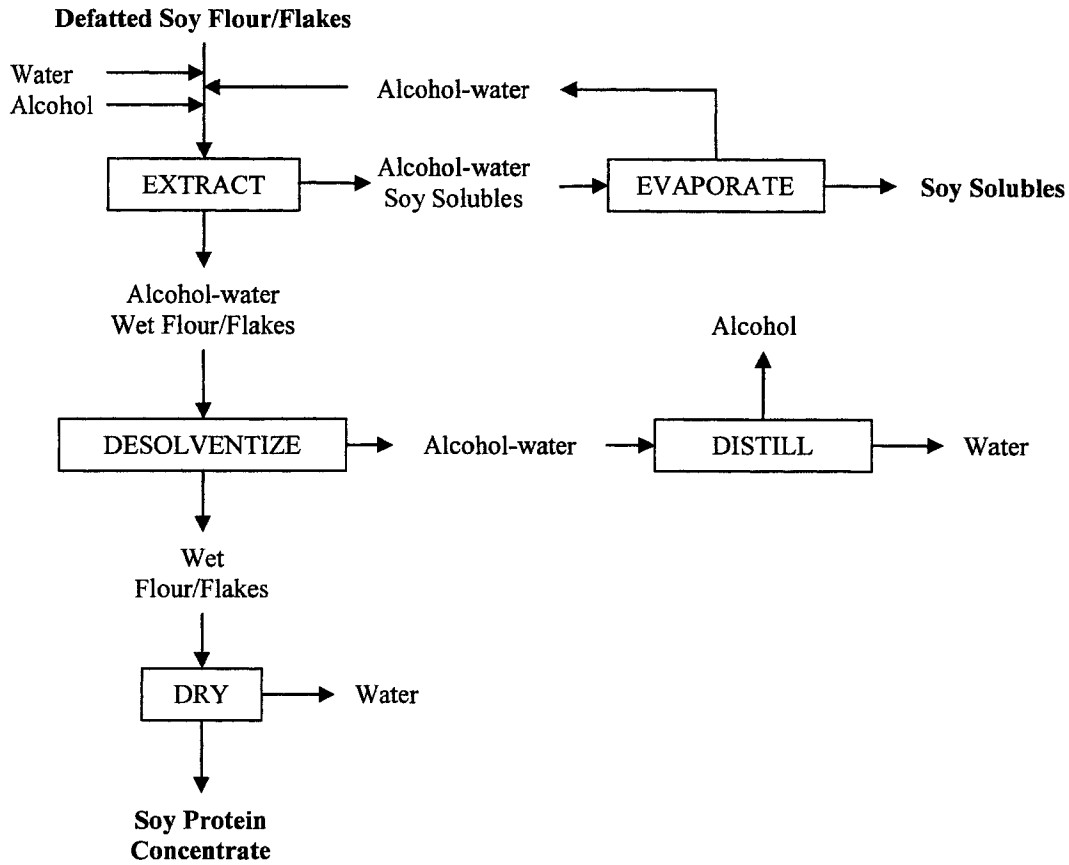


Figure 6 – Ethanol-washed soy protein concentrate flow diagram. Adapted from Lusas and Rhee (1995).

Soy protein isolates

Soy protein isolates (SPIs) contain >90% protein on dry-weight basis (N x 6.25). Commercial yields are approximately 33% of the initial solids, corresponding to approximately 60% of the protein recovered in SPI (Sathe and others 1989). Traditional SPI production is described by Wolf (1983). Defatted soy flakes or flour is extracted with water at 1:10 to 1:20 solids-to-solvent ratio at 60°C under alkaline conditions (pH 8 to 11) adjusted with sodium hydroxide (Fig. 7). The insoluble fiber is removed by centrifuging and the extract is acidified to between pH 4 and 5 with HCl. The resulting slurry is centrifuged and the precipitate (curd) collected is washed and centrifuged again. The washed curd is neutralized with sodium or calcium hydroxide and spray-dried, or dried without neutralization. Some processors may extract the fiber a second time to improve protein yield. High pH and temperatures favor production of lysinoalanine. This compound is formed at

expense of lysine and cysteine in soy protein, diminishing its biological value. Lysinoalanine causes kidney disease (nephrocytomegaly) in rats, while its effect on humans is uncertain (Lusas and Rhee 1995). Soy protein isolates usually contain 90.0 to 92.0% protein, 0.5 to 1.0% fat, 0.1 to 0.2% crude fiber, < 0.2% soluble fiber, < 0.2% insoluble fiber, 4.0 to 5.0% ash, and 3.0 to 4.0% carbohydrates, on a moisture-free basis (Endres 2001).

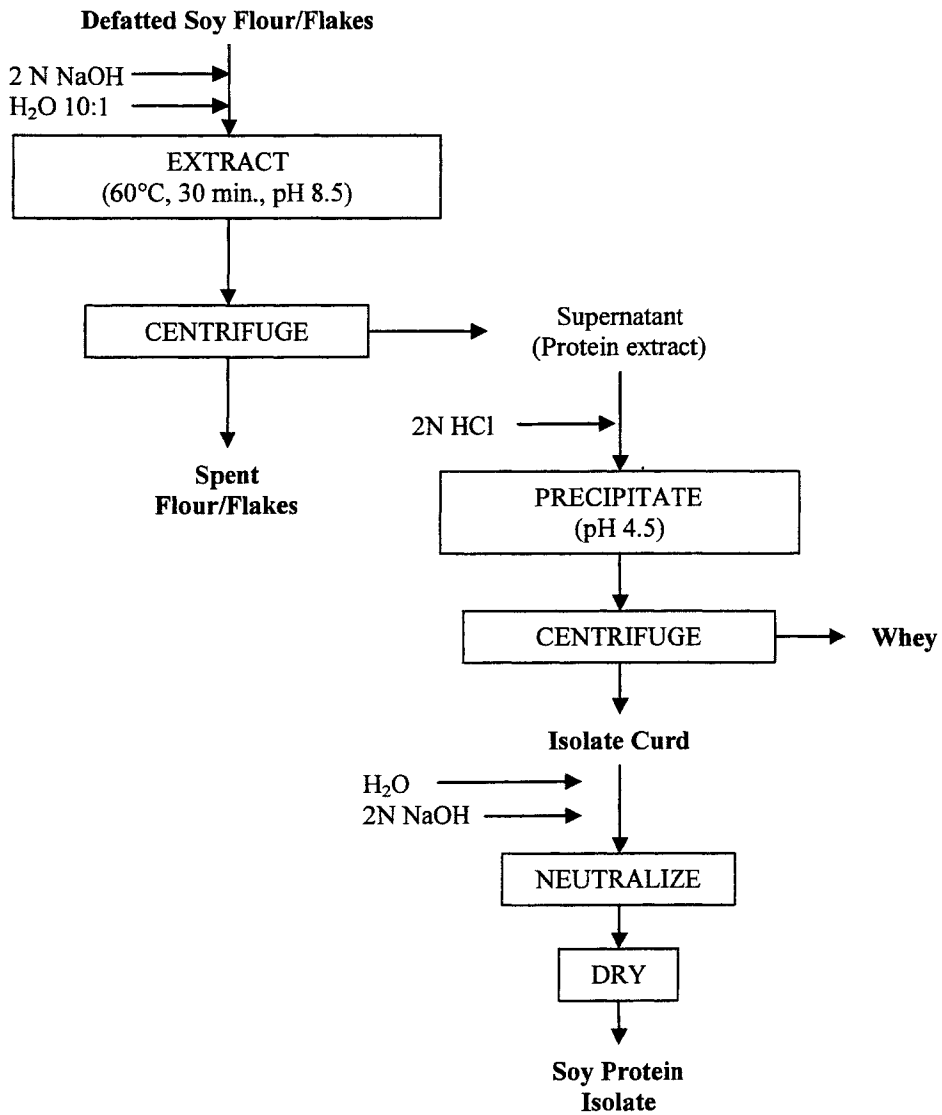


Figure 7 – Soy protein isolate flow diagram

SPI is used as ingredients in high-protein foods including dairy foods, nutritional supplements, meats, infant formulas, nutritional beverages, soups, sauces, and snacks. SPI utilization is based on the wide range of highly desirable functional properties such as solubility, hydrophobicity, emulsification, foaming, fat and water absorption, gelling, and viscosity control.

Some SPI processes may use combinations of salts (Saio and others 1975), addition of reducing agents (Hirotsuka and others 1998), electro-acidification (Bazinet and others 2000), membrane filtration (Lawton and others 1979), hydrothermal processing, enzyme treatments, among others, but specific details of commercial processes are usually not fully disclosed and vary among manufacturers.

Fractionated soy proteins

One of the main bodies of soy foods research has focused on fractionating individual storage proteins (glycinin and β-conglycinin, which comprise nearly 70% of the total protein in soybeans) and relating them to important functional properties and health benefits. While earlier research has focused on obtaining pure glycinin and β-conglycinin to study structure-function relationships, the recent increase in popularity of soy protein is due to its potential health benefits (Messina 1999), which continue to drive soy protein research and commercial development of new soy-protein-based food products and ingredients.

Several laboratory methods to fractionate soy proteins are reported in the literature (Wolf 1956, Roberts and Briggs 1965, Wolf and Sly 1967, Eldridge and Wolf 1967, Koshiyama 1965, 1968a, 1968b, 1972, Thanh and Shibasaki 1976, Saio 1973, 1974, 1975, Nagano and others 1992, Wu and others 1999, and Rickert and others 2004a). One of the first attempts to fractionate soy proteins was by using low temperatures. Wolf (1956) reported the recovery of a glycinin-rich fraction and named it cold-insoluble fraction (CIF). Others described this method as cryoprecipitation and some authors termed "glycinin" as the cryoprotein from soybeans (Wolf and Sly 1967). These methods focused on recovering the glycinin-rich fraction and most authors did not even address the β-conglycinin protein.

Probably the most extensively used laboratory method for simultaneous fractionation of glycinin and β-conglycinin is one described by Thanh and Shibasaki (1976). The

fractionation of these globulins of soy protein was accomplished by extracting soybean meal with Tris-buffer solution containing β-mercaptoethanol at pH 7.8, centrifuging to remove the insoluble material, adjusting the pH of the supernatant to 6.6, dialyzing, centrifuging to produce crude glycinin and β-conglycinin-rich fractions, isoelectrically precipitating the β-conglycinin fraction, washing, and freeze-drying. To complete this purification, column chromatography was required.

Koshiyama (1965) reported on a method utilizing calcium ions to precipitate the remaining glycinin contaminant after obtaining a CIF, obtaining a purified β-conglycinin-rich fraction after subsequent gel filtrations of the crude protein with Sephadex G-100 and G-200. This further purification resulted in an ultracentrifugally pure fraction. The author did not report fraction yields, but in a later study (Koshiyama 1972) on an improved purification method, where the calcium salt was replaced by NaCl at 0.6 M, ultracentrifugally pure β-conglycinin was obtained (after subsequent gel filtrations) yielding 16% of the protein from the starting material. Saio and others (1973, 1974, 1975) reported on a method where calcium salt was added as the extraction buffer and the fiber was first extracted to obtain a β-conglycinin-rich supernatant and the precipitate was redisolved and centrifuged to obtain an glycinin-rich fraction. The glycinin-rich fraction yielded 39% and the β-conglycinin-rich fraction yielded 21.4% of the total protein from the starting soy flour. The purities obtained, on ultra-centrifugal basis, were about 61.4% for the glycinin-rich fraction and 68% for the β-conglycinin fraction (Saio and others 1973). Other experimental methods to fractionate soybean proteins have been reported. Roberts and Briggs (1965) reported a method to obtain 90% ultracentrifugally pure β-conglycinin, after ammonium sulfate precipitation of a soy protein extract and subsequent gel filtration steps, the reported yields for this fraction was only 4% of the β-conglycinin originally present in the extract. Eldridge and Wolf (1967) report on a method to obtain ultracentrifugally pure glycinin after subsequent cold precipitations and gel filtration on Sephadex G-200. Nagano and others (1992) reported a method using three precipitation steps where a soy protein extract was added with sodium bisulfite, pH adjusted to 6.4, and cooled in ice bath to obtain a glycinin-rich fraction, then the resulting supernatant was added with NaCl and pH adjusted to 5 to obtain an intermediate mixture of glycinin and β-conglycinin, the resulting supernatant was diluted with water and

pH adjusted to 4.8 to obtain a β-conglycinin-rich fraction. The yields reported for glycinin and β-conglycinin-rich fraction were 10 and 6% of the starting material, and purities measured by densitometry of SDS-polyacrylamide gel electrophoresis were above 90% for both proteins. This method differs from the methods previously reported (Koshiyama 1965, 1972, Thanh and Shibasaki 1976, Roberts and Briggs 1965) in that it uses simple precipitation steps, does not use further column purification of the individual fractions, and replaces the use of β-mercaptoethanol for sodium bisulfite.

Wu and others (1999) successfully scaled up to pilot plat production scale the method developed by Nagano and others (1992) to obtain kg quantities of the individual storage proteins (Fig. 8). This process was a relatively simple procedure based on differences in solubility behaviors of glycinin and β-conglycinin, with the addition of NaCl to salt-in the β-conglycinin, after precipitation of the glycinin-rich fraction, and carefully adjusting the pH of the resulting supernatant to obtain an intermediate mixture with the objective of precipitating the remaining glycinin in the solution, after the intermediate fraction was precipitated the resulting extract was diluted with water two-fold to salt-out the β-conglycinin present in this extract and pH adjusted to isoelectric point of this protein. Relatively high yields of the individual protein fractions were obtained both at pilot-scale (11.2 and 10.9%) and lab-scale (12.9 and 9.8) for glycinin and β-conglycinin-rich fractions, respectively. The purities of the fractions obtained at pilot-scale were lower than those for lab-scale as measured by urea-SDS-PAGE. The researchers further analyzed the different fractions protein components native state finding that the contaminant β-conglycinin in the glycinin-rich fraction was completely denatured, while only one-half of the contaminant glycinin in the β-conglycinin-rich fraction was denatured. The intermediate fraction's protein components were had the lowest percentage of native structure as analyzed rocket immunoelctrophoresis gels. Later, this fractionation process was improved by decreasing the solvent to flake ration from 15:1 to 10:1 and changing the extraction temperature of the starting material from 20 to 45°C (Rickert and others 2004a) also obtaining three protein fractions, a β-conglycinin-rich, a glycinin-rich, and an intermediate fraction (mixture of the former two proteins along with a significant amount of lipoxygenase). Two waste streams were produced, spent flakes and

whey. Phytochemical recovery (Rickert and others 2004a) and functional properties of the fractions obtained (Rickert and others 2004b) were improved, but purity was not enhanced.

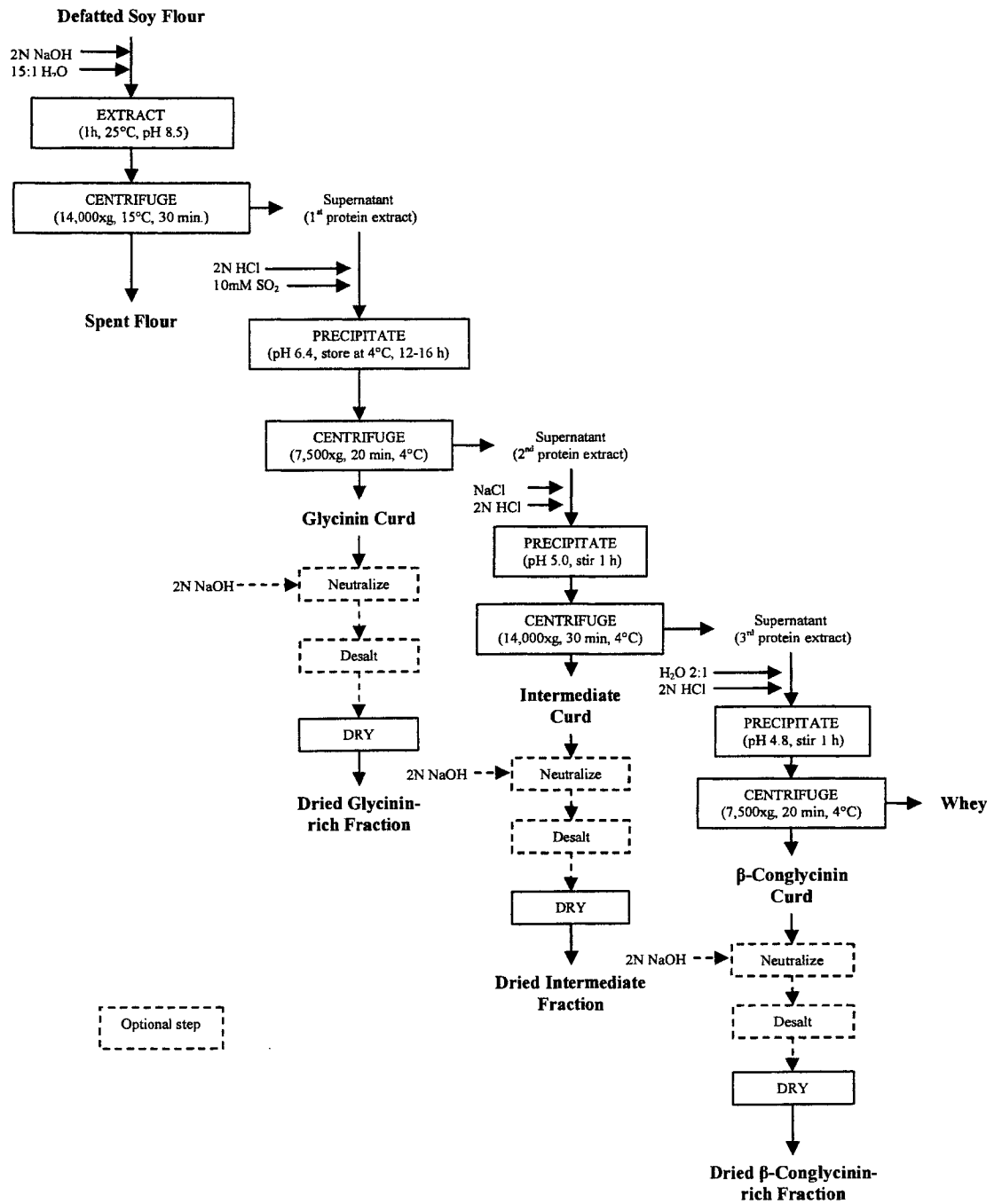


Figure 8 – Three-step soy storage protein fractionation flow diagram. Adapted from Wu and others (1999).

Wu and others (2000) also reported a simplified method to fractionate soy proteins at pilot-scale obtaining two protein fractions, a glycinin-rich and a β-conglycinin-rich by using a membrane filtration step to obtain a β-conglycinin-rich fraction, after previous precipitation of the glycinin-rich fraction. The yield of the β-conglycinin-rich fraction was improved, but at expense of purity. Thiering and others (2001) reported a fractionation method using pressurized carbon dioxide as a volatile electrolyte, and careful adjustments of pH to fractionate a glycinin-rich, a β-conglycinin-rich and an intermediate fractions. They reported a 28% yield for the glycinin-rich fraction with 95% electrophoretic purity and 21% yield for the β-conglycinin-rich fraction with 80% purity.

Recently, Saito and others (2001) reported on a method where soy protein extract was treated with phytase to hydrolyze phytic acid. They hypothesized that the phytate hydrolysis will disrupt the phytate-protein complexes and improve fractionation by means of pH adjustment. After enzyme treatment, two fractions were obtained by adjusting the pH to 6 for precipitating a glycinin-rich fraction and pH 5 for precipitating a β-conglycinin-rich fracition. They reported ~22 and ~ 36% protein yields in their β-conglycinin-rich and glycinin-rich fractions, respectively. The purities for both fractions were about 80% as determined by densitometric analysis after SDS-PAGE.

Several patents claim methods for glycinin and β-conglycinin fractionation. Howard and others (1983) disclosed a method to fractionate soy storage proteins by means of pH adjustments in the presence of sulfite ions and water-soluble salts. Lehnhardt and others (1983) disclosed a method to fractionate glycinin and β-conglycinin from an isoelectrically precipitated mixture of them. Hirotsuka and others (1988) disclosed a method for fractionating soy proteins by reduction and isoelectric precipitation of the proteins achieving ingredients with improved functional properties. Masahiko and others (1994), Samoto and others (1996), Savolainen and others (1999), and Kohno and others (2001) disclosed methods to fractionate soy proteins claiming industrial uses for the fractions obtained. Using a slightly different approach, Bringe (2001) disclosed method to obtain food ingredients with increased proportions of glycinin or β-conglycinin by means of starting with genetically modified lines that are rich in one of the above-mentioned proteins.

Functionality of soy protein ingredients

Broadly speaking, the properties of proteins that determine their uses in foods are collectively called functional properties. "Functional properties denote those physicochemical properties of food proteins that determine their behavior in foods during processing, storage, preparation, and consumption" (Kinsella and others 1985). Furthermore, Pour-El (1981) defined functionality as "any property of a food or food ingredient, except its nutritional ones, that influences its utilization." The specific functional property required depends upon the specific food system in which the ingredient is used and the value of such ingredient results from its effectiveness to deliver such properties. The functionalities of soy protein ingredients have been widely studied and published. The amount of literature available on this subject is vast, yet in some cases contradictory and quite complex to interpret. The main reasons for this is the complexity of soy proteins and protein ingredients including production variables and the lack of widely accepted reference methods to measure protein function.

Factors affecting protein functionality

Several factors affect the functional applicability of proteins. These factors can be grouped into three factors, intrinsic, environmental, and processing factors. There are three major intrinsic factors, composition of protein (structure, amino acid composition), conformation of the protein (native or denatured), and mono- or multi-component (pure or mixture of several different proteins). Environmental factors are pH, oxidation-reduction status, ionic strength, presence of specific salts, water content of the system, and presence of carbohydrates, lipids and surfactants. Processing treatment also influence the protein behavior in the system and factors commonly introduced while processing are heating, drying, pH, ionic strength, presence of reducing agents, storage conditions, and physical, enzymatic or chemical modifications (Kinsella 1979). Due to the number and complexity of these factors and their interactions, a multiplicity of variables have to be taken into account when assessing functionality of a specific protein or group of proteins in a given food system.

Soy protein and drying methods

Freeze-drying is widely used in scientific research when studying proteins. The process involves the removal of bulk water from a frozen protein dispersion by sublimation under vacuum, followed by controlled heating to moderate temperatures to remove the remaining water. Residual moisture levels are often <1%. Freeze-drying is believed to be the best method to stabilize protein functionality (Fagain 1997).

Spray-drying, on the other hand, is the primary method used by food and related industries, especially in the production of milk powder, dairy products, and food ingredients such as SPI. Spray-drying rapidly dries solutions or slurries to particulate form by atomizing the liquid in a heated chamber. Spray-drying typically consists of pre-concentrating the liquid (for more economic operation), atomizing (creation of droplets), drying in a stream of hot, dry gas (usually air), separating the powder from moist gas, cooling, and packaging.

Solubility

Solubility is recognized as one of the most important functional properties of proteins, since most other functional properties will be affected by this functional property. "Protein solubility is, thermodynamically, the protein concentration in the solvent in a single- or two-phase system at the equilibrium state" (Vojdani 1996). All of the above-mentioned factors as well as the amino acid and non-amino acid components of the protein affect solubility behavior of a given protein. A functional protein ingredient must have high solubility in order to be able to have good emulsification, foaming, and gelation properties (Morr and others 1985).

Soy protein solubility has been widely studied and is an important factor in processing soy proteins for several reasons. The extraction and separation of individual soy proteins depend on solubility and how it changes with pH, ionic strength, reducing agents, and calcium ions. In addition, the qualities of soy ingredients are often graded on their NSI and PDI, and have been related to digestibility (Boonvisut and Whitaker 1976, Kella and others 1986). For some end uses, such as high-protein drinks, a highly soluble protein is an important factor of product quality.

Solubility of soy proteins is highly pH-dependent. Soy protein isolates exhibit a typical U shaped curve when solubility is plotted against pH (Fig. 9). This solubility behavior is significantly influenced by a multiplicity of factors. Soy protein isolates were more soluble at their isoelectric point (~4.5) in the presence of 0.5M NaCl than in water. At low pHs this tendency was reversed and the protein without addition of NaCl remained more soluble at pH 2 (~70%) than the salt added (~20%). Solubilities of dialyzed soy protein isolate curds were significantly higher than non-dialyzed ones, especially at pH 6-8 and 2-3 (Shen 1976).

Changes in solubility during processing have been observed for soy protein isolates. Petrucelli and Anon (1994a) noticed that thermal treatments at 98°C lead to solubility loss and correlated this solubility decrease with the degree of aggregation observed by non-denaturing electrophoresis. They also observed that heat treatments at 80 and 92°C had little effect on solubility and attributed these conflicting results to the short time of heating. Boatright and Hettiarachchy (1995) found that spray-dried soy protein isolates were more soluble than freeze-dried ones.

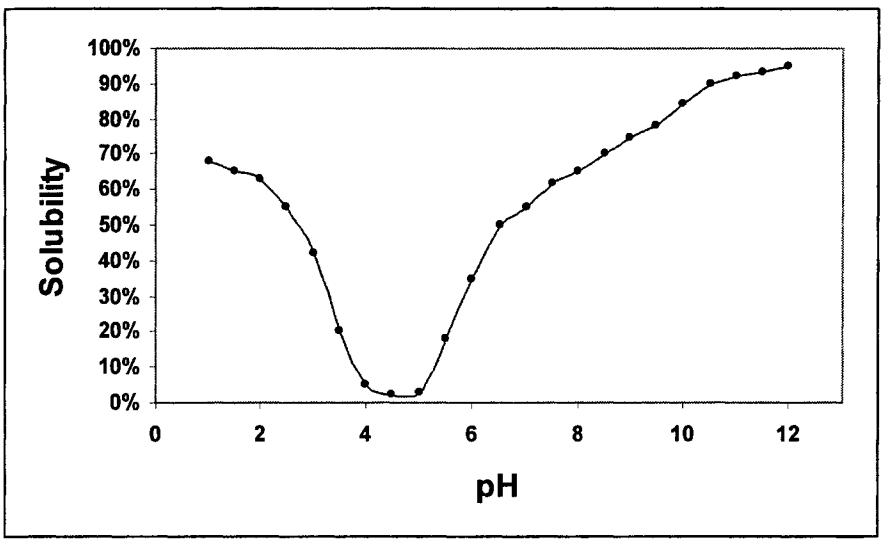


Figure 9- pH-solubility profile of soy protein isolate in water. Aadapted from Shen (1976).

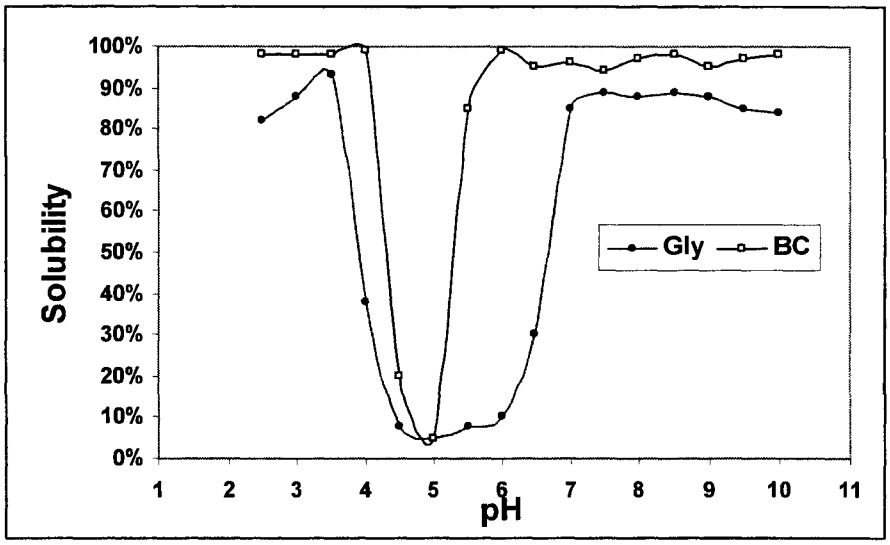


Figure 10- pH-solubility profile of soy glycinin (Gly) and β-conglycinin (BC) in water. Adapted from Yuan and others (2002).

Solubility behavior of the individual storage proteins has also been reported (Yuan and others 2002, Bian and others 2003, Rickert and others 2004b). In a study reported by Yuan and others (2002), the results showed that glycinin precipitated over a much wider pH range, compared to β-conglycinin (Fig. 10). This behavior was attributed to the greater Van der Waals and hydrophobic forces among glycinin molecules. These researchers also observed a salting-in effect for both proteins when NaCl was added to the system at a 0.3M concentration. Similar observations were reported by Bian and others (2003) and Rickert and others (2004b). Dias and others (2003) characterized the solubility behavior of acidic and basic polypeptides isolated from a glycinin sample by using two different reducing agents. While acidic polypeptides were more soluble than the original glycinin, basic polypeptides were highly insoluble in a pH range of 2-10.

Surface hydrophobicity

Many of the molecular and functional properties of food proteins are related to their contents of hydrophobic and hydrophilic amino acids, and their distribution in the primary structure (Damodaran 1988). The amount of hydrophobic regions exposed by a given protein significantly affects intermolecular interactions, such as binding of small ligands, or the association with other macromolecules, including protein-protein or protein-lipid

interactions, which in turn affect surface-active functional properties. Wagner and others (2000) found a positive correlation between solubility and surface hydrophobicity from a set of samples from both commercial and laboratory-prepared soy protein isolates. Although these observations seem contradictory because proteins with high surface hydrophobicity would be expected to aggregate and remain insoluble, the researchers grouped their samples based on both solubility and denaturation degree since the values reported were not observed to follow a sole trend. Furthermore, other considerations, such as presence of denaturants, salts, reducing agents and protein composition of the samples, were needed for a more accurate data interpretation. Contrasting with these results, Hayakawa and Nakai (1985) found a positive correlation between surface hydrophobicity and protein insolubility when Phenil Sephalose and ANS were used as probes. Petruccelli and Anon (1994a) reported on the effect of pH on the surface hydrophobicity of soy protein isolates. Surface hydrophobicity increased with increased pH treatment of the isolates (from ~33 at pH 6 to ~74 at pH 11) as measured with an ANS probe. This behavior was attributed to protein unfolding and subunit dissociation. Scilingo and Anon (2004) found that surface hydrophobicity was reduced with the increased addition of calcium ions to protein isolates and attributed this behavior to the existence of specific calcium-soy protein interactions. Some of this conflicting results may be attributed to the fact that surface hydrophobicity as determined by fluorometry only measure the surface hydrophobicity of the soluble portion of a given protein sample.

Wu and others (1999) reported surface hydrophobicity of glycinin-rich and β-conglycinin-rich soy protein isolates produced at pilot-scale. Glycinin had lower surface hydrophobicity than β-conglycinin, but both proteins had lower hydrophobicities when compared to traditional soy protein isolate made from the same soybeans. Rickert and others (2004b) found that β-conglycinin had lower surface hydrophobicity than glycinin when using the Wu procedure and attributed this difference to thermal behavior. When analyzing the optimized process, glycinin-rich and β-conglycinin-rich fractions were not different from each other but were significantly different from soy protein isolate made from the same soybeans.

Emulsification properties

Proteins are often used to aid emulsion formation and increase emulsion stability in foods. Proteins are much larger and more complex than simple emulsifier molecules. The emulsifying properties of a protein depend on two factors, the ability to reduce interfacial tension because of its adsorption to the interface and the ability to form a film, which would act as an electrostatic, structural, and mechanical barrier (Petruccelli and Anon 1994b). The formation of protein-stabilized emulsions requires that the protein molecule migrate to the water/lipid interface and unfold so that its hydrophobic regions can contact the lipid phase (Wagner and Gueguen 1999b). In order to achieve this, protein molecules must have both hydrophilic and hydrophobic regions and retain flexibility in order to unfold. Emulsions are thermodynamically unstable. Once formed, an emulsion can undergo a number of changes. It is of interest to know not only how efficient a protein dispersion is in emulsifying but also the stability of the resulting emulsion. Emulsion formation depends on a fast desorption, unfolding in the interface, and reorientation, whereas stability is determined by a decrease of the interface free energy and also by the film rheological properties (Petrucelli and Anon 1994b).

The type and method of protein preparation affect the formation and stability of emulsions. Soy protein isolates are superior to soy protein concentrates. The pH and ionic strength of the aqueous phase affect the emulsifying properties of soy proteins. The emulsification properties of soy protein isolate as well as isolated glycinin and β-conglycinin showed typical responses to pH, following solubility profile and the maximum emulsifying activity was found to be at pH 10 (Aoki and others 1980). Petruccelli and Anon (1994b) found better emulsifying properties for soy protein isolates at pH 9 than at pH 7, and that protein isolates enriched in β-conglycinin emulsified significantly better than did traditional isolates and glycinin-rich isolates. Wagner and Gueguen (1999b) found a positive correlation between emulsification stability and surface hydrophobicity in a set of glycinin samples that had been modified either by heat treatment under mild acidic condition with or without disulfide bond reduction. These researchers reported that when disulfide bond reduction was induced, glycinin was a poor emulsifier, even at high ionic strength, but the

same samples exhibited high emulsification stability. From this study, it was concluded that emulsification capacity and stability are controlled by different mechanisms.

Bian and others (2003) studied the influence of pH on emulsification capacity of glycinin-rich and β-conglycinin-rich fractions. Interestingly, the glycinin-rich fraction exhibited maximum emulsification capacity at pH 2 and emulsified significantly more oil than did the β-conglycinin-rich fraction. In contrast, at pH 7 and 9.5, β-conglycinin emulsified more oil than did glycinin and the emulsion formed was more stable. Similar observations were reported by Rickert and others (2004b) while comparing glycinin-rich and β-conglycinin-rich fractions from two different processes. Dias and others (2003) reported on the emulsification behavior of reduced acidic and basic polypeptides from a glycinin-rich fraction. The original glycinin, the reduced basic polypeptides and the high molecular weight acidic polypeptides emulsified similar amounts of oil, whereas the low-molecular-weight acidic subunits emulsified significantly higher amounts of oil per g of protein.

Foaming properties

Proteins are polymers of amino acids that have hydrophilic and hydrophobic side chains. The amphipathic character that these side chains confer to proteins is responsible for their adsorption at interfaces. To form foam efficiently, a protein needs to adsorb rapidly during the transient stage of foam formation. The adsorption of proteins at interfaces is controlled by three processes, the transport from bulk solution to the interface, penetration into the surface layer, and reorganization of the protein structure in the adsorbed layer.

Two types of interfacial deformation are relevant for protein mediated foam formation, interactions between proteins and the surface density of the proteins present at the surface. Foams are subjected to destabilization processes like disproportionation, drainage, and coalescence. A gel-like adsorbed film with a finite yield stress can slow disproportionation. During film drainage surface shear rheological properties seem to be important because a correlation was found between surface rheological properties and foam properties. The higher the apparent surface shear viscosity, the slower drainage and the more stable the foam (Prins 1999). A surface tension gradient is prerequisite for stability against coalescence during foaming because coalescence requires film rupture and the rate of rupture

depends on the film thickness and on its mechanical properties, in particular on the stress at which the film breaks (Wilde 2000).

Wagner and Gueguen (1999a) studied the foaming properties of native and chemically modified glycinin. Glycinin was modified by means of combined treatments of cold or hot acidic treatment with or without disulfide bridges reduction. A positive relationship was observed between surface behavior and foaming properties. Dissociation, deamidation, and reduction produced structural changes on glycinin (increased surface hydrophobicity, increased net charge, decreased molecular size) which enhanced the adsorption and anchorage of proteins at the air-water interface and, as a consequence improved the foam forming and stability.

Bian and others (2003) studied the foam forming capacity, stability and rate of foaming of glycinin-rich and β-conglycinin-rich isolates. They found no differences in foaming capacities between fractions produced by using a laboratory procedure, but foaming capacity of a glycinin-rich fraction produced at pilot-scale was higher than that of the β-conglycinin fraction produced by the same procedure. For both procedures reported, the β-conglycinin-rich fraction formed more stable foams and was faster forming them, compared to the glycinin-rich fraction. In contrast, Rickert and others (2004b) comparing individual storage protein fractions produced at pilot plant scale, found that, in general, the glycinin-rich fraction was a better foaming agent than the β-conglycinin-rich fraction.

Viscosity

Viscosity can be defined as the resistance of a protein dispersion to flow. Fluids may be studied by subjecting them to continuous shearing at a constant rate. The concentration and inherent physicochemical properties and conformations of each protein species affect the viscosity of the protein solution. Viscosity is an important functional property of fluid foods, such as beverages and batters, and the design of processing lines (Snyder and Kwon 1987). Rheology is often the only means that may be used to describe with confidence the state or performance of complex food systems such as soy protein isolates (Wagner and others 1992).

Petruccelli and Anon (1994a) studying the relationships between processing parameters and the structural and functional properties of soy protein isolates found that

solubility correlated negatively with viscosity. Wagner and others (1992) studied the influence of different factors (degree of denaturation and aggregation due to processing treatments, responses to NaCl and Na_2SO_3 addition) on viscosity and rheological behavior of commercial soy isolates. Thermal treatment lead to higher viscosity in the protein dispersions, the increase in viscosity was observed even in dispersions that were previously treated with salt addition. Additions of salt and reducing agent affected water imbibing capacity and, as a consequence, the viscosity. Salt and reducing agent lead to decreased viscosity, especially in samples that were previously heated. Increased protein concentrations significantly increased viscosity of the isolated soy protein dispersions. Apparent viscosity correlated positively with water imbibing capacity.

Bian and others (2003) found that β-conglycinin dispersions resulted were more viscous when compared to glycinin dispersions. This observation was valid for all concentrations (5, 7, and 9%), temperatures (5, 25, and 50°C), and pHs (3, 6, and 9) tested. In contrast, Rickert and others (2004b) measuring dynamic viscosities of glycinin-rich and β-conglycinin-rich fractions found no differences. Dias and others (2003) compared viscosities of low- and high-molecular-weight acidic polypeptides and basic polypeptides produced from a glycinin-rich fraction by two different reducing treatments (β-mercaptoethanol and sodium bisulfite as reducing agents). The highest viscosity was observed for the basic polypeptides produced by sodium bisulfite treatment. Comparing all the rest of the samples the high-molecular-weight acidic polypeptides were the most viscous. The polypeptides produced with sodium bisulfite treatment were consistently more viscous than those produced with β-mercaptoethanol.

Other important functional properties

Gelation or gel formation is a protein aggregation phenomenon in which attractive and repulsive forces are balanced and a well-ordered tertiary network of matrix is formed, which is capable of holding significant amounts of water (Hermansson 1978). Gelation consists of two steps: 1. conformational change or partial denaturation of protein molecules and 2. gradual association or aggregation of protein molecules (Matsumura and Mori 1996).

Water- and fat-holding capacities are affected by protein composition and conformation. Both of these properties are of utmost importance in food systems, since they not only determine the acceptability of a food but also profit margin (Barbut 1996).

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CHAPTER 2. EFFECTS OF REDUCING AGENT CONCENTRATION ON SOY PROTEIN FRACTIONATION AND FUNCTIONALITY

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Abstract

The concentration of the reducing agent SO_2 significantly affected fraction yields, purities, and compositions during soy protein fractionation, especially the purity of the glycinin-rich fraction. The optimum amount of reducing agent was 5 mM SO_2 when considering protein yield, purity and functional properties. With no SO_2, the glycinin-rich fraction contained 28.8% of the total protein with only 63.4% glycinin, and the β-conglycinin-rich fraction contained 10.4% of the total protein with 93.5% β-conglycinin; whereas, by adding 5 mM SO_2,the glycinin-rich fraction contained 23.4% of total protein with 81.5% glycinin, and the β-conglycinin-rich fraction contained 16.8% of the total protein with 83.7% β-conglycinin. Increasing amounts of storage proteins were lost in the whey fraction as SO_2 concentration increased. The thermal behaviors of the fractions were only slightly affected by using higher amounts of SO_2. The functional properties of the two major fractions were greatly influenced by the addition of SO_2. The solubility and hydrophobicity of the glycinin-rich fraction decreased with increasing SO_2 concentration, whereas the solubility of the β-conglycinin-rich fraction increased. Emulsification properties of the glycinin-rich fraction were adversely affected by higher SO_2 concentrations, whereas, the β-conglycinin-rich fraction was improved. Maximum foaming properties were observed at 5 mM SO_2. The β-conglycinin-rich fractions had better emulsification properties than did the glycinin-rich fractions.

Introduction

Soybean seeds contain between 35 and 46% protein at maturity (Nagano and others 1996). This protein is a heterogeneous group that may be classified in terms of their biological function (metabolic and storage proteins), solubilities, or sedimentation rate in fractional centrifugation (Thiering and others 2001). The two major storage proteins, the 7S

globulins or β-conglycinin (37-39% of total protein) and 11S globulins or glycinin (31-44% of total protein) have different intrinsic properties giving rise to different functional behaviors (Bazinet and others 2000). Considerable efforts have been made to fractionate these two proteins into relatively pure fractions in order to study their inherent properties and evaluate their potential food and industrial applications. There are no commercially available soy protein fractions today, however, because the fractionation procedures are expensive and small changes during processing cause considerable variation in properties of the fractions produced.

One successful procedure was first described by Nagano and others (1992) and later modified by Wu and others (1999), and recently further improved by Rickert and others (2004a). This procedure is relatively simple and based on extracting the protein from defatted soybeans by alkali leaching and precipitating the different proteins by precisely changing pH and ionic strength of the medium. This process yields three different fractions, a glycinin-rich fraction, a β-conglycinin-rich fraction, and an intermediate fraction as a mixture of both proteins. One key factor for successful fractionation is the addition of a reducing agent prior to the first protein precipitation (glycinin). The reducing agent of choice is SO_2, which is added in the form of $NaHSO_3$.

The reducing agent is believed to prevent co-precipitation of glycinin and β-conglycinin (Thiering and others 2001). Wolf (1993) concluded that adding a reducing agent to precipitate glycinin significantly improved this fraction's purity when using the fractionation method described by Thanh and Shibasaki (1978). Suitable reducing agents for use as protein fractionation aids are any sulfite compound that yields SO_2 in solution, glutathione, cysteine, and β-mercaptoethanol. It is also recognized that adding a reducing agent during fractionation may modify the functional properties of the protein. Although reducing agents are widely used in protein fractionation procedures, their precise mechanism of action is unknown.

Kim and Kinsella (1987) found that the exposing soy glycinin to a reducing agent improved its surface-active properties. Abtahi and Aminlari (1997) found that solubility increased with added reducing agents to a soybean milk base and attributed this effect to the cleavage of disulfide bonds. Boonvisut and Whitaker (1976) and Kella and others (1986)

found that cleaving disulfide bonds also improved solubility, surface hydrophobicity and, as a consequence, *in-vitro* digestibility of soy proteins. Further studies have been conducted by Petrucelli and Anon (1995) and Wagner and Gueguen (1995, 1999a, 1999b) in which they related altered surface activity to reduction of disulfide bonds. In general, the above mentioned studies started with isolated protein products and subjected them to a reducing treatment without considering other processing factors such as previous thermal history, changes in ionic strength and pH. The objectives of the present study were to evaluate the addition of $NaHSO_3$ during soy protein fractionation and characterize the resulting products by determining the effects of SO_2 concentration on yields, purity, and key functional properties.

Materials and Methods

Soy flour

Soy protein fractions were produced from air-desolventized, hexane-defatted white flakes (IA 2020 variety, 1999 harvest) produced in the extraction pilot plant of the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). The defatted flakes were milled with a Krups grinder (Distrito Federat, Mexico) to achieve 100% of the material passing through a 50-mesh screen by using small quantities (10 g) to preserve the native protein state. The protein content of the flour was 57.3% on a dry-weight basis with a protein dispersibility index (PDI) of 93.8 as determined by Silliker Laboratories (Minnetonka, MN). The flour was stored in sealed containers at 4°C until used.

Protein fractionation

The basis for the soy protein fractionation procedure utilized in this study has been reported by Wu and others (1999) and is a modification to methods of Nagano and others (1992). About 80 g of defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 1 h at 25°C. After centrifuging at 14,300 x g and 15°C for 30 min, the protein extract was decanted and the amount of insoluble fiber residue was determined and sampled for proximate composition. Sufficient $NaHSO_3$ was added to the protein extract to achieve a

range of SO_2 concentrations (0, 5, 10, 20, and 30 mM), and the pH of the extract was adjusted to 6.4 with 2N HCl. The resulting slurry was stored at 4°C for 12-16 h and centrifuged at 7,500 x g and 4°C for 20 min. A glycinin-rich fraction was obtained as a precipitated curd. This fraction was redisolved in de-ionized water and adjusted to pH 7 with 2N NaOH, sampled, and stored in sealed containers at -80°C until freeze-drying. To the supernatant (protein extract), NaCl was added to achieve 0.25 M, the pH was adjusted to 5.0 with 2N HCl, and the slurry was stirred for 1 h. The slurry was then centrifuged at 14,000 x g and 4°C for 30 min. An intermediate fraction, a mixture of glycinin and β-conglycinin, was obtained as the precipitated curd; this fraction was treated as described above. The supernatant (protein extract) was combined with de-ionized water to achieve a three-fold increase in volume and the pH was adjusted to 4.8. The slurry was centrifuged at 7,500 x g and 4°C for 20 min. A β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as described above, and the amount of supernatant (whey) was determined and sampled for proximate composition. Each SO_2 treatment was replicated three times and means reported.

Proximate analyses and mass balances

Nitrogen contents of the soy flour, isolated protein fractions and byproduct streams were measured by using the combustion or Dumas method (AOAC 1995a) with a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). The nitrogen values were converted to Kjeldahl nitrogen by using the conversion formula of Jung and others (2003). All measurements were determined at least three times and means reported. The factor used to convert percentage nitrogen to protein content was 6.25. Moisture was determined by oven-drying for 3 h at 130°C (AOAC 1995b). Mass balances of solids and protein were determined. Analyses were replicated in triplicate and means reported.

Protein profile and subunit composition

Urea-sodium dodecylsulfate-polyacrylamide gel electrophoresis (urea-SDS-PAGE) was performed using the methods of Rickert and others (2004a) to quantify the protein component profiles of the fractions. Soybean storage proteins were identified by comparing

to a pre-stained SDS-PAGE molecular-weight standard, low range (Bio-Rad Laboratories, Hercules, CA). Glycinin and β-conglycinin subunit bands were confirmed by using purified standards produced according to methods of O'Keefe and others (1991). Densitometry was carried out by using the Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). SDS-PAGE results were calculated as % composition; total storage protein in a given fraction = [(sum of storage protein subunit bands)/(sum of all bands)] x 100, fraction purity/composition = [(sum of subunit bands)/(sum of storage protein bands)], and subunit composition of a specific protein = [(subunit band)/(sum of subunits for the specific protein)]. All analyses were replicated at least four times and means reported.

Thermal behavior

Thermal behaviors of the isolated proteins were assessed by using differential scanning calorimetry (DSC). Sample dispersions (15-20 mg) of 10% (w/w, dry basis) protein were hermetically sealed in aluminum pans. A sealed empty pan was used as reference. The samples were heated from 25 to 120°C at 10°C/min using an SII Exstar 6000 (Seiko Instrument, Inc., Tokyo, Japan). All samples were analyzed at least three times and means were reported.

Solubility

Solubility was evaluated according to methods of Rickert and others (2004b). The samples were tested at pH 7.0. Solubility was calculated as: % Solubility = (amount of protein in supernatant/amount of initial protein in the sample) x 100. All samples were analyzed at least three times and means reported.

Surface hydrophobicity

Surface hydrophobicity was measured by using methods of Wu and others (1999) with modifications. Protein dispersions were prepared as in the solubility test and aliquots of the soluble protein (supernatant) were serially diluted to obtain 6.25 to 100 μg/mL protein concentrations with 0.1 M phosphate buffer (pH 7.0) as diluent. To 3-mL aliquots of each

dilution, 40 μL of 1-anilino-8-naphthalene sulfonic acid magnesium salt monohydrate (ANS, ICN Biomedicals, Inc., Aurora, OH) (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was dispersed. Fluorescence intensities (FI) were measured by using a Turner Quantech® spectrophotometer (Barnstead Thermolyne, Dubuque, IA) with 440- (excitation) and 535-nm (emission) filters. FI were standardized using a solution of 40 μL of ANS in 3 mL of phosphate buffer as the zero point and 15 μL of ANS in 3 mL of methanol assigned an arbitrary value of 80 FI. FIs were plotted against percentage protein concentration. The slope of the regression line was reported as surface hydrophobicity. Samples were run in triplicate and means reported.

Emulsification properties

Emulsification capacity was measured according to the methods of Bian and others (2003) with modifications. Twenty-five mL of a 2% (w/w, dry basis) sample dispersion adjusted to pH 7.0 with 2 N HCl or NaOH was transferred to a 400-mL plastic beaker. Soybean oil dyed with approximately 4 μg/mL Sudan Red 7B (Sigma, St. Louis, MO) was continuously blended into the dispersion at 37 mL/min flow rate by using a Bamix wand mixer (ESGE AG Model 120, Mettlen, Switzerland) at the low setting until phase inversion. Emulsification capacity (g oil/g sample) was calculated as g of oil used to cause inversion multiplied by 2. Emulsification activity and emulsification stability index were measured according to methods of Rickert and others (2004b). All analyses were replicated at least three times and means reported.

Foaming properties

Foaming properties were measured according to methods of Sorgentini and others (1995) with modifications developed by Rickert and others (2004b). A 0.5% (w/w, dry basis) sample dispersion was prepared and the pH adjusted to 7.0. A 95-mL aliquot was loaded into a custom-designed glass column (58.5 cm x 2 cm) with a coarse fritted glass at the bottom, and N_2 was purged through the sample at 100 mL/min flow rate. Time for the foam to reach 300-mL volume, time for one-half of the liquid incorporated into the foam to drain back, and

volume of the liquid incorporated into the foam were measured. Three parameters were calculated:

Foaming capacity (FC) = $V_f/(f_r \times t_f)$

Specific rate constant of drainage (K) = $1/(V_{max} \times t_{1/2})$

Rate of liquid conversion to foam (V_i) = V_{max}/t_f

where V_f = a fixed volume of 300 mL, f_r = the flow rate of the gas, t_f = time to reach V_f, V_{max} = volume of liquid incorporated into foam, and $t_{1/2}$ = time to drain one-half of the liquid incorporated into the foam. Samples were run in triplicate and means reported.

Statistical analysis

The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM), and Least Significant Differences (LSD) were calculated at the 5% level to compare treatment means using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

Results and Discussion

Mass balances and fraction yields

All five SO_2 treatments were successful in obtaining three different fractions, a glycinin-rich fraction, a β-conglycinin-rich fraction, and an intermediate mixture of both proteins (Table 1). The protein contents for all glycinin-rich and β-conglycinin-rich fractions exceeded 90%, with very little variability among treatments for a given fraction. The protein contents of the intermediate fractions were about 80% for all treatments. The protein curds of each fraction had unique characteristics. At 0 mM SO_2, the curd of the glycinin-rich fraction was a white, chalk-like, loose precipitate that was quite difficult to decant, while the curd of the β-conglycinin-rich fraction for the same treatment was a very compact, rubbery textured precipitate that was very difficult to re-dissolve. As the amount of SO_2 increased, the curd of the glycinin-rich fraction became increasingly easier to manipulate at low temperatures, somewhat runny and more yellow in color. At the same time the curds of the β-conglycinin-rich fraction were compact, white, and easy to manipulate and re-dissolve. There were no observable differences for the intermediate fractions at different SO_2 concentrations and the intermediate curds were usually tan in color.

Table 1-Fraction yields and storage protein compositions of starting materials and fractionated products

Fraction/ Treatment	Fraction Yield (%)		Storage Protein Content and Composition (%)		
	Solids	Protein	Total	β-conglycinin	Glycinin
Defatted Flour	100.0	100.0	72.9 ± 0.8	39.4 ± 0.5	60.6 ± 0.5
Protein Extract	68.4 ± 1.4	74.2 ± 2.2	79.6 ± 0.6	41.7 ± 1.0	58.3 ± 1.0
Glycinin-rich Fraction					
0 mM SO_2	16.5^{a}	28.8^{a}	75.1^{c}	36.6^{a}	63.4^{c}
5 mM SO_2	12.6^{b}	23.4^{b}	84.7^{b}	18.5^{b}	81.5^{b}
10 mM SO_2	13.8^{b}	23.1^{b}	91.8^{a}	17.4^{b}	82.6^{b}
20 mM SO_2	13.8^{b}	26.9^{a}	84.4^{b}	15.2^{b}	84.8^{b}
30 mM SO_2	13.3^{b}	27.5^{a}	84.8^{b}	7.0^{c}	93.0^{a}
LSD	1.9	3.3	2.1	4.5	4.5
Intermediate Fraction					
0 mM SO_2	17.7^{a}	28.5^{a}	70.2^{c}	56.8^{a}	43.2^{c}
5 mM SO_2	17.9^{a}	$27.2^{a,b}$	79.3^{a}	39.2^{c}	60.8^{a}
10 mM SO_2	18.1^{a}	$26.4^{a,b}$	78.1^{a}	40.2^{c}	59.8^{a}
20 mM SO_2	12.4^{b}	23.7^{b}	74.4^{b}	45.2^{b}	54.8^{b}
30 mM SO_2	13.6^{b}	23.1^{b}	74.3^{b}	45.1^{b}	54.9^{b}
LSD	2.3	4.2	3.5	4.5	4.5
β-Conglycinin-rich Fraction					
0 mM SO_2	8.1^{a}	10.4^{c}	87.7^{b}	93.5^{a}	6.5^{b}
5 mM SO_2	10.3^{a}	16.8^{a}	87.3^{b}	83.7^{b}	16.3^{a}
10 mM SO_2	10.7^{a}	18.4^{a}	87.9^{b}	83.9^{b}	16.1^{a}
20 mM SO_2	9.3^{a}	13.8^{b}	89.6^{b}	84.8^{b}	15.2^{a}
30 mM SO_2	7.7^{b}	$13.1^{b,c}$	100.0^{a}	83.5^{b}	16.5^{a}
LSD	2.8	3.3	2.6	1.6	1.6
Whey					
0 mM SO_2	35.4^{a}	8.7^{b}	66.0^{d}	25.8^{c}	74.2^{a}
5 mM SO_2	34.3^{a}	$11.8^{a,b}$	$67.3^{c,d}$	$26.8^{b,c}$	$73.2^{a,b}$
10 mM SO_2	35.2^{a}	12.4^{a}	67.8^{c}	25.5^{c}	74.5^{a}
20 mM SO_2	36.1^{a}	13.5^{a}	72.9^{b}	$29.7^{a,b}$	$70.3^{b,c}$
30 mM SO_2	35.9^{a}	14.6^{a}	83.2^{a}	30.2^{a}	69.8^{c}
LSD	3.3	3.5	1.4	3.7	3.7

LSD denotes least significant difference; means within each fraction followed by different superscripts are statistically different. n=3.

The addition of SO_2 significantly affected both yields of solids and total protein in the various fractions (Table 1). For the glycinin-rich fraction, yields of solids and total protein were highest when no SO_2 was used but the purity was low. Very little SO_2 was needed to significantly improve purity of the glycinin-rich fraction. There were no significant differences among the different levels of SO_2 for yields of solids in the glycinin-rich fraction. In contrast, the total protein yields of the glycinin-rich fractions initially dropped with added SO_2 and then increased as SO_2 concentration increased (20 and 30 mM). The initial drop in yields (from 0 mM to 10 mM SO_2) could be attributed to significantly improved purities of these fractions when sulfites were used (Table 1). The consequential increase in protein yields of the glycinin-rich fraction (between 5-10 mM and 20-30 mM) was attributed to increased purity at 20-30 mM SO_2.

At higher SO_2 concentrations in the protein extract, the glycinin subunit composition of the glycinin-rich fraction became enriched in basic polypeptides indicating that β-conglycinin was associated with the glycinin acidic subunits and increased amounts of sulfites disrupted this association (Table 2). This behavior was counter-intuitive since increasing amounts of reducing agent should cleave more disulfide bonds favoring glycinin solubility (Kella and others 1985), but is in agreement with observations of Damodaran and Kinsella (1982) where once acidic and basic polypeptides are separated by reduction, the basic polypeptides aggregate and precipitate, while the acidic ones remain in solution. Another possible explanation for this phenomenon is that cold precipitation of the glycinin subunits is retained even though they exist as individual polypeptides (Wolf 1993).

The yields of the β-conglycinin-rich fractions were also affected by higher concentrations of SO_2 (Table 1). Excluding the treatment without SO_2 that yielded low solids and very low protein, low SO_2 concentrations (5 and 10 mM) yielded higher amounts of total protein than did higher SO_2 concentrations (20 and 30 mM), but only the 30mM SO_2 treatment yielded significantly less solids.

The intermediate fraction (a mixture of glycinin and β-conglycinin) was also affected by the addition of SO_2 having significantly lower yields of solids at 20 and 30 mM SO_2 (Table 1). Similar yields of solids and protein were observed in the intermediate fractions of the 0, 5, and 10 mM treatments. The 20 and 30 mM SO_2 treatments yielded significantly less

Table 2-Subunit compositions of the starting materials and soy protein fractions

Fraction/ Treatment	β-Conglycinin Subunit Composition (%)			Glycinin Subunit Composition (%)	
	α'	α	β	Acidic	Basic
Defatted Flour	34.7 ± 0.6	34.3 ± 0.9	31.0 ± 0.2	59.9 ± 0.7	40.1 ± 0.7
Protein Extract	31.6 ± 0.1	33.4 ± 1.6	35.0 ± 1.5	54.2 ± 1.4	45.8 ± 1.4
Glycinin-rich Fraction					
0 mM SO_2	20.6^{a}	25.8^{c}	53.6^{c}	56.0^{a}	44.0^{c}
5 mM SO_2	0.0^{b}	43.5^{a}	56.5^{c}	55.7^{a}	44.3^{c}
10 mM SO_2	0.0^{b}	43.3^{a}	56.7^{c}	48.1^{b}	51.9^{b}
20 mM SO_2	0.0^{b}	37.1^{b}	62.9^{b}	42.4^{b,c}	57.6^{a,b}
30 mM SO_2	0.0^{b}	0.0^{d}	100.0^{a}	39.4^{c}	60.6^{a}
LSD	0.7	4.9	5.1	6.8	6.8
Intermediate Fraction					
0 mM SO_2	28.3^{a}	32.0^{c}	39.7^{b}	41.3^{b}	58.7^{b}
5 mM SO_2	25.6^{a,b}	38.2^{a}	36.2^{c}	34.0^{c}	66.0^{a}
10 mM SO_2	24.6^{b}	36.0^{b}	39.4^{b}	29.3^{c}	70.7^{a}
20 mM SO_2	23.3^{b}	30.8^{c}	45.9^{a}	43.6^{b}	56.4^{b}
30 mM SO_2	24.9^{b}	30.9^{c}	44.2^{a}	49.0^{a}	51.0^{c}
LSD	3.4	4.0	2.4	4.9	4.9
β-Conglycinin-rich Fraction					
0 mM SO_2	31.6^{a,b}	33.4^{a,b}	35.0^{b}	0.0^{e}	100.0^{a}
5 mM SO_2	28.7^{b}	36.7^{a}	34.6^{b}	33.6^{d}	66.4^{b}
10 mM SO_2	28.8^{b}	37.1^{a}	34.1^{b}	45.5^{c}	54.5^{c}
20 mM SO_2	32.3^{a}	34.8^{a,b}	32.9^{b}	58.2^{b}	41.8^{d}
30 mM SO_2	30.7^{a,b}	31.7^{b}	37.6^{a}	62.8^{a}	37.2^{e}
LSD	2.9	3.9	2.3	2.3	2.3
Whey					
0 mM SO_2	0.0	43.2^{b}	56.8^{a}	46.4^{a,b}	53.6^{a,b}
5 mM SO_2	0.0	50.1^{a}	49.9^{b}	44.2^{b}	55.8^{a}
10 mM SO_2	0.0	46.8^{a,b}	53.2^{a,b}	44.2^{b}	55.8^{a}
20 mM SO_2	0.0	45.5^{a,b}	54.5^{a,b}	49.7^{a}	50.3^{b}
30 mM SO_2	0.0	50.1^{a}	49.9^{b}	49.5^{a}	50.5^{b}
LSD	ND	4.9	4.9	3.5	3.5

LSD denotes least significant difference; means within each fraction followed by different superscripts are statistically different. N=3.

total protein compared to 0 mM SO_2, but similar amounts to 5 and 10 mM SO_2.

There were no differences in solids loss to the whey fraction (a waste stream in this process) among all treatments (Table 1). Larger amounts of protein were lost to the whey fraction at10, 20, or 30 mM SO_2. Protein losses tended to increase as SO_2 increased (significant at α=0.1 with and LSD of 1.6 but not significant at α=0.05), which was consistent with the trend of higher solubility at higher SO_2 concentration. This loss was mainly due to increased amounts of storage proteins going with the whey.

The glycinin-rich and intermediate fractions had approximately similar yields of solids and protein (14 and 15.9%, and 25.9 and 25.8%, respectively), whereas the yields of solids and protein of the β-conglycinin-rich fractions were less (9.2 and 14.5%, respectively), which is consistent with the lower amount of β-conglycinin in the defatted flour (Table 1). When pooling all protein fractions for a given treatment (sum of glycinin-rich + intermediate + β-conglycinin-rich fractions), less solids and protein were recovered as the SO_2 concentration increased. The combined yields of solids decreased from 42.3% at 0 mM SO_2 to 34.6% at 30 mM SO_2; the combined protein yield decreased from 67.7% at 0 mM SO_2 to 63.7% at 30 mM SO_2.

Nagano and others (1992) reported the yields of solids for their glycinin-rich and β-conglycinin-rich fractions to be 10 and 6%, respectively. Wu and others (1999) reported solids yields of 11-12.5% and 10-11% for glycinin-rich and β-conglycinin-rich fractions, respectively, for a similar procedure, but at pilot plant scale. Rickert and others (2004a) reported solids content for their glycinin-rich and β-conglycinin-rich fractions 12.5 and 14%, and 7.7 and 13.6%, respectively, for two different processes. The second amount corresponded to what they called "optimized" Wu procedure (changing Wu's extraction temperature from 25 to 45°C and the flake-to-solvent ratio from 15:1 to 10:1). Our yields of solids for the glycinin-rich fractions were similar to those of Wu and others (1999) and Rickert and others (2004a) but higher than those of Nagano and others (1992). Our solids yields for the β-conglycinin-rich fractions were also similar to those previously reported for this procedure, but significantly lower than those reported by Nagano and others (1992). When comparing the yields of solids and protein of our intermediate fractions to those reported by Wu and others and Rickert and others, we obtained significantly higher amounts

(almost twice as much). The rationale to explain this behavior is somewhat difficult since Nagano and others did not report their yields for this fraction and none of the above-mentioned studies reported the protein profile of their soy flours. One possible explanation is that all four studies started with different soybean varieties and glycinin and β-conglycinin contents vary widely (Fehr and others 2003). In addition, our soy flour had 93.8 PDI while Rickert and others (2004) started with soy flakes with 84 PDI. These differences would account for the higher yields observed in our study.

Protein profile and subunit composition

SDS-PAGE was used to compare the relative amounts of total storage proteins, glycinin, β-conglycinin (Table 1) and subunit compositions for each fraction (Table 2). The amount of total storage proteins was the sum of subunit bands for both glycinin and β-conglycinin. The amounts of total storage proteins in the glycinin-rich fractions increased as SO_2 concentration increased. The purity of the glycinin-rich fraction was also greatly affected by SO_2 concentration. With as little as 5 mM SO_2, the purity of the glycinin-rich fraction was improved by 18.1% (increasing from 63.4 to 81.5%). Increased SO_2 concentration improved the glycinin contents of the glycinin-rich fraction. The use of reducing agent avoids co-precipitation of β-conglycinin with glycinin in the first precipitation. The acidic and basic polypeptide distribution of glycinin in the glycinin-rich fraction was also affected. Higher SO_2 concentrations increased the amounts of basic polypeptides precipitated with the glycinin-rich fraction (Table 2). The subunit distribution of the β-conglycinin contaminant in the glycinin-rich fraction was also affected by SO_2 concentration. The concentration of β subunits of β-conglycinin increased as SO_2 concentration increased, following the pattern of the basic polypeptide precipitation. This phenomena was probably due to association of the basic polypeptides of glycinin with the β subunit of β-conglycinin (Utsumi and others 1984). None of the glycinin-rich fractions treated with sulfites were contaminated with α' subunits, while the contamination of α subunits decreased with increased SO_2 concentration. This observation suggests that, in the absence of reducing agent glycinin is associated through its acidic polypeptides to the α or α' subunit component of β-conglycinin.

Purity of the β-conglycinin-rich fraction was affected by SO_2 concentration. The total yield of storage protein in the β-conglycinin-rich fraction was significantly greater at 30 mM SO_2 but at the expense of yields of total solids and protein (Table 1). When no SO_2 was used, the purity of the β-conglycinin-rich fraction was higher, but this was at the expense of yields of solids and protein. The principal contaminant in all cases was glycinin. Higher SO_2 concentrations did not lead to higher purities. The subunit distribution of the contaminant glycinin was affected by using higher SO_2 concentrations. The amount of acidic glycinin subunits increased as SO_2 concentration increased (Table 2). β-Conglycinin subunit distribution in this fraction was not affected by SO_2 concentration.

The total yields of storage proteins in the intermediate fraction were significantly affected by SO_2 concentration (Table 1). Significantly higher protein yields were obtained with the use of SO_2, but the higher concentrations did not lead to higher amounts of total storage proteins (maximums were observed at 5 and 10 mM SO_2). The ratio of glycinin to β-conglycinin was also influenced by SO_2 concentration. The best results were obtained at 5 and 10 mM SO_2, since we wanted to precipitate as much of the remaining glycinin as possible in order to obtain higher purity and yield of β-conglycinin. Subunit distributions for β-conglycinin followed the same pattern ($\alpha' < \alpha < \beta$), with the exception of the 5 mM SO_2 where the α subunit content was higher than that of the β subunit. This increased amount of the β subunit was consistent with reports of Utsumi and others (1984) as discussed earlier. For the glycinin component of this fraction the amount of basic polypeptides decreased as SO_2 concentration increased, probably due to higher recoveries of this particular polypeptide in the glycinin-rich fraction. This was consistent with the increased recoveries of acidic polypeptides in the β-conglycinin-rich fractions (Table 2).

The total amount of storage protein lost to the whey fraction was significantly affected by SO_2 concentration. As SO_2 concentration increased, the amount of total storage protein lost in the whey also increased. The ratio of glycinin to β-conglycinin remained the same at 0, 5, and 10 mM SO_2, but the amount of β-conglycinin lost increased at 20 and 30 mM SO_2. The subunit distributions for both proteins remained constant over the range of SO_2 concentrations tested.

Thermal properties

The thermal behaviors of the different protein fractions were significantly affected by SO_2 concentration (Table 3). For the glycinin-rich fraction, the temperature of denaturation of the contaminant β-conglycinin was lower when no SO_2 was used and there were no significant differences among treatments when SO_2 was used. The temperature of denaturation for the glycinin component ranged between 89.1 and 90.6°C. The enthalpy of denaturation for the contaminant β-conglycinin decreased when SO_2 was used. This observation was consistent with purity. There were no significant differences in denaturation enthalpy for the glycinin component of this fraction indicating that SO_2 did not affect the thermal denaturation of glycinin.

The denaturation temperatures of the β-conglycinin-rich fractions were not significantly affected by SO_2 for any of the proteins. The enthalpy of denaturation was lowest with no SO_2, but no differences were observed for the other treatments. The enthalpy for the contaminant glycinin did not change up to 20 mM SO_2, and a maximum value was observed at 30 mM, which was consistent with purity. In spite of being the purest fraction, the protein product produced at 0 mM SO_2 had the lowest denaturation enthalpy for the β-conglycinin component.

The denaturation temperatures for the intermediate fractions were not different among treatments for any of the proteins measured. The glycinin component of this fraction denatured at significantly higher temperature compared to the behavior of glycinin in any of the other fractions. This was attributed the high amount of salt being present (Table 3). The enthalpy of denaturation for both proteins in this fraction showed a decreasing trend as the SO_2 concentration increased, but these differences were only significant at α=0.1 (LSD=0.35 and 0.5 mJ/mg of protein, for β-conglycinin and glycinin, respectively). This decrease in enthalpy is probably due to action of the reducing agent in disrupting larger aggregates, probably liberating native glycinin and β-conglycinin (Petrucelli and Anon 1995).

When comparing all treatments, the glycinin-rich fraction had the highest enthalpy of denaturation with a mean of 16.65 mJ/mg, followed by the β-conglycinin-rich fraction at 10.12 mJ/mg, and the intermediate fraction at 4.73 mJ/mg. When comparing the thermal behaviors of our fractions to those reported by Wu and others (1999) and Rickert and others

Table 3-Thermal behaviors of soy protein fractions

Fraction/ Treatment	Denaturation Temperature (°C)		Enthalpy of Denaturation (mJ/mg of protein)	
	β-Conglycinin	Glycinin	β-Conglycinin	Glycinin
Glycinin-rich Fraction				
0 mM SO_2	73.6[b]	90.6[a]	1.56[a]	15.89[a]
5 mM SO_2	75.0[a]	90.2[a,b]	0.28[b]	15.55[a]
10 mM SO_2	74.7[a]	89.1[d]	0.32[b]	15.65[a]
20 mM SO_2	75.0[a]	89.5[c,d]	0.23[b]	16.74[a]
30 mM SO_2	75.1[a]	89.8[b,c]	0.18[b]	16.90[a]
LSD	0.6	0.5	0.13	1.37
Intermediate Fraction				
0 mM SO_2	75.8[a]	93.3[a]	1.71[a]	5.00[a]
5 mM SO_2	76.2[a]	93.9[a]	1.65[a]	3.38[b]
10 mM SO_2	74.8[a]	93.7[a]	1.48[a]	2.91[b]
20 mM SO_2	75.7[a]	93.9[a]	1.22[a]	2.87[b]
30 mM SO_2	76.2[a]	94.0[a]	1.07[a]	2.35[b]
LSD	2.5	0.9	0.80	1.10
β-Conglycinin-rich Fraction				
0 mM SO_2	76.8[a]	90.9[a,b]	8.22[b]	0.15[b]
5 mM SO_2	75.9[a,b]	91.3[a]	10.97[a]	0.16[b]
10 mM SO_2	75.1[b]	89.0[b]	10.64[a]	0.06[b]
20 mM SO_2	75.1[b]	89.9[a,b]	10.27[a]	0.24[b]
30 mM SO_2	75.1[b]	90.3[a,b]	9.59[a,b]	0.55[a]
LSD	1.1	2.0	1.52	0.25

LSD denotes least significant difference; means within each fraction followed by different superscripts are statistically different. N=3.

(2004b), we found some discrepancies. Their glycinin-rich fractions were devoid of native β-conglycinin and they reported lower enthalpies for the intermediate and β-conglycinin-rich fractions. This was probably due to their defatted soy flour probably having harsher previous heat treatment as evidenced by the lower PDI in the Rickert study. In spite of these differences, their hypothesis that the intermediate fraction yields significant amount of denatured protein is still valid since this fraction in our study also had the lowest enthalpies for both proteins. In our case, there was probably significant co-precipitation of native protein with denatured protein.

Solubility

The solubilities of the glycinin-rich fractions slightly decreased as the amount of SO_2 increased (Table 4). Maximum values were observed for 0 and 5 mM SO_2. There were no differences among all other treatments for this fraction. These observations are in contrast with thermal behavior, since 5 mM SO_2 yielded the glycinin-rich fraction with lowest denaturation enthalpy. These solubility differences can be partially attributed to the fact that the treatment fractions that had the highest solubilities were also those that had the highest contents of glycinin acidic polypeptides and the fractions with lower solubilities were those with higher contents of glycinin basic polypeptides. Liu and others (1999) and Dias and others (2003) characterized the solubility behavior of acidic polypeptide components of glycinin and found that these polypeptides were more soluble, even more than native glycinin.

The solubilities of the intermediate fractions were not significantly affected by SO_2 concentration and the intermediate fraction had the lowest solubilities among all fractions. This fraction also had the lowest denaturation enthalpies (Table 3) indicating substantial denaturation.

The solubility of the β-conglycinin-rich fraction was greatly influenced by SO_2 concentration (30% higher when SO_2 was used). These results are consistent with thermal behavior. It seems that acid precipitation of β-conglycinin in absence of reducing agent caused more denaturation. Increased SO_2 concentration did not affect the solubility behavior of this fraction, probably because β-conglycinin structure is not stabilized by disulfide bridges (Thanh and Shibasaki 1979). As a consequence, SO_2 did not induce structural changes to this protein.

When comparing all fractions regardless of SO_2 treatment, the glycinin-rich fraction was the most soluble (mean of all treatments, 89.9%), followed by the β-conglycinin-rich fraction (mean, 86.0%) and the intermediate fraction (mean, 41.8%; LSD=3.5%). This trend changes when the 0 mM SO_2 treatment, with mean values for solubility of 92.4, 89.4, and 41.8% for β-conglycinin-rich, glycinin-rich and intermediate fractions, respectively (LSD = 2.7%), is excluded from the data analysis. When excluding 0 mM SO_2, our data were

consistent with those of Bian and others (2003) and Rickert and others (2004b), where fractions solubility followed the same trend as thermal behavior.

Table 4-Solubilities and surface hydrophobicities of soy protein fractions

Fraction/Treatment	Solubility (%)	Surface Hydrophobicity
Glycinin-rich Fraction		
0 mM SO_2	$92.1^{a,b}$	191^{a}
5 mM SO_2	93.2^{a}	159^{b}
10 mM SO_2	88.1^{c}	160^{b}
20 mM SO_2	$88.2^{b,c}$	140^{b}
30 mM SO_2	88.1^{c}	143^{b}
LSD	4.0	26
Intermediate Fraction		
0 mM SO_2	$43.2^{a,b}$	158^{b}
5 mM SO_2	$41.1^{a,b}$	167^{b}
10 mM SO_2	39.7^{b}	156^{b}
20 mM SO_2	43.4^{a}	195^{a}
30 mM SO_2	$42.9^{a,b}$	189^{a}
LSD	3.7	21
β-Conglycinin-rich Fraction		
0 mM SO_2	60.2^{b}	131^{b}
5 mM SO_2	92.6^{a}	197^{a}
10 mM SO_2	93.8^{a}	187^{a}
20 mM SO_2	92.0^{a}	179^{a}
30 mM SO_2	91.2^{a}	195^{a}
LSD	3.6	23

LSD denotes least significant difference; means within each fraction followed by different superscripts are statistically different. N=3.

Surface hydrophobicity

The surface hydrophobicity of the glycinin-rich fraction was highest with no SO_2. Higher concentrations of SO_2 (20 and 30 mM) gave lower surface hydrophobicities (significant at $p<0.1$, but not at $p<0.05$). The surface hydrophobicity of the β-conglycinin-rich fraction increased as SO_2 concentration increased, but was not affected at >5 mM SO_2. This was in contrast to thermal behavior (Table 3). Apparently, SO_2 introduced structural changes without affecting denaturation enthalpy. The surface hydrophobicity of the

intermediate fraction increased after adding 20 mM and 30 mM SO_2, probably because these samples had the lowest denaturation enthalpies among all intermediate fractions. There were no differences in hydrophobicity at 0, 5, and 10 mM SO_2.

When comparing each fraction regardless of SO_2 concentration, the glycinin-rich fractions had the lowest mean surface hydrophobicity (159) and the intermediate and β-conglycinin-rich fractions had higher mean values (173 and 178, respectively). Our results contrast somewhat with those of earlier reports. Wu and others (1999) reported that the β-conglycinin-rich fraction had the highest surface hydrophobicity and the intermediate and glycinin-rich fractions were similar. Using a similar procedure, Rickert and others (2004b) found the β-conglycinin-rich fraction to have the lowest surface hydrophobicity and no differences for their intermediate and glycinin-rich fractions. Our results and those of Wu and others (1999) partially agree in that β-conglycinin is more hydrophobic than glycinin (Hayakawa and Nakai 1985). Discrepancies in results when discussing the intermediate fraction are not surprising, since the ANS probe test only measures the surface hydrophobicity of the soluble portion of a given sample and the solubilities of the intermediate fractions were only about 40%.

Emulsification properties

The emulsification capacities of the glycinin-rich fractions were significantly affected by SO_2 concentration (Table5). The glycinin-rich fraction obtained without using reducing agent had the highest emulsification capacity, probably because this fraction was significantly contaminated with β-conglycinin, which is a better emulsifier. The glycinin-rich fractions produced at low SO_2 concentrations were more effective emulsifying agents than those fractions obtained at high SO_2 concentrations. This observation was probably due to the fact that increased SO_2 concentrations yielded glycinin-rich fractions with decreased acidic polypeptide components, which is an excellent emulsifier (Dias and others 2003, Liu and others 1999).

The emulsification capacities of the β-conglycinin-rich fractions were also affected by SO_2 concentration. The β-conglycinin-rich fraction obtained without SO_2 was a very poor emulsifier, probably due to the low solubility and surface hydrophobicity of this fraction. All

levels of SO_2 improved emulsification capacity of the β-conglycinin-rich fractions but higher SO_2 did not further improve emulsification capacity. The fractions obtained when using SO_2 emulsified almost twice as much oil as did the β-conglycinin-rich fraction prepared without SO_2.

The emulsification capacity of the intermediate fraction was also affected by the usage of SO_2. The intermediate fraction obtained without SO_2 had significantly higher emulsification capacity. All fractions obtained when using SO_2 had similar emulsification capacities that were lower than was obtained for the intermediate fraction at 0 mM SO_2. This was probably because the intermediate fraction obtained at 0 mM SO_2 was significantly less denatured than when SO_2 was used (Table 3).

Regardless of SO_2 concentration, the β-conglycinin-rich fraction had the highest mean emulsion capacity (546 g of oil/g of product), followed by the glycinin-rich fraction (351 g/g) and the intermediate fraction (235 g/g). When the same comparison was made without the 0 mM SO_2treatment, the differences between the glycinin and β-conglycinin were larger (308 vs. 195) and the values for glycinin and intermediate fractions were closer (68 vs. 116). Our results agree with those of Rickert and others (2004b) and Bian and others (2003) in that β-conglycinin is a significantly better emulsifier than glycinin. For differences between the glycinin-rich fractions and intermediate fractions, the results are more difficult to compare. Our results agree with those by Rickert and others (2004b) for the Wu-process and the "optimized" process of Rickert and others. Bian and others did not find differences in emulsification capacities between these two fractions.

Emulsification activities and stability indices for the glycinin-rich fractions were significantly affected by SO_2 concentration (Table 5). Emulsification activity was highest for the glycinin-rich fraction when not using SO_2. All other treatments were significantly lower but similar among themselves. Emulsification stability was highest for the glycinin-rich fractions produced at low SO_2 concentrations, intermediate for glycinin-rich fractions prepared without SO_2, and lowest for glycinin-rich fractions prepared at high SO_2 concentrations. This behavior was attributed to lower acidic polypeptide content as SO_2 concentration increased. Acidic polypeptides not only emulsified more oil, but also formed

more stable emulsions (Dias and others 2003) compared to the glycinin-rich fraction from which they were isolated. Similar results were reported by Liu and others (1999).

Table 5-Emulsification properties of soy protein fractions

Fraction/ Treatment	Emulsification Properties		
	Capacity (g oil/ g product)	Activity (Absorbance 500 nm)	Stability Index (min)
Glycinin-rich Fraction			
0 mM SO_2	591[a]	0.182[a]	69[b]
5 mM SO_2	347[b]	0.145[b]	82[a]
10 mM SO_2	351[b]	0.152[b]	84[a]
20 mM SO_2	235[c]	0.157[b]	54[c]
30 mM SO_2	232[c]	0.152[b]	53[c]
LSD	72	0.015	12
Intermediate Fraction			
0 mM SO_2	282[a]	0.282[a]	71[a]
5 mM SO_2	222[b]	0.200[b,c]	69[a]
10 mM SO_2	232[b]	0.168[c]	62[a]
20 mM SO_2	210[b]	0.217[b]	81[a]
30 mM SO_2	228[b]	0.175[c]	72[a]
LSD	38	0.035	20
β-Conglycinin-rich Fraction			
0 mM SO_2	332[b]	0.223[d]	66[b]
5 mM SO_2	582[a]	0.298[c]	182[a]
10 mM SO_2	586[a]	0.306[b,c]	194[a]
20 mM SO_2	623[a]	0.319[a,b]	204[a]
30 mM SO_2	607[a]	0.332[a]	222[a]
LSD	53	0.019	74

LSD denotes least significant difference; means within each fraction followed by different superscripts are statistically different. N=3.

For the β-conglycinin-rich fraction, increasing SO_2 concentration significantly increased emulsification activity. The samples treated with SO_2 formed more stable emulsions, but increasing SO_2 concentration did not further influence stability. The low values for this fraction obtained without SO_2 were probably due to low solubility, hydrophobicity and denaturation enthalpy. We attributed the higher emulsification activities

obtained at higher SO_2 concentrations to the higher contents of glycinin acidic subunits in this fraction.

The emulsification activities of the intermediate fractions were highest for the fraction prepared without SO_2, probably due to the higher proportion of β-conglycinin content and higher denaturation enthalpies. All other products had significantly lower emulsification activities and they were similar to each other. Emulsification stability indices for this fraction were the same for all treatments.

When comparing the different fractions regardless of reducing agent treatment, the β-conglycinin-rich fraction had the highest emulsification activity and stability index (0.296 and 173, respectively), followed by the intermediate fraction (0.208 and 71, respectively) and the glycinin-rich fraction (0.158 and 68, respectively). When the comparison was made without the 0 mM SO_2 treatment, the trends were similar with higher values for the β-conglycinin-rich fraction (0.314 and 200, respectively) and the same results for the intermediate and glycinin-rich fractions. These results compare well with findings of Rickert and others (2004b) and Bian and others (2003) for the β-conglycinin-rich fraction, with the exception of the "optimized" fractionation of Rickert and others (2004b) in which intermediate fraction had the highest emulsification activity and stability.

Foaming properties

The foaming properties of the glycinin-rich fractions were significantly affected by the using SO_2 during fractionation (Table 6). Foaming capacity was highest for the glycinin-rich fraction obtained at 5 mM SO_2. Foaming capacity was lower for the glycinin-rich fractions obtained at 0 and 30 mM SO_2. Foaming stability index was lowest for the glycinin-rich fractions obtained with 5 mM SO_2 (the higher the value, the less stable the foam), followed by the 10 and 20 mM SO_2 treatments, and the glycinin-rich fractions obtained with 0 and 30 mM SO_2 were the most stable foams. All glycinin-rich fractions had low foaming rates when using SO_2 whereas the glycinin-rich fraction obtained without SO_2 was the fastest foam-forming agent among the glycinin-rich fractions.

The β-conglycinin-rich fraction obtained at 5 mM SO_2 had the best foaming capacity. All other SO_2 treatments for this fraction yielded fractions with similar foaming capacities.

Foaming stability index was highest for the β-conglycinin-rich fraction obtained at 0 mM SO_2, all other treatments for this fraction had similar foaming stability indices. The rate of foaming was the property most affected by SO_2 concentration for the β-conglycinin-rich fraction. As SO_2 concentration increased, the rate of foaming decreased.

Table 6-Foaming properties of soy protein fractions

Fraction/ Treatment	Foaming Properties		
	Capacity (mL of foam/mL of 0.5% product dispersion)	Stability (K= 1/mL min)	Rate (mL/min)
Glycinin-rich Fraction			
0 mM SO_2	1.03^{c}	0.045^{c}	4.8^{a}
5 mM SO_2	1.22^{a}	0.115^{a}	2.6^{b}
10 mM SO_2	$1.16^{a,b}$	0.089^{b}	2.1^{b}
20 mM SO_2	$1.16^{a,b}$	0.080^{b}	2.4^{b}
30 mM SO_2	$1.05^{b,c}$	0.058^{c}	2.3^{b}
LSD	0.13	0.018	0.6
Intermediate Fraction			
0 mM SO_2	0.94^{a}	0.007^{a}	13.6^{c}
5 mM SO_2	1.01^{a}	0.006^{b}	20.8^{a}
10 mM SO_2	0.96^{a}	0.004^{c}	18.2^{b}
20 mM SO_2	0.95^{a}	0.004^{c}	17.8^{b}
30 mM SO_2	0.94^{a}	0.004^{c}	17.9^{b}
LSD	0.13	0.001	2.0
β-Conglycinin-rich Fraction			
0 mM SO_2	1.06^{b}	0.004^{b}	15.5^{a}
5 mM SO_2	1.40^{a}	0.018^{a}	17.5^{a}
10 mM SO_2	1.07^{b}	0.018^{a}	12.4^{b}
20 mM SO_2	1.09^{b}	0.020^{a}	9.5^{c}
30 mM SO_2	1.08^{b}	0.019^{a}	6.5^{d}
LSD	0.19	0.005	2.5

LSD denotes least significant difference; means within each fraction followed by different superscripts are statistically different. N=3.

The foaming capacities of the intermediate fractions were not affected by SO_2 concentration. Foaming stability index, on the other hand, was significantly affected with more stable foams obtained at 10, 20, or 30 mM SO_2 (Table 6). The rate of foaming for the

intermediate fraction was highest for those obtained with 5 mM SO_2. All other SO_2 concentrations yielded intermediate fractions with similar foaming rates and the intermediate fraction obtained without SO_2 yielded the lowest foaming rate.

When comparing the different fractions, regardless of reducing agent treatment, the glycinin-rich and β-conglycinin-rich fractions had high foaming capacities (1.12 and 1.14, respectively) compared with the intermediate fraction (0.96). On the other hand, the intermediate fraction formed the most stable foams (FSI= 0.005), followed by the β-conglycinin-rich fraction (0.016), and the glycinin rich fraction (0.077). The same order was observed for rate of foaming with the intermediate fraction being the fastest (18.7 mL/min), followed by the β-conglycinin-rich fraction (12.3 mL/min) and the glycinin-rich fraction (2.8 mL/min). Eliminating the treatment prepared without SO_2, like for the other functional properties tested, did not significantly alter these results. Comparing these results to those reported earlier in the literature we found several differences. Rickert and others (2004b) found no differences between the intermediate and glycinin-rich fractions for foaming capacity, while their β-conglycinin-rich fractions had the lowest foaming capacity, similar results for rate of foaming, and no differences among fractions and treatments for foaming stability. On the other hand, Bian and others (2003) reported no differences in foaming capacity between the intermediate and β-conglycinin-rich fractions but both were lower than for their glycinin-rich fractions. Their results for both foaming stability and rate of foaming followed the same pattern, their intermediate fraction scored the highest, followed by their β-conglycinin-rich fraction and their glycinin rich fraction.

Proposed mechanism for SO_2 action during soy protein fractionation

Although most soy protein fractionation procedures utilize reducing agents, no mechanism for the effect of reducing agents during fractionation has been proposed. Wolf (1993) showed that a reducing agent was necessary to achieve effective fractionation and indicated that the reducing agents used during fractionation preferentially break the inter-chain disulfide bonds since these bonds are readily accessible to the reducing agent. Intra-chain disulfide bonds, on the other hand, are shielded in the protein molecule and also need a denaturing agent to expose them to the reducing agent for cleavage. The reason for why

breaking disulfide bond between the acidic and basic polypeptide components of glycinin subunits improves protein fractionation remains unanswered.

Fairly strong protein-phytate interaction occurs in aqueous soy protein extracts at alkaline pHs and calcium mediates between phytate and protein (Omosaiye and Cheryan 1979) (Fig. 1A). Furthermore, the amount of phytate that could be removed from the aqueous extract decreased with as pH increased and attributed this observation to increasing strength of the salt-mediated linkage. Several ultrafiltration steps were needed to effectively remove phytate from the extract and attributed this to the sequential removal of calcium followed by dilution steps, suggesting that calcium concentration was important for calcium-mediated linkages to occur. Kroll (1984) reported that about 30% of the calcium and 20% of the phosphorus present in soy protein isolates are bound to the protein. Chen and Morr (1985) also suggested protein-phytate interactions and reported that the isoelectric point from 4.2-4.5 to 4.8-5.0 shifted in phytate-reduced soy protein extracts. Brooks and Morr (1985) reported that both glycinin and β-conglycinin co-eluted in a gel-filtration procedure along with significant amounts of calcium and phosphorus, and suggested that these salt-mediated linkages interfere with soy protein fractionation and characterization, especially for the β-conglycinin component.

Based on our observations and the current understanding of glycinin (Nielsen 1985) and β-conglycinin structures (Thanh and Shibasaki 1978, 1979), we propose the following mechanism for reducing agent action during soy protein fractionation (Fig. 1B). Reducing agent was essential to obtain the glycinin-rich fraction in high purity. We believe that 5 to 30 mM SO_2 break disulfide bonds between the acidic and basic polypeptides of glycinin. Native glycinin and β-conglycinin complex with phytate through calcium bridges and this interaction is between the acidic polypeptide and the α or α' subunits of β-conglycinin. β-Conglycinin is a heterogeneous mixture of different molecular species resulting from various combinations of three subunits (α, α', and β). The α and α' subunits share a core region with the β subunit (~74% of the molecule) and an extension region composed of 155 amino acid residues at the N terminus, which contains numerous acidic residues. This region is probably near the surface of the molecule and may lie on one face of the trimer (Mills and others 2001). At the pH of SO_2 addition (~ 8.5), these components are able to complex with calcium

to form bridges with phytate. We believe that the electrostatic forces involved in this calcium bridge preferentially expose these particular glycinin subunits for action of SO_2. Once the disulfide bridge is broken, the acidic polypeptide remains in solution with β-conglycinin, and the basic polypeptide precipitates with the glycinin-rich fraction. This model fits our data for glycinin polypeptide partitioning and observations of Damodaran and Kinsella (1982). Once the pH is adjusted and the glycinin-rich fraction precipitated, 0.25 M NaCl is added, which disrupts the calcium bridges and salts-in most of the native β-conglycinin and acidic polypeptides of glycinin. The subsequent adjustment to pH 5 precipitates species that are not salt-sensitive, mainly denatured glycinin and β-conglycinin (Wu and others 1999, Rickert and others 2004b) and those β-conglycinin trimers rich in β subunits, since they have higher affinity with the glycinin component (Utsumi and others 1984). After precipitation of the intermediate fraction, the remaining proteins in solution (mainly β-conglycinin) are precipitated by reducing the ionic strength of the medium by diluting with two-fold de-ionized water and adjusting the pH to 4.8, which precipitates β-conglycinin (Thanh and Shibasaki 1979).

Conclusions

The use of a reducing agent is necessary to fractionate soy protein into a glycinin-rich and a β-conglycinin-rich fractions having high yields and purities. The addition of >20 mM SO_2 yields significantly less protein and solids in the purified fractions. The 5 mM SO_2 level gave the best combination of yields, purities, and functional properties for both the glycinin-rich fraction and the β-conglycinin-rich fraction. The addition of SO_2 during soy protein fractionation significantly modifies the functional properties of the fractionated soy proteins. Solubility, emulsification capacity, emulsion stability, and foaming capacity of the glycinin-rich fraction decrease, and foam stability increases as SO_2 concentration increases. Surface hydrophobicity, emulsification activity, and rate of foaming of the glycinin-rich fraction significantly decrease due to the addition of SO_2 but this decrease does not depend on concentration. The emulsification activity of the β-conglycinin-rich fraction increases while foaming capacity and the rate of foaming decrease with increased SO_2 concentration.

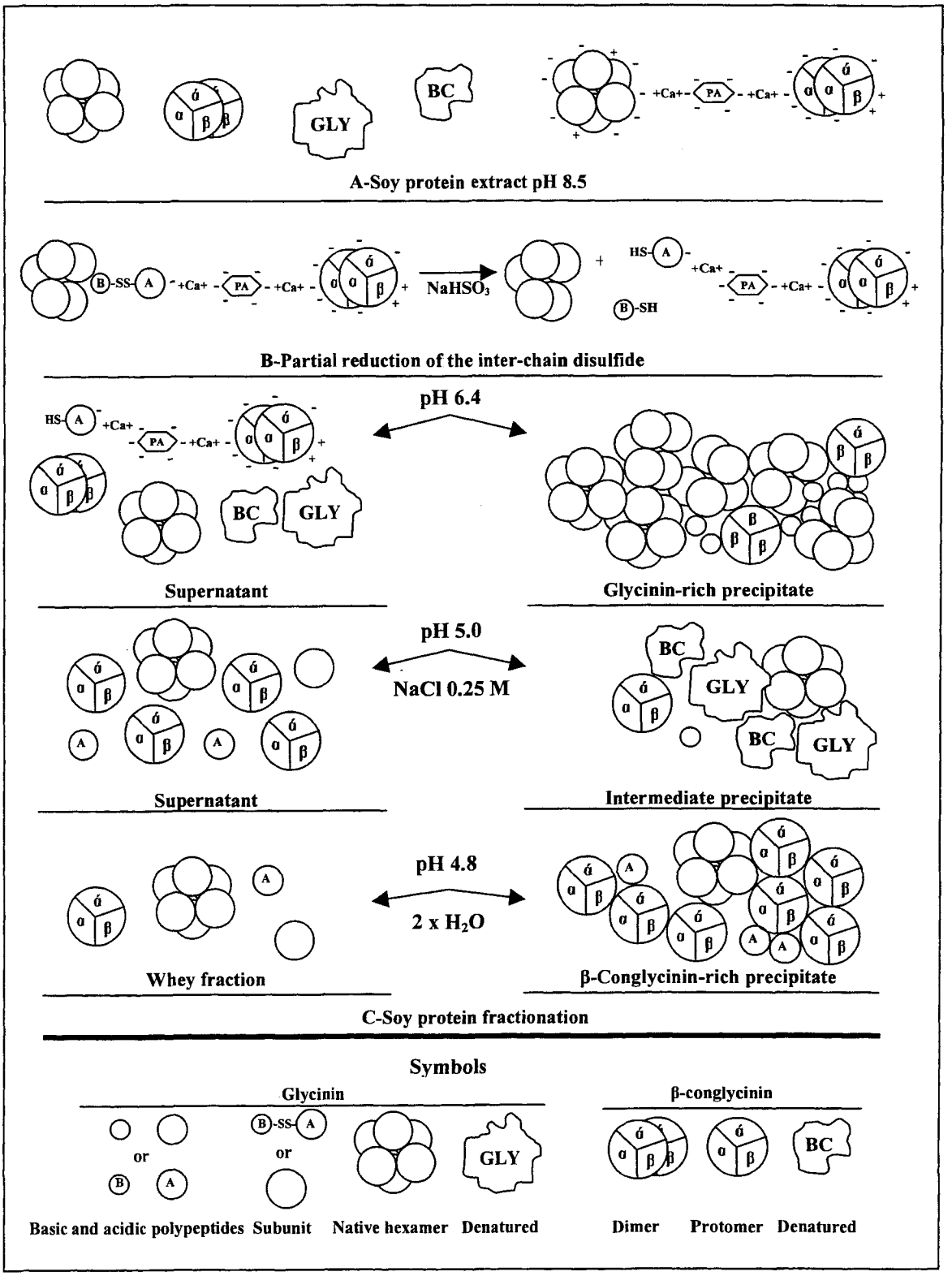

Figure 1. Schematic representation of soy protein fractionation procedure.

Solubility, surface hydrophobicity, emulsification capacity, and emulsion stability increase while foaming stability of the β-conglycinin-rich fraction decreases with the addition of SO_2, but these properties are not affected by SO_2 concentration.

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CHAPTER 3. EFFECTS OF NACL CONCENTRATION ON SALTING-IN AND DILUTION DURING SALTING-OUT ON SOY PROTEIN FRACTIONATION

A paper to be submitted to the *Journal of Food Science*

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Abstract

Glycinin and β-conglycinin are the main storage proteins in soybeans that can be fractionated by using alkali extraction, SO_2, salting-in with NaCl, salting-out by dilution and pH adjustment to produce a glycinin-rich fraction, a β-conglycinin-rich fraction, and an intermediate fraction, which is a mixture of the two proteins. Two different strategies were employed to optimize the procedure to achieve high efficiency in recovering the β-conglycinin-rich fraction. The first strategy was to optimize salting-in effects of NaCl and the effects of NaCl concentration on the yields and purities of the protein fractions were investigated. The maximum protein yield of the β-conglycinin-rich fraction was obtained at 500 mM NaCl, but at the expense of purity. The optimum NaCl concentration was 250 mM, at which good protein yield (18.5%) and purity (84.5%) were achieved. At higher NaCl concentrations, the protein yields of the intermediate fractions were significantly lower, and the protein loss in the whey fraction increased. The second strategy was to improve the salting-out step for the β-conglycinin-rich fraction. At 0- and 0.5-fold dilution, the purities and yields of the β-conglycinin-rich fractions were significantly lower than at 1.0- and 2.0-fold dilution. There were no differences in protein yields or purities when using 1.0- or 2.0-fold dilution. Based on these results the recommended NaCl concentration for the salting-in step is 250 mM and the dilution factor for salting-out is 1.0.

Introduction

Soybeans have become an important world commodity because they are ubiquitous, inexpensive, and have unique chemical composition, good nutritional value, versatile uses, and recently recognized functional health benefits. Yet, less than 5% of the available soybean protein is used for food, but this percentage is likely to grow since the perception of soy as a

healthy food is strong (Golbitz 2003). One of the main bodies of soy foods research has focused on the individual storage proteins (glycinin and β-conglycinin, which are complex and comprise nearly 70% of total soybean seed protein) and relating them to important functional properties and health benefits. The recent increase in popularity of soy protein is due to its potential health benefits (Messina 1997), which continue to drive soy protein research and commercial development of new soy-protein-based food products and ingredients.

There is considerable interest in obtaining purified β-conglycinin fractions, because this specific soy protein is believed to be responsible for health benefits observed in populations consuming high amounts of soy protein. These benefits include reducing blood cholesterol (Adams and others 2004, Duranti and others 2004, Manzoni and others 2003) and plasma triglyceride levels (Aoyama and others 2001, Baba and others 2004, Moriyama and others 2004), which impact cardiovascular health. Another study by Tsuruki and others (2003) reports that a peptide derived from β-conglycinin may be anticarcinogenic.

β-Conglycinin is a trimeric protein having molecular weight of 126-171 kDa and is composed of three subunits, α' (~57 kDa), α (~57 kDa), and β (~42 kDa) (Thanh and Shibasaki 1977). Several combinations of these subunits are found ($\alpha'\beta_2$, $\alpha\beta_2$, $\alpha\alpha'\beta$, $\alpha_2\alpha'$, $\alpha_2\beta$, α_3, and β_3) providing heterogeneity (Mills and others 2001). In spite of intense interest, these individual proteins or their enriched fractions are not commercially available nor easily obtained for clinical trials. The basis for isolating β-conglycinin from the other soybean storage protein, glycinin, in defatted soy flour is salting-in, followed by adjusting pH and salting-out to obtain a protein fraction enriched in β-conglycinin. The fractionation method of choice today is a procedure reported by Wu and others (1999), which is a relatively simple procedure based on differences in solubilities of glycinin and β-conglycinin at different pH and ionic strength combinations. The objectives of the current work were to optimize the NaCl concentration in the salting-in step and evaluate effects on β-conglycinin yield, purity and subunit distribution; to optimize the salting-out step and evaluate effects on β-conglycinin yield, purity and subunit distribution; and to identify potential cost-saving alternatives in the isolation of a β-conglycinin-rich fraction.

Materials and Methods

Soy flour

Soy protein fractions were produced from air-desolventized, hexane-defatted white flakes (IA 2020 variety, 1999 harvest) produced in the extraction pilot plant of the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). The defatted flakes were milled with a Krups grinder (Distrito Federal, Mexico) to achieve 100% of the material passing through a 50-mesh screen by using small quantities (10 g) to preserve the native protein state. The protein content of the soy flour was 57.3% on dry-weight basis with 93.8 protein dispersibility index (PDI) as determined by Silliker Laboratories (Minnetonka, MN). The flour was stored in sealed containers at 4°C until used.

Protein fractionation

The control soy protein fractionation procedure utilized in this study has been reported by Wu and others (1999) and is a modification to methods of Nagano and others (1992) (Fig. 1). About 200 g of defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 1 h. After centrifuging at 14,300 x g and 15°C for 30 min, the supernatant protein extract was decanted and the amount of insoluble fiber residue was determined and sampled for proximate composition. Sufficient $NaHSO_3$ was added to the protein extract to achieve 10 mM SO_2 and the pH was adjusted to 6.4 with 2N HCl. The resulting slurry was stored at 4°C for 12-16 h and centrifuged at 7,500 x g and 4°C for 20 min. A glycinin-rich fraction was obtained as the precipitated curd. This fraction was redisolved in de-ionized water, adjusted to pH 7 with 2N NaOH, sampled, and stored in sealed containers at -80°C until freeze-drying. NaCl was added to the supernatant and the pH was adjusted to 5 with 2N HCl and the slurry stirred for 1 h. The slurry was centrifuged at 14,000 x g and 4°C for 30 min. An intermediate mixture of glycinin and β-conglycinin was obtained as the precipitated curd; this fraction was treated as described for the glycinin-rich precipitate. Deionized water was added to the resulting supernatant (using a 2:1 dilution factor) and the pH adjusted to 4.8. The resulting slurry was centrifuged at 7,500 x g and 4°C for 20 min. A β-conglycinin-rich fraction

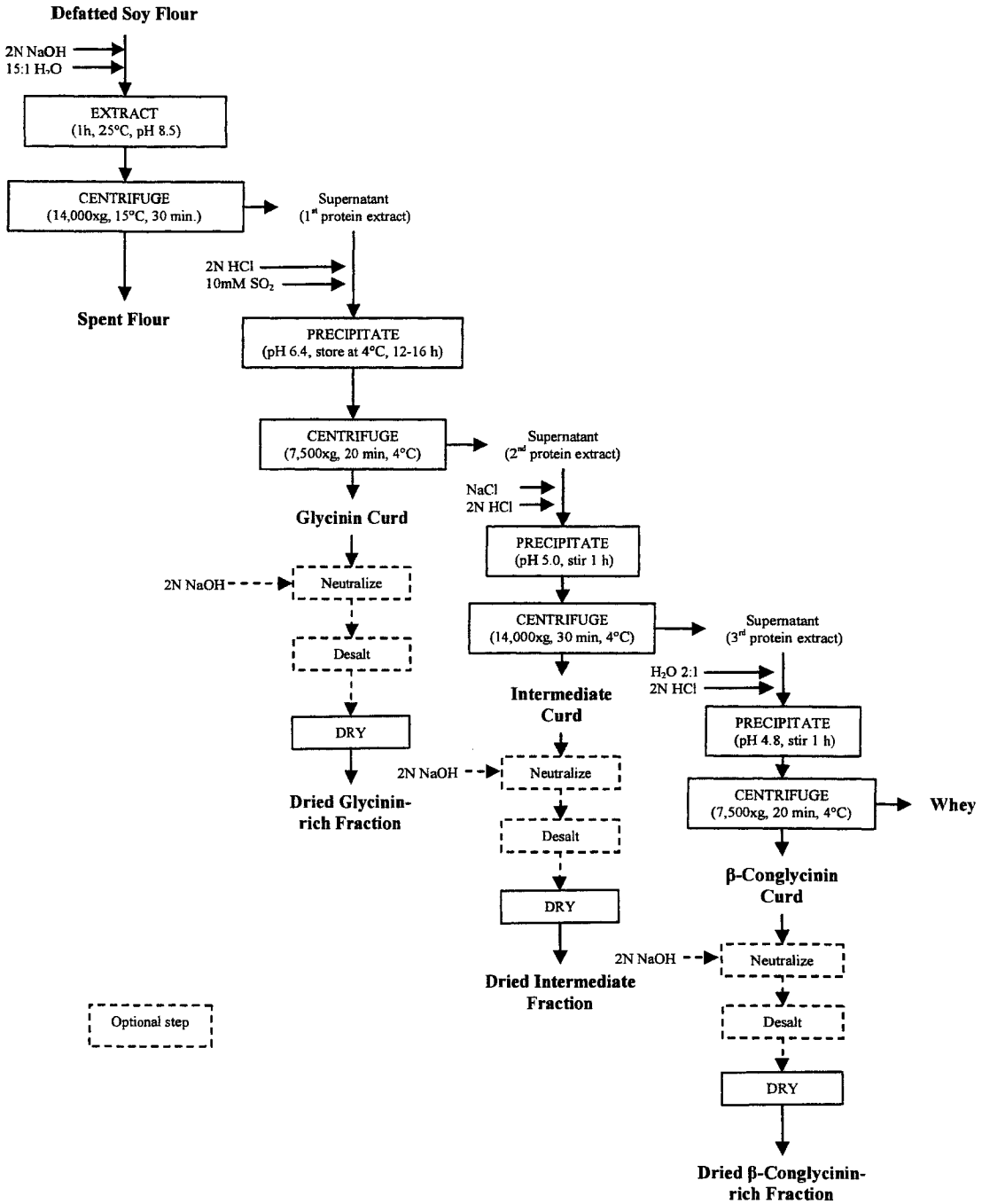

Figure 1-Flow diagram of Wu's fractionation procedure (Wu and others 1999).

was obtained as the precipitated curd; this fraction was treated as described for the glycinin-rich fraction. The amount of supernatant (whey) was determined and sampled for proximate composition.

Determining salting-in effects on fractionation

For the salting-in study, the control fractionation procedure described above was followed with modifications. The protein extract after precipitating the glycinin-rich fraction was the starting point for this study and was divided into 9 aliquots of ~150g each. Sodium chloride was added to each aliquot to obtain 0, 10, 20, 50, 100, 200, 250, 500, and 1000 mM NaCl concentrations and the fractionation procedure described for the control above was followed. The dilution factor used was 2:1 in all cases. Proximate analysis and protein compositions for the protein extract, glycinin-rich fraction and the resulting supernatant after glycinin-rich fraction precipitation are shown in Table 1. All procedures were replicated in duplicate and means reported.

Determining salting-out effects on fractionation

For the salting-out study, the control fractionation procedure described above was followed with 250 mM NaCl addition as the optimum salt concentration. The resulting protein extract after precipitating the intermediate fraction was the starting point for this study and this extract was divided into four 150-g aliquots. Deionized water was added to each aliquot to achieve 0-, 0.5-, 1-, and 2-fold dilution (1+0, 1+0.5, 1+1, and 1+2 volume of third extract plus volume of water, respectively), and the control fractionation procedure described above was followed. Proximate analysis and protein compositions of the starting materials are shown in Table 2. All procedures were replicated in duplicate and means reported.

Proximate analysis

Nitrogen contents of the soy flour, protein fractions, and byproduct streams were measured by using the combustion or Dumas method (AOAC 1995a) and a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). The nitrogen values were converted to

Table 1-Fraction yields and storage protein compositions due to salting-in soy protein fractionation

Fraction	Flour (%)	Protein Extract (%)	Glycinin-rich Fraction (%)	Glycinin Supernatant (%)
Yields				
Solids	100.0	68.9 ± 1.1	11.6 ± 1.9	57.3 ± 0.8
Protein	100.0	75.1 ± 2.0	24.8 ± 0.3	50.3 ± 0.6
Storage Protein Content and Protein Compositions				
Storage Protein	73.7 ± 0.3	77.7 ± 0.2	85.6 ± 0.3	79.8 ± 1.2
Glycinin	62.9 ± 0.2	57.2 ± 1.7	86.7 ± 0.5	31.8 ± 1.1
Acidic	57.3 ± 2.0	59.6 ± 4.1	56.2 ± 0.5	43.2 ± 0.6
Basic	42.7 ± 2.0	40.4 ± 4.1	43.8 ± 0.5	56.8 ± 0.6
β-Conglycinin	37.1 ± 0.2	42.8 ± 1.7	13.3 ± 0.5	68.2 ± 1.1
α'	31.9 ± 0.3	28.8 ± 1.3	0.0 ± 0.0	28.1 ± 0.9
α	36.1 ± 0.3	35.4 ± 0.9	42.5 ± 0.7	34.8 ± 0.3
B	32.1 ± 0.4	35.8 ± 0.3	57.5 ± 0.7	37.1 ± 0.6

Means ± one standard deviation.

Table 2-Fraction yields and storage protein compositions due to salting-out during soy protein fractionation

Fraction	Flour (%)	Protein Extract (%)	Glycinin-rich Fraction (%)	Glycinin Sup. (%)	Intermediate Fraction (%)	Intermed. Sup. (%)
Yields						
Solids	100.0	71.7 ± 1.3	12.3 ± 0.4	59.5 ± 0.9	18.0 ± 0.7	41.4 ± 1.3
Protein	100.0	73.8 ± 1.9	22.1 ± 0.9	51.8 ± 1.1	27.1 ± 0.8	24.7 ± 0.9
Storage Protein Composition						
Storage Protein	73.1 ± 0.5	79.0 ± 0.6	86.0 ± 0.4	79.8 ± 1.2	78.5 ± 3.6	81.3 ± 2.1
Glycinin	62.4 ± 0.5	58.1 ± 0.7	93.2 ± 0.2	31.7 ± 1.1	56.8 ± 2.5	7.2 ± 3.0
Acidic	58.1 ± 1.5	57.4 ± 8.5	52.3 ± 2.7	43.2 ± 0.6	49.6 ± 1.9	36.1 ± 5.1
Basic	41.9 ± 1.5	42.6 ± 8.5	47.7 ± 2.7	56.8 ± 0.6	50.4 ± 1.9	63.9 ± 5.1
β-Conglycinin	37.6 ± 0.5	41.9 ± 0.7	6.8 ± 0.2	68.3 ± 1.1	43.2 ± 2.5	92.8 ±3.0
α'	33.5 ± 0.1	29.8 ± 1.0	0.0 ± 0.0	28.0 ± 0.9	21.9 ± 0.9	34.8 ± 0.9
α	36.1 ± 0.5	35.4 ± 0.7	0.0 ± 0.0	35.4 ± 0.3	31.8 ± 0.7	38.1 ± 1.3
β	30.4 ± 0.4	34.7 ± 0.4	100.0 ± 0.0	36.6 ± 0.6	46.3 ± 1.6	27.1 ± 1.1

Means ± one standard deviation. Glycinin sup. denotes the resulting supernatant after glycinin-rich fraction precipitation; Intermed. Sup. denotes the resulting supernatant after precipitating the intermediate fraction.

Kjeldahl nitrogen using the conversion factor of Jung and others (2003). The conversion factor used to convert percentage nitrogen to protein content was 6.25. Moisture was determined by oven drying for 3 h at 130°C (AOAC 1995b). Mass balances of solids and

protein were determined for all treatments. All measurements were determined at least three times and means reported.

Urea-SDS-PAGE

Urea-sodium dodecylsulfate-polyacrylamide gel electrophoresis (urea-SDS-PAGE) was performed by using methods of Rickert and others (2004) to quantify the protein compositions of the products. The soybean storage proteins were identified by using a pre-stained SDS-PAGE molecular-weight standard, low range (Bio-Rad Laboratories, Hercules, CA). Glycinin and β-conglycinin subunit bands were confirmed by using purified standards produced according to methods of O'Keefe and others (1991). Densitometry was carried out by using the Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) with scanned images produced by a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). SDS-PAGE results were calculated as % composition of total storage protein in a given fraction = [(sum of storage protein subunit bands)/(sum of all bands)] x 100, fraction purity/composition = [(sum of subunit bands)/(sum of storage protein bands)], and subunit composition of a specific protein = [(subunit band)/(sum of subunits for the specific protein)]. All sample measurements were replicated at least four times and means reported.

Statistical analysis

The data were analyzed by using Analysis of Variance (ANOVA) and General Linear Model (GLM), and Least Significant Differences (LSD) were calculated at the 5% level to compare treatment means using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

Results and Discussion

Salting-in effects

Starting protein extract. The supernatant obtained after precipitating the glycinin-rich fraction was the starting point to evaluate the salting-in effects by NaCl addition. The fractionation procedure is shown in Figure 1 and the results for proximate analysis and protein compositions are shown in Table 1. This protein extract contained 57% of the solids and approximately 50% of the protein originally present in the starting flour. Almost 80% of

this protein was storage protein. About 70% of the storage protein present in this supernatant was β-conglycinin and the remaining 30% was glycinin.

Intermediate fraction. The objective of precipitating an intermediate fraction is to precipitate as much residual glycinin as possible from the supernatant obtained after precipitating the glycinin-rich fraction with the goal of enriching the resulting extract in β-conglycinin. The yields of protein and solids of the intermediate fraction decreased with increasing NaCl concentration, especially at 50 mM NaCl and higher (Table 3). The total storage protein content in the intermediate fraction remained relatively constant up to 250 mM NaCl (maximum at 200 mM) but significantly declined at >200 mM NaCl. These observations are in agreement with those reported by Yuan and others (2002) for glycinin and β-conglycinin solubility in the presence of salt. The glycinin contents of the intermediate fractions were larger than for the β-conglycinin contents of the intermediate fractions obtained at 200 mM NaCl and higher; this proportion being inverse for 100 mM and less (Table 3, Fig. 2a). These observations are consistent with the reversible and irreversible association-dissociation behaviors of β-conglycinin in this pH and ionic strength ranges (Thanh and Shibasaki 1979). Interestingly, the amount of β-conglycinin precipitated in the intermediate fraction exceeded that of glycinin at 1000 mM NaCl. Lakemond and others (2000) reported solubility for glycinin at high ionic strengths and pH 5.0 to be >80%.

The subunit distribution of the β-conglycinin component of the intermediate fraction varied inconsistently with ionic strength (Table 4, Fig. 2b), all treatments had different subunit distributions. The β subunit content of β-conglycinin was higher at 100 to 250 mM NaCl than at other NaCl concentrations (Table 4). This preferential association of the β-conglycinin β_3 homotrimers with glycinin has been reported before (Utsumi and others 1984). The glycinin subunit distribution in the intermediate fraction was also affected by NaCl concentration (Table 4). Nearly equal amounts of acidic and basic subunits were recovered in the intermediate fraction at 20 mM NaCl and less, whereas most other NaCl concentrations gave more basic subunits than acidic subunits in the intermediate fraction.

Table 3-Fraction yields and storage protein yield and compositions soy protein fractions due to salting-in

Fraction/NaCl Concentration (mM)	Fraction Yields (%)		Storage Protein Content and Composition (%)		
	Solids	Protein	Total	β-Conglycinin	Glycinin
Intermediate					
0	25.8^{a}	41.8^{a}	79.5^{a,b}	64.1^{b}	35.9^{e}
10	23.9^{a,b}	41.5^{a}	80.4^{a,b}	61.1^{b,c}	38.9^{d,e}
20	24.5^{a,b}	40.5^{a}	79.6^{a,b}	63.0^{b}	37.0^{e}
50	22.4^{b}	35.1^{b}	79.1^{a,b}	59.1^{c,d}	40.9^{c,d}
100	18.9^{c}	34.6^{b}	79.3^{a,b}	56.6^{d}	43.4^{d}
200	17.1^{c}	27.8^{c}	82.0^{a}	43.9^{e}	56.1^{b}
250	17.8^{c}	26.8^{c}	78.9^{b}	40.4^{f}	59.6^{a}
500	6.2^{d}	13.6^{d}	66.3^{c}	39.5^{f}	60.5^{a}
1000	4.4^{d}	10.6^{e}	64.2^{c}	67.5^{a}	32.5^{f}
LSD	3.0	2.5	3.1	3.4	3.4
β-Conglycinin-rich					
0	3.2^{e}	2.9^{f}	82.2^{c}	89.9^{b}	10.1^{e}
10	2.4^{e}	3.0^{f}	80.9^{c}	83.7^{d}	16.3^{c}
20	3.3^{d,e}	4.1^{e,f}	84.4^{b}	88.2^{b,c}	11.8^{d,e}
50	4.8^{d}	5.9^{e}	82.0^{c}	86.5^{c}	13.5^{d}
100	6.6^{c}	8.8^{d}	88.9^{a}	95.6^{a}	4.5^{f}
200	8.4^{b}	14.9^{c}	81.6^{c}	86.8^{c}	13.2^{d}
250	10.7^{a}	18.5^{b}	85.5^{b}	84.5^{d}	15.5^{c}
500	11.2^{a}	24.8^{a}	82.1^{c}	58.8^{e}	41.2^{b}
1000	7.8^{b,c}	16.6^{b,c}	74.3^{d}	38.8^{f}	61.2^{a}
LSD	1.6	2.1	2.1	1.9	1.9
Whey					
0	28.3^{f}	9.2^{e}	60.0^{e}	23.4^{d,e}	76.6^{b,c}
10	30.9^{e,f}	9.3^{e}	64.1^{d}	20.0^{e,f}	80.0^{a,b}
20	29.5^{e,f}	9.3^{e}	57.2^{f}	20.9^{e}	79.1^{b}
50	30.1^{e,f}	9.8^{d,e}	61.1^{e}	19.1^{e,f}	80.9^{a,b}
100	31.8^{d,e}	10.4^{d,e}	61.1^{e}	17.5^{f}	82.5^{a}
200	34.3^{c,d}	11.1^{c,d}	60.3^{e}	26.2^{d}	73.8^{c}
250	36.9^{c}	12.7^{c}	69.4^{c}	36.0^{c}	64.0^{d}
500	39.9^{b}	15.5^{b}	76.3^{b}	68.3^{b}	31.7^{e}
1000	45.1^{a}	26.6^{a}	78.6^{a}	74.0^{a}	26.0^{f}
LSD	2.8	1.8	1.6	3.0	3.0

Means within a column followed by different superscripts are significantly different at $P<0.05$.

Table 4-Glycinin and β-conglycinin subunit compositions of soy protein fractions due to salting-in

Fraction/NaCl Concentration	β-Conglycinin Subunit Composition (%)			Glycinin Subunit Composition (%)	
(mM)	α'	α	β	Acidic	Basic
Intermediate					
0	28.0^{b}	34.8^{b}	37.2^{c}	45.9^{c}	54.1^{c}
10	32.8^{a}	$36.4^{a,b}$	30.8^{e}	50.3^{b}	49.7^{d}
20	29.3^{b}	38.1^{a}	$32.6^{d,e}$	50.7^{b}	49.3^{d}
50	27.5^{b}	31.1^{c}	41.4^{b}	40.0^{d}	60.0^{b}
100	24.6^{c}	28.7^{d}	46.7^{a}	$38.0^{d,e}$	$62.0^{a,b}$
200	27.8^{b}	28.7^{d}	$43.5^{a,b}$	$38.9^{d,e}$	$61.1^{a,b}$
250	29.2^{b}	28.2^{d}	42.6^{b}	43.6^{c}	56.4^{c}
500	29.1^{b}	$36.1^{a,b}$	$34.8^{c,d}$	60.4^{a}	39.6^{e}
1000	$26.8^{b,c}$	35.8^{b}	37.4^{c}	35.8^{e}	64.2^{a}
LSD	2.9	2.1	3.3	3.5	3.5
β-Conglycinin-rich					
0	19.2^{d}	64.3^{a}	16.5^{f}	100.0^{a}	0.0^{d}
10	20.2^{d}	59.2^{b}	20.6^{d}	100.0^{a}	0.0^{d}
20	20.3^{d}	58.6^{b}	21.1^{d}	100.0^{a}	0.0^{d}
50	$30.6^{a,b}$	50.9^{c}	18.5^{e}	60.1^{b}	39.9^{c}
100	30.8^{a}	40.5^{d}	28.7^{c}	55.6^{c}	44.4^{b}
200	28.3^{c}	36.1^{e}	35.6^{b}	55.6^{c}	44.4^{b}
250	28.5^{c}	35.6^{e}	35.9^{b}	45.5^{d}	54.5^{a}
500	32.1^{a}	31.5^{f}	36.4^{b}	49.2^{d}	50.8^{a}
1000	$29.2^{b,c}$	29.5^{g}	41.3^{a}	49.4^{d}	50.6^{a}
LSD	1.6	2.0	1.9	4.2	4.2
Whey					
0	0.0^{c}	0.0^{e}	100.0^{a}	$60.8^{a,b}$	$39.2^{d,e}$
10	0.0^{c}	0.0^{e}	100.0^{a}	64.2^{a}	35.8^{e}
20	0.0^{c}	0.0^{e}	100.0^{a}	59.8^{b}	40.2^{d}
50	0.0^{c}	0.0^{e}	100.0^{a}	54.6^{c}	45.4^{c}
100	0.0^{c}	0.0^{e}	100.0^{a}	$49.2^{d,e}$	$50.8^{a,b}$
200	0.0^{c}	37.4^{c}	62.6^{b}	$51.8^{c,d}$	$48.2^{b,c}$
250	0.0^{c}	46.8^{a}	53.2^{c}	45.1^{e}	54.9^{a}
500	30.8^{b}	42.1^{b}	27.1^{e}	59.0^{b}	41.0^{d}
1000	34.1^{a}	30.5^{d}	35.4^{d}	59.1^{b}	40.9^{d}
LSD	0.6	1.9	1.6	4.3	4.3

Means within a column followed by different superscripts are significantly different at $P<0.05$.

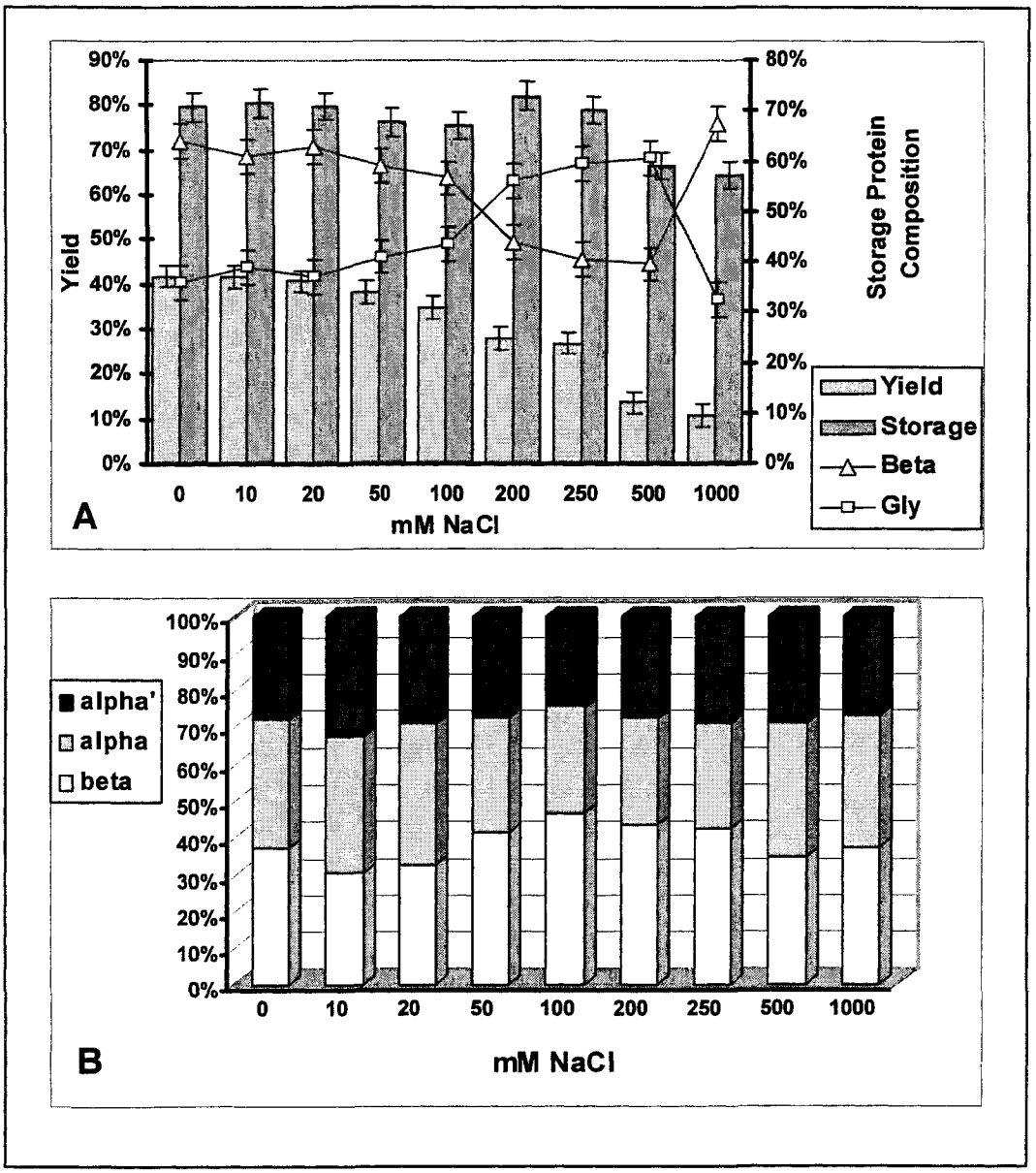

Figure 2-Protein yields, storage protein yields and compositions (A), and β-conglycinin subunit distributions in the intermediate fractions due to salting-in (B). Beta denotes β-conglycinin and Gly, glycinin.

β-Conglycinin-rich fraction. The yields of protein and solids for the β-conglycinin-rich fraction increased as NaCl concentration increased with maximum yields occurring at 500 mM NaCl. The low yields of β-conglycinin-rich fraction at low ionic strengths were consistent with results obtained for the intermediate where these treatments yielded a significant amount of β-conglycinin. These results are in agreement with observations of Maruyama and others (2002a, 2002b) for the solubility behavior of β-conglycinin homo- and hetero-timers at low ionic strength. The protein and solids yields decreased (Table 3) at 1000 mM NaCl, probably caused by the salting-in phenomena that soy storage proteins undergo (Yuan and others 2002). In addition, our yields of β-conglycinin-rich fraction agree with the model for β-conglycinin association-dissociation and precipitation behaviors reported by Thanh and Shibasaki (1979). The total storage protein contents of the β-conglycinin-rich fractions were >80% for all treatments (maximum at 100 mM NaCl), with the exception of 1000 mM NaCl that gave 74.3% total storage protein. The purity of the β-conglycinin-rich fraction was >80% for up to 250 mM NaCl. At 500 mM NaCl and higher, purity decreased (Table 3, Fig. 3a), probably due to poor recovery of the contaminant glycinin during precipitation of the intermediate protein fractions.

The subunit composition of β-conglycinin was affected by NaCl concentration (Table 4, Fig. 3b, Fig. 7). There were no significant differences in subunit compositions of β-conglycinin at NaCl concentrations 50 mM and less, and the α subunit predominated. These results contrast with those reported by Maruyama and others (2002a) for α_3 homotrimer, but data interpretation is difficult because these treatments yielded very little protein in the β-conglycinin-rich fraction. As NaCl concentrations increased, the yields of α' and α subunits increased and β subunit decreased, which is in agreement with observations of Maruyama and others (2002a, 2002b) for solubilities of β-conglycinin. At 200 and 250 mM NaCl, the subunit compositions of the β-conglycinin component of this fraction were the same. At 1000 mM NaCl, the predominant subunit was β (41.3%), probably because this fraction had the greates glycinin contamination and β subunit of β-conglycinin associated with the glycinin basic polypeptide component (Utsumi and others1984). The subunit composition of the contaminant glycinin was also affected by NaCl concentration (Table 4). At 20 mM NaCl and less, the contamination was comprised of only the acidic subunits of glycinin. At 50 to

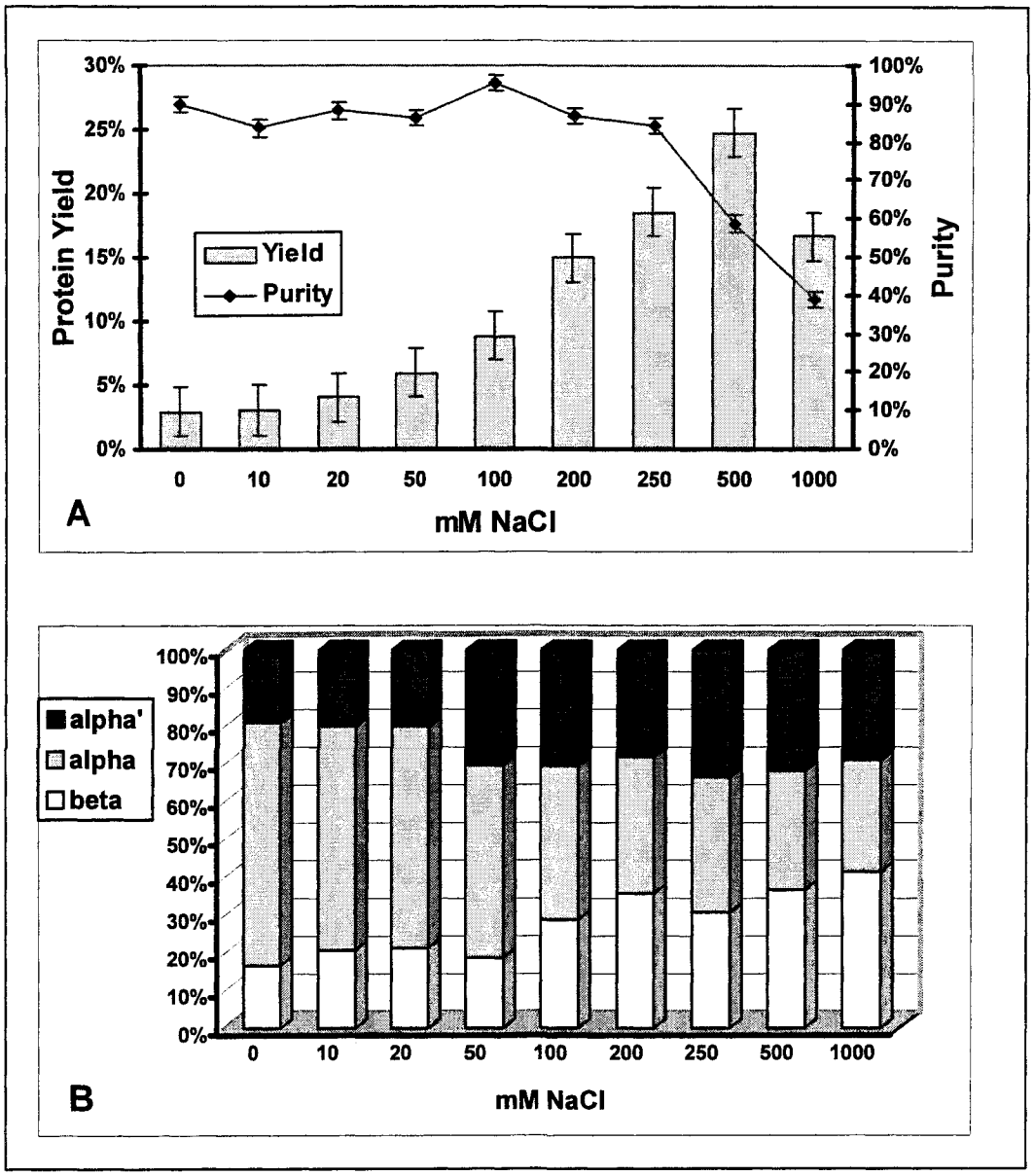

Figure 3-Fraction protein yields, purities (A), and β-conglycinin subunit compositions of the β-conglycinin-rich fractions due to salting-in (B).

200 mM NaCl, the glycinin contamination was about 60:40 acidic-to-basic subunits. At 250 to 1000 mM NaCl, the glycinin contamination contained nearly equal contents of acidic and basic subunits. This proportional decrease in the relative content of acidic polypeptides was probably due to the only glycinin component remaining in solution at low ionic strengths was the acidic polypeptide and, as the amount of salt increased, so did the glycinin contaminanation. The soulubilities of the acidic polypeptide components was reported by Liu and others (1999) and Dias and others (2003) and agree with our observations.

Whey fraction. Soy whey is a dilute stream that is normally considered to be waste and low solids and protein in this fraction are desirable. The losses of protein and solids in the whey fraction increased as NaCl concentration increased (Table 3). These losses began to increase at 50 mM NaCl and became quite large at 250 mM NaCl and higher. The primary loss is protein but increased salt levels contribute to the solids in the whey (Table 3, Fig. 4a). Total storage protein loss also increased as NaCl concentration increased. At 500 mM NaCl and above, the major storage protein lost to the why was β-conglycinin; for NaCl concentrations <500 mM, the major component was glycinin. This increase in protein loss was due to the salting-in effect due to the salt addition. This behavior is based on electrostatic interaction between the charged residues of the soy proteins and the ions of the salt in solution (Yuan 2002).

The subunit composition of β-conglycinin in the whey fraction was affected by NaCl concentration (Table 4, Fig. 4b, Fig. 8). At <100 mM NaCl and less, the only subunit of β-conglycinin detected in the whey fraction was the β subunit. This observation is inconsistent with reports of Maruyama and others (2002a) about the solubility behavior of native β-conglycinin β_3 homo-trimer. One possible explanation for our results is that these subunits are present as individual free soluble subunits and not as trimers, but this explanation does not fit the model presented by Thanh and Shibasaki (1979). A more probable explanation for this phenomenon at low ionic strengths is the model presented by Damodaran and Kinsella (1982) in which glycinin basic polypeptides preferentially form soluble aggregates with the β subunits of β-conglycinin. Utsumi and others (1984) has confirmed this preferential association between subunits. The whey fractions produced at 200 and 250 mM NaCl were composed of the α and β subunits. The last subunit to be salted-in was α', requiring 500 mM

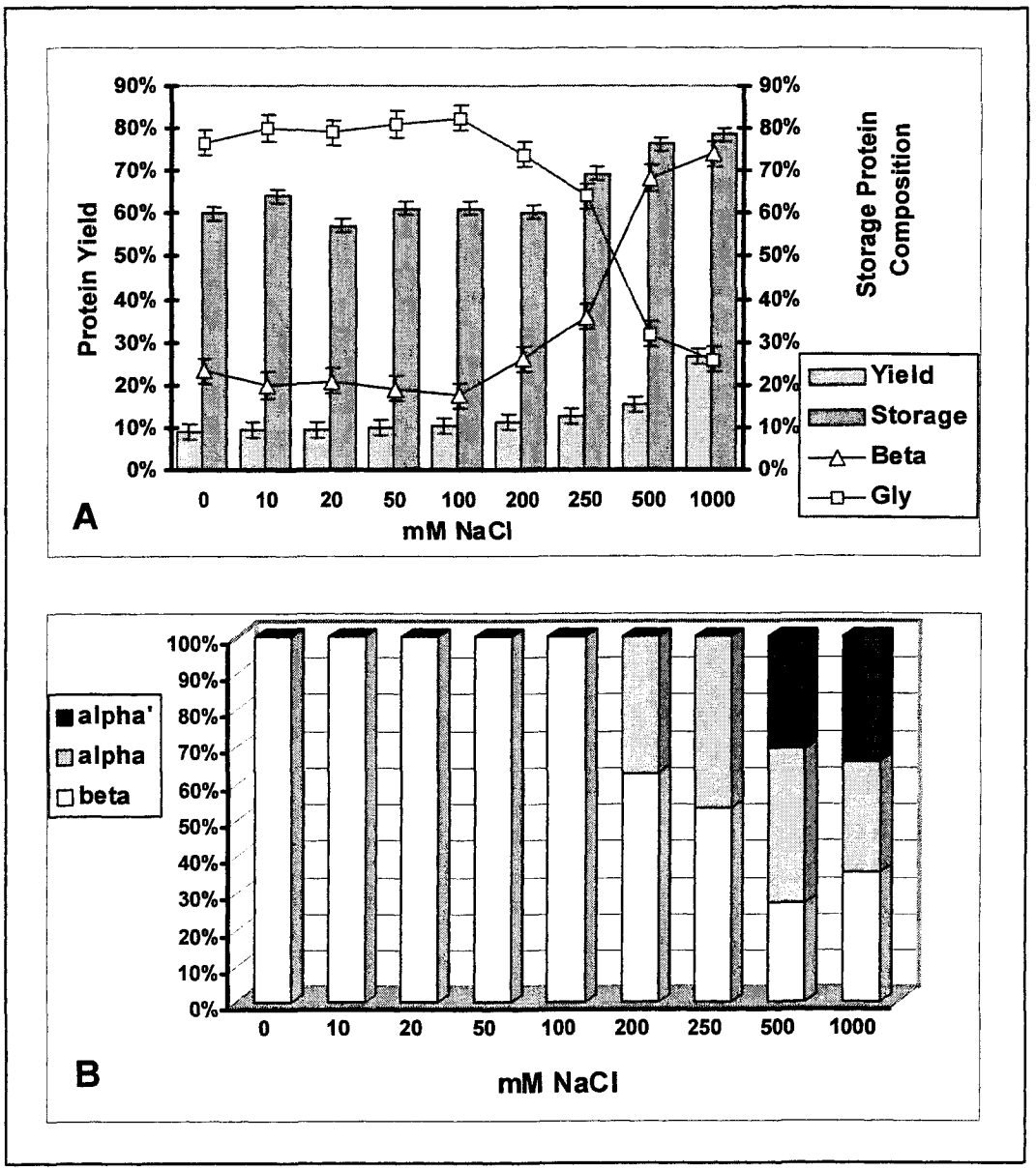

Figure 4-Total protein yields, storage protein yields and compositions (A), and β-conglycinin subunit distributions of the whey fractions due to salting-in (B). Beta denotes β-conglycinin and Gly, glycinin.

NaCl before appearing in the whey fraction. These preferential salting-in behaviors of the individual β-conglycinin subunits are somewhat conflicting with findings of Maruyama and others (2002a, 2002b) for β-conglycinin homo- and hetero-trimers, but their studies were carried out on purified trimers. In our case, we are dealing with mixtures of proteins, which are known to interact in solution (Damodaran and Kinsella 1982, Utsumi and others 1984, Petrucelli and Anon 1995). The glycinin subunit composition was also affected by NaCl concentration. At low (0-50 mM) and high (500 and 1000 mM) NaCl concentrations, the predominant glycinin components were the acidic polypeptides, which was not surprising since they are more soluble than the basic polypeptides (Liu and others 1999, Dias and others 2003). At 100 to 250 mM NaCl, the acidic and basic subunits were evenly distributed.

Salting-out effects

Starting protein extract. The supernatant obtained after precipitating the intermediate fraction was the starting point to evaluate the salting-out effects by changing dilution factors. The fractionation procedure followed is shown in Fig. 1 and the results for proximate analysis and protein compositions up to obtaining the intermediate fraction supernatant are shown in Table 1. This protein extract contained 41% of the solids originally present in the flour and approximately 25% of the protein originally present in the flour. About 81% of the protein in this extract was storage protein. About 93% of the storage protein present in this supernatant was β-conglycinin and the remaining 7% was glycinin. The objective of the dilution step was to salt-out the β-conglycinin and precipitating it.

β-Conglycinin-rich fraction. The β-conglycinin-rich fraction has the highest potential value and high yields and purity are desired. The yields of protein and solids for the β-conglycinin-rich fraction increased as dilution factor increased, with maximum yields of protein and solids achieved at 2-fold dilution (Table 5, Fig. 5a). The total storage protein contents of the β-conglycinin-rich fractions were >85% for all treatments with maximums at 0.5- and 1-fold dilutions. Fraction purity was highest at 1-fold dilution (89.1%), but all dilution factors gave >82% purities (Table 5, Fig. 5a). The main contaminant in all cases was glycinin. Significant cost savings can be achieved if the dilution factor is reduced from 2 to 1

because of 33% less final volume. Considering both protein yield and purity, there were no differences in the amount of β-conglycinin recovered between 2- and 1-fold dilution.

Table 5- Fraction yields and storage protein yields and compositions in the whey and β-conglycinin-rich fractions due to salting-out

Fraction/Dilution Factor	Fraction Yields (%)		Storage Protein Content and Composition (%)		
	Solids	Protein	Total	B-Conglycinin	Glycinin
β-Conglycinin-rich					
0	6.9[d]	2.7[d]	86.9[b]	86.0[b]	14.0[b]
0.5	8.2[c]	11.2[c]	90.7[a]	82.4[c]	17.6[a]
1	9.6[b]	16.7[b]	90.3[a]	89.1[a]	10.9[c]
2	10.7[a]	18.5[a]	85.1[c]	83.9[b]	16.1[b]
LSD	0.9	1.5	1.6	2.7	2.7
Whey					
0	41.8[a]	21.7[a]	89.4[a]	73.4[a]	26.6[d]
0.5	36.4[b]	16.0[b]	79.5[b]	61.3[b]	38.7[c]
1	37.7[b]	13.5[c]	77.1[b]	56.7[c]	43.3[b]
2	36.9[b]	12.7[c]	72.8[c]	30.7[d]	69.3[a]
LSD	2.9	1.4	2.8	3.9	3.9

Means within a column followed by different superscripts are significantly different at $P<0.05$.

The β-conglycinin subunit composition was also affected by dilution factor (Fig. 5b, Fig. 7). At 0-fold dilution, the principal subunit component of the β-conglycinin-rich fraction was β (45.3%). This was probably due to all the glycinin contaminant in the β-conglycinin-rich fraction at this dilution factor was comprised of basic polypeptides, and these two peptides preferentially associate (Utsumi and others 1984). There were no significant differences in β-conglycinin subunit compositions for 0.5- and 1-fold dilutions. At 2-fold dilution, the α subunit content significantly increased (37.1%). At 0.5- to 2-fold dilution, the β-conglycinin subunit compositions were approximately evenly distributed. The subunit composition of the contaminant glycinin was also affected by dilution. For no dilution, the contamination was only basic polypeptides of glycinin. For 0.5-fold dilution, the glycinin contaminant contained 84.2% basic polypeptide. These amounts of basic polypeptides at lower dilutions were probably due to the basic polypeptide content of the supernatant used as

starting point for this study had ~64% basic polypeptides (although low in total glycinin content) and the acidic polypeptides were lost to the whey fraction. For 1- and 2-fold dilution, the subunit distributions of the contaminant glycinin were approximately evenly distributed between acidic and basic polypeptides, which was consistent with our findings for the control procedure (250 mM NaCl and 2-fold dilution factor).

Whey fraction. The losses of protein and solids to the whey decreased as dilution increased (Table 5). This phenomenon was attributed to more effective salting-out of the protein that increased the yield of the β-conglycinin-rich fraction at higher dilution factors. Losses of total storage protein also decreased with more dilution (Fig. 6a). With no dilution, 89.4% of the protein lost in the whey was storage protein, in contrast to only 72.8% at 2-fold dilution. At 2-fold dilution, the main component of the storage protein lost was glycinin, whereas, the main component was β-conglycinin at all other dilution factors (Table 5, Fig. 6a). The β-conglycinin subunit composition in the whey fraction was affected by dilution factor (Table 6, Fig. 6b, Fig. 8). The α' subunit was absent from the whey obtained after 2-fold dilution at the salting-out step of the process. 1-Fold dilution resulted in unique subunit composition with 28.2, 45.1, and 26.7% contents for α', α, and β, respectively.

Table 6-Glycinin and β-conglycinin subunit compositions in the β-conglycinin-rich and whey fractions due to salting-out

Fraction/Dilution Factor	β-Conglycinin Subunit Composition (%)			Glycinin Subunit Composition (%)	
	α'	α	β	Acidic	Basic
β-Conglycinin-rich					
0	24.3^{b}	30.4^{d}	45.3^{a}	0.0^{d}	100.0^{a}
0.5	31.5^{a}	$33.2^{c,d}$	35.3^{b}	15.8^{c}	84.2^{b}
1	31.2^{a}	$35.4^{b,c}$	33.4^{b}	54.5^{a}	45.5^{d}
2	$28.7^{a,b}$	37.1^{a}	34.2^{b}	45.5^{b}	54.5^{c}
LSD	5.2	3.4	4.6	2.6	2.6
Whey					
0	33.1^{a}	35.4^{c}	31.5^{b}	76.8^{a}	23.2^{d}
0.5	28.2^{b}	51.3^{a}	20.5^{d}	61.8^{b}	38.2^{c}
1	28.2^{b}	45.1^{b}	26.7^{c}	50.0^{c}	50.0^{b}
2	0.0^{c}	46.8^{b}	53.2^{a}	27.6^{d}	72.4^{a}
LSD	2.3	3.9	4.0	3.3	3.3

Means within a column followed by different superscripts are significantly different at $P<0.05$

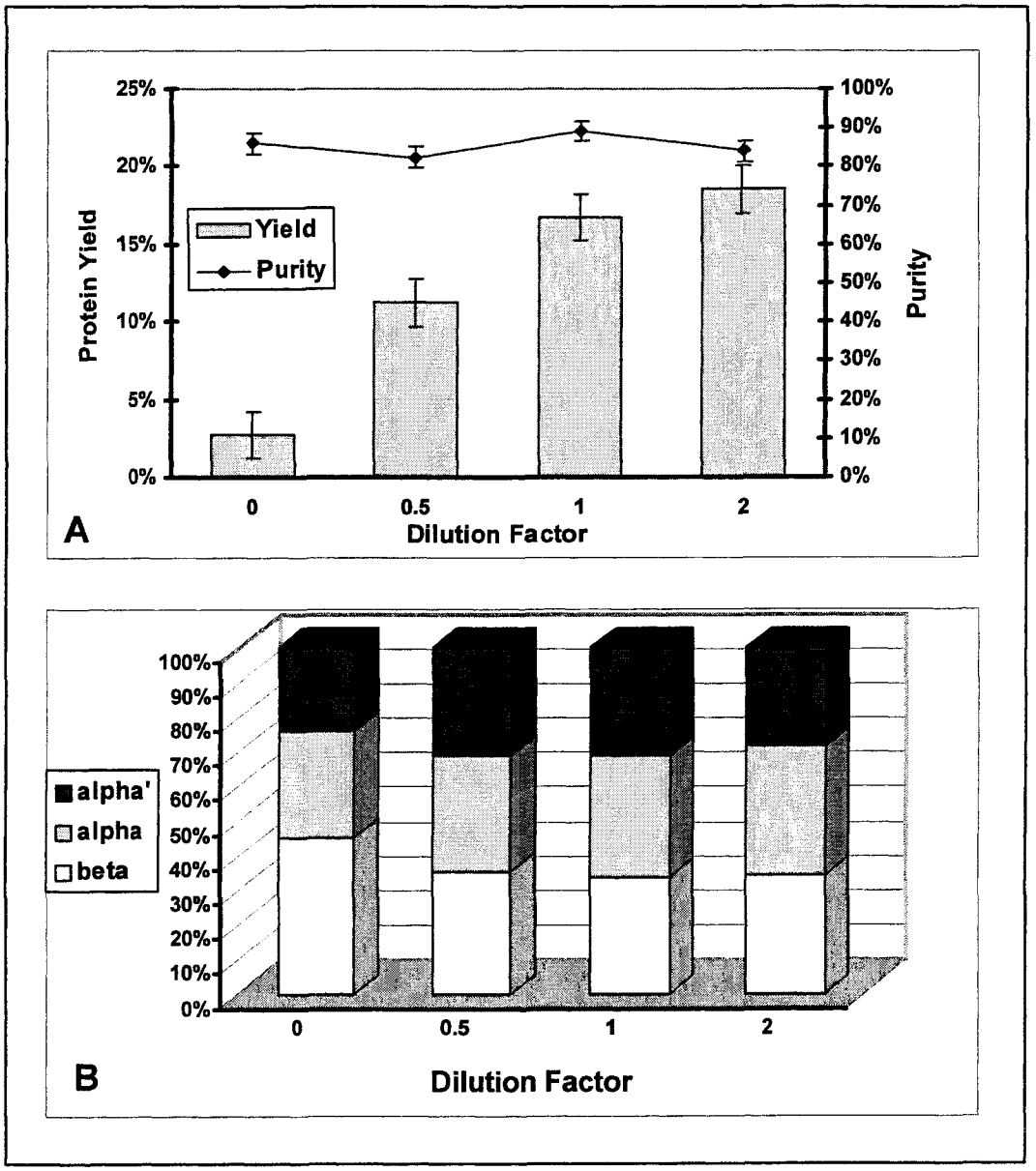

Figure 5-Fraction protein yields, purities (A), and β-conglycinin subunit distributions of the β-conglycinin-rich fractions due to salting-out (B).

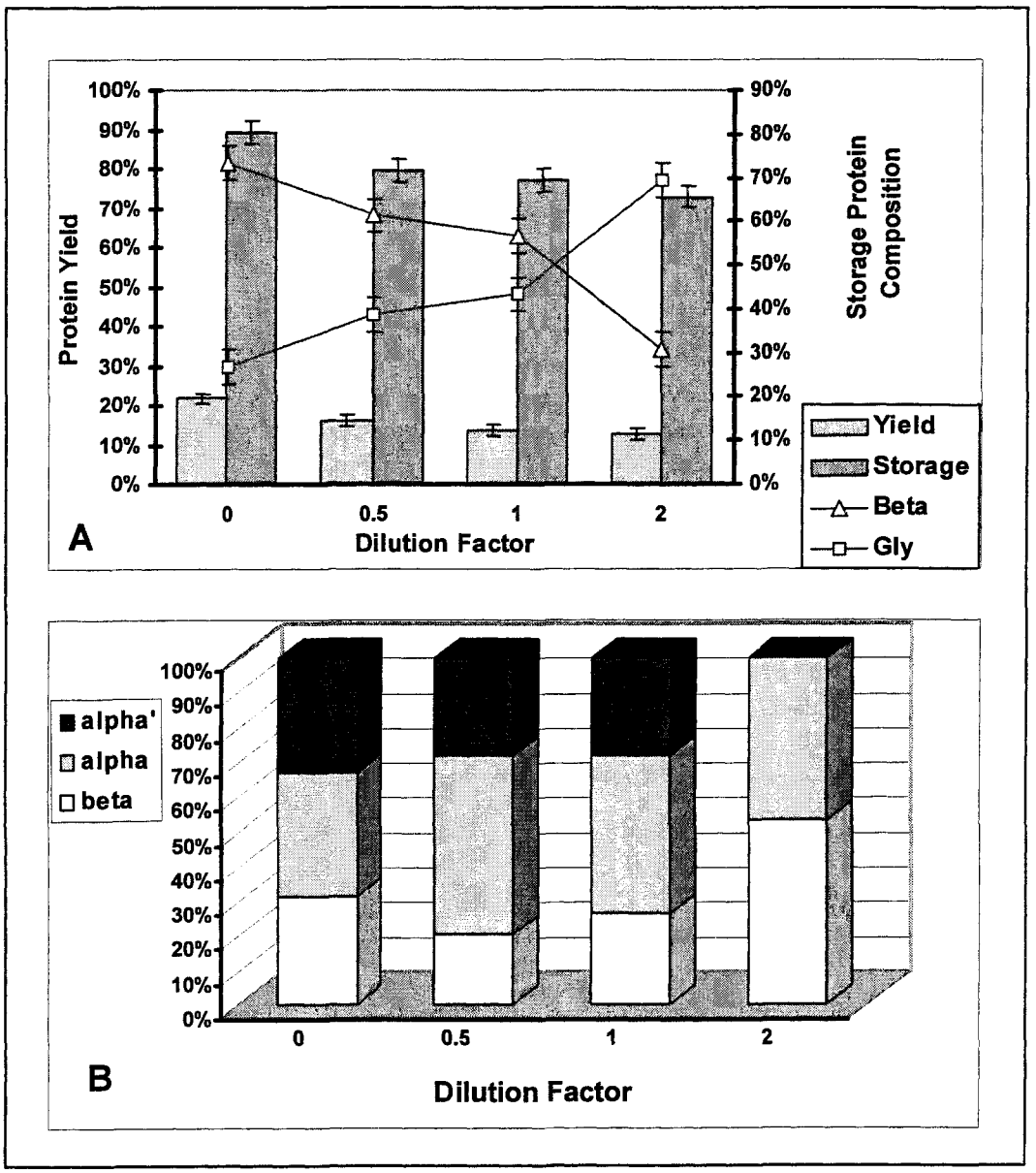

Figure 6-Total protein yields, storage protein yields and compositions (A), and β-conglycinin subunit distributions of the whey fractions due to salting-out (B). Beta denotes β-conglycinin and Gly, glycinin.

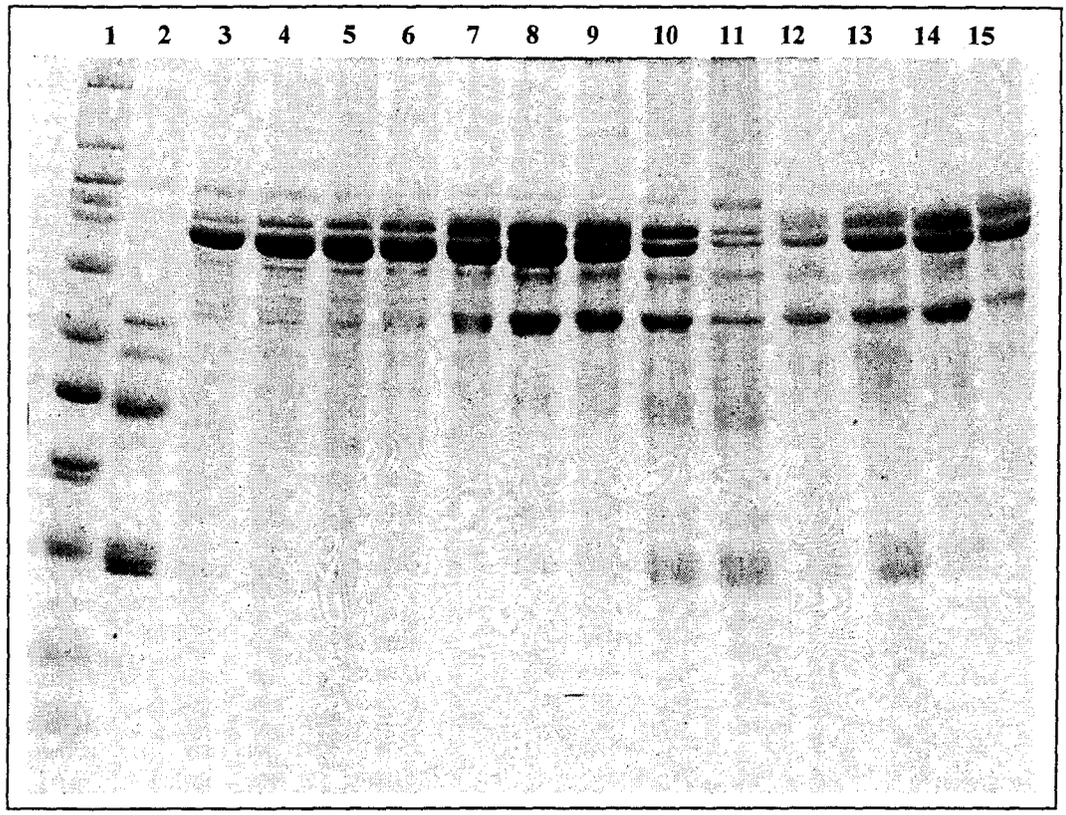

Figure 7-Urea-SDS-PAGE of β-conglycinin-rich fractions; Lane 1, molecular weight standard; lane 2 glycinin standard; lane 3-11, β-conglycinin-rich fractions after adding; 0, 10, 20, 50, 100, 200, 250, 500, and 1000 mM of NaCl, respectively; lane 12-14, β-conglycinin-rich fractions at 0, 0.5, and 1 factors of dilution, respectively; lane 15, β-conglycinin standard.

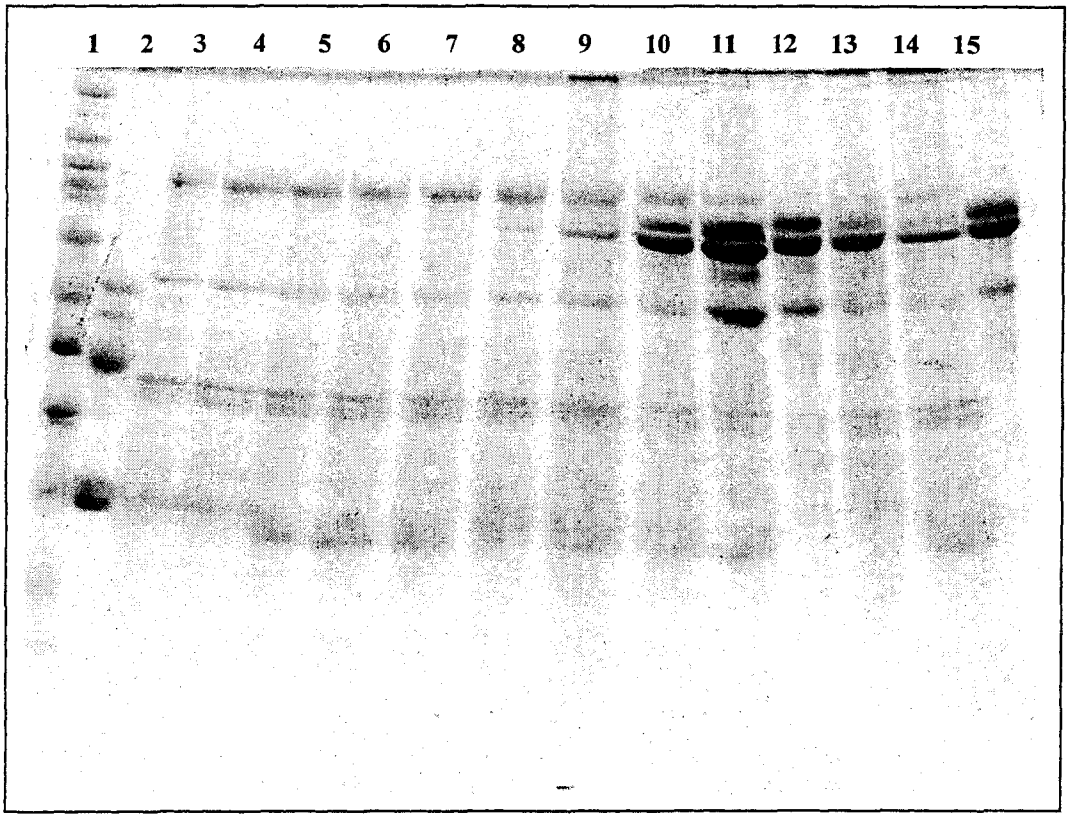

Figure 8-Urea-SDS-PAGE of whey fractions; Lane 1, molecular weight standard; lane 2, glycinin standard; lane 3-11, whey fractions after adding; 0, 10, 20, 50, 100, 200, 250, 500, and 1000 mM of NaCl, respectively; lane 12-14, whey fractions at 0, 0.5, and 1 factors of dilution, respectively; lane 15, β-conglycinin standard.

Conclusions

The optimum NaCl concentration for salting-in soy protein during fractionation was 250 mM. Considerable cost savings can be achieved by reducing the dilution factor from 2 to 1 to salt-out soy protein with a minimal yield loss, but with similar β-conglycinin protein recovery. The different trimers of β-conglycinin have different salting-in and salting-out behaviors. Those β-conglycinin trimers rich in β subunits are the first to salt-in and the last to salt-out. Those β-conglycinin trimers rich in α subunits salts-in and salts-out second, and those β-conglycinin trimers rich in α' subunits are the last to salt-in and the first to salt-out. The glycinin basic polypeptide was generally associated with the β subunit of β-conglycinin. When using 250 mM NaCl for salting-in and 1-fold dilution for salting-out, slightly lower yields of solids and total protein were achieved, compared to 2-fold dilution for salting-out. When yield of storage protein and purity of the β-conglycinin fraction were factored-in, both processes yielded the same amount of this particular storage protein (~13%) with similar subunit composition. Losses of protein and solids to the whey fractions were also the same for both dilutions.

Acknowledgment

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CHAPTER 4. FRACTIONATING SOYBEAN STORAGE PROTEINS USING CALCIUM AND $NAHSO_3$

A paper to be submitted to the *Journal of Food Science*

Nicolas A. Deak, Patricia A. Murphy, and Lawrence A. Johnson

Abstract

Individual soybean storage proteins have been identified as food ingredients having nutraceutical properties, especially β-conglycinin. Several methods to fractionate soy proteins on industrial scale have been published, but there are no commercial products of fractionated soy proteins. The present study addresses this problem by using calcium salts to achieve glycinin-rich and β-conglycinin-rich fractions in high yield and purity. A well-known three-step fractionation procedure that uses SO_2, NaCl and pH adjustments was evaluated with $CaCl_2$ and $CaSO_4$ as substitutes for NaCl. The use of $CaSO_4$ was limited because of its low solubility. Calcium was effective in precipitating residual glycinin (after precipitating a glycinin-rich fraction) into the intermediate fraction at 5 to 10 mM $CaCl_2$ and pH 6.4, eliminating the contaminant glycinin from the β-conglycinin-rich fraction. Purities of 100% β-conglycinin with unique subunit compositions were obtained after prior precipitating the glycinin-rich and intermediate fractions. The use of 5 mM SO_2 in combination with 5 mM $CaCl_2$ in a two-step fractionation procedure produced the highest purities in the glycinin-rich (85.2%) and β-conglycinin-rich (80.9%) fractions. The glycinin in the glycinin-rich fraction had a unique acidic (62.6%) to basic (37.4%) subunit distribution. The β-conglycinin-rich fraction was approximately evenly distributed among the β-conglycinin subunits (30.9, 35.8, and 33.3%, for α', α, and β subunits, respectively). Yields of solids and protein as well as purities and subunit compositions were highly affected by pH and SO_2 and $CaCl_2$ concentrations.

Introduction

Soy protein isolates (SPI) contain >90% protein on dry-weight basis (N x 6.25) and commercial yields are approximately 33% of the soy flour mass, corresponding to approximately 60% of the protein (Sathe and others 1989). Recently recognized potential

health benefits (Messina 1997), as well as, demand by enlightened consumers for new soy-protein-based food products and ingredients, are now driving soy protein research and commercial development.

Soybean proteins are not a homogeneous group. Soy proteins have been traditionally classified by their sedimentation coefficients into four groups, 2S, 7S, 11S, and 15S with peak molecular weights of approximately 25, 126-171, 350, and 600 kDa, respectively. A typical commercial soy isolate process yields approximately 22% 2S, 37% 7S, 31% 11S, and 11% 15S proteins, but these amounts may vary significantly depending on variety, crop year, handling and thermal treatment (Liu 1999). Furthermore, these sedimentation-coefficient-based protein groups are also heterogeneous mixtures of different proteins. The major storage proteins in soybeans are glycinin (also referred to as 11 S) and β-conglycinin (also referred to as 7S). Consumption of β-conglycinin has been identified as lowering cholesterol and blood triglycerides (Adams and others 2004, Duranti and others 2004, Manzoni and others 2003). β-Conglycinin is a trimeric protein about 126-171 kDa in molecular wieght. This protein is composed of three subunits: α' (~57 kDa), α (~57 kDa), and β (~42 kDa) (Thanh and Shibasaki 1977). Several different combinations of these subunits have been found (α′ β_2, α β_2, αα′β, $\alpha_2\alpha'$, $\alpha_2\beta$, α_3, and β_3) providing heterogeneity (Mills and others 2001).

Complex laboratory procedures have been developed to fractionate these major soybean storage proteins from each other. Some techniques are difficult to reproduce because small variations in the procedures significantly alter the compositions of the fractions obtained. Probably the most frequently used laboratory procedure for fractionating glycinin and β-conglycinin is one described by Thanh and Shibasaki (1976). This method uses β-mercaptoethanol and complete purification is achieved after several column chromatography steps. This process is much too complex to produce food ingredients. Other experimental methods to fractionate soy storage proteins have been reported by Roberts and others (1965), Eldrige and others (1967), Nagano and others (1992), Wu and others (1999), and Thiering and others (2001) as well as others. These fractionation methods are too expensive for industrial purposes and, in some cases, utilize chemicals that are not food-grade.

Several patents claim methods for glycinin and β-conglycinin fractionation. Howard and others (1983) disclosed a method to fractionate soy proteins by means of pH adjustments

in the presence of sulfite ions and water-soluble salts. Lehnhardt and others (1983) disclosed a method to fractionate glycinin and β-conglycinin from an isoelectrically precipitated mixture. Hirotsuka and others (1988) disclosed a method to fractionate soy proteins by reduction and isoelectric precipitation achieving ingredients with improved functional properties. Masahiko and others (1994), Samoto and others (1996), Savolainen and others (1999), and Kohno and others (2001) disclosed methods to fractionate soy proteins claiming industrial uses for the fractions obtained. Using a slightly different approach, Bringe (2001) disclosed a method to produce food ingredients with increased proportions of glycinin or β-conglycinin by using soybean varieties that were genetically modified to be rich in one protein.

Several studies have focused on the effects of calcium salts to fractionate or purify soy proteins and calcium salts are commonly used to produce tofu. The first report was by Koshiyama (1965) where 250 mM of $CaCl_2$ was utilized to purify the supernatant remaining after cold precipitation of a glycinin-rich fraction, but yields and purities of this crude β-conglycinin fraction were not determined, and the author suggested that further column chromatography purification was required. Another method was reported by Saio and others (1973) where 10 mM of $CaCl_2$ was added to the extraction buffer and defatted soybean meal was first extracted to obtain a 7S-rich supernatant and the precipitate was redisolved and centrifuged to obtain an 11S-rich fraction. The purities on ultracentrifugal basis were about 60%, which were much lower than purities reported for other fractionation methods.

Other researchers have focused on determining specific mechanisms of Ca^{2+} binding by the 7S fraction of soybean proteins (Rao and Rao 1976), interaction of 11S fraction of soybean protein with Ca^{2+} (Sakakibara and Noguchi 1977), the effect of pH on Ca^{2+} binding by soybean proteins (Kroll 1984), and the effect of pH and Ca^{2+}-induced associations of soybean proteins (Yuan and others 2002). This later study concluded that the amount of Ca^{2+} necessary to precipitate a mole of β-conglycinin was much larger than the amount required to precipitate a mole of glycinin, and related these findings to the charge density per surface area of the proteins. This work suggested that differential precipitation of these two storage proteins could be achieved by changing the pH of the medium with the presence of Ca^{2+} ions.

We have previously reported on the effects of NaCl on the salting-in and salting-out of the β-conglycinin-rich fraction during soybean storage protein fractionation (Deak and others 2004). The objectives of the present work were to determine if NaCl could be replaced with calcium salts in fractionating soy proteins and to develop new simplified fractionation methods by utilizing calcium salts as fractionating agents. We further hypothesized that the addition of a reducing agent in our procedure would significantly decrease the co-precipitation problems experienced in previous studies.

Materials and Methods

Soy flour

Air-desolventized, hexane-defatted white flakes (IA 2020 variety, 1999 harvest) were extracted in the pilot plant of the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). The defatted flakes were milled with a Krups grinder (Distrito Federat, Mexico) to achieve 100% of the material passing through a 50-mesh screen by using small quantities (10 g) to preserve the native protein state. The protein content of the flour was 57.3% on dry-weight basis with 93.8 protein dispersibility index (PDI) as determined by Silliker Laboratories (Minnetonka, MN). The flour was stored in sealed containers at 4°C until used.

Soy protein fractionation (Wu procedure)

The soy protein fractionation procedure utilized as the control procedure was reported by Wu and others (1999), which is a modification of Nagano and others (1992) (Fig. 1). About 200 g defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the slurry was stirred for 1 h. After centrifuging at 14,300 x g and 15°C for 30 min, the protein extract was decanted and the mass of insoluble fiber residue was determined and sampled for proximate composition. Sufficient $NaHSO_3$ was added to the protein extract to achieve 10 mM SO_2 and the pH was adjusted to 6.4 with 2N HCl. The resulting slurry was stored at 4°C for 12-16 h and centrifuged at 7,500 x g and 4°C for 20 min. A glycinin-rich fraction was obtained as the precipitated curd. This fraction was redisolved in de-ionized water and adjusted to pH 7 with

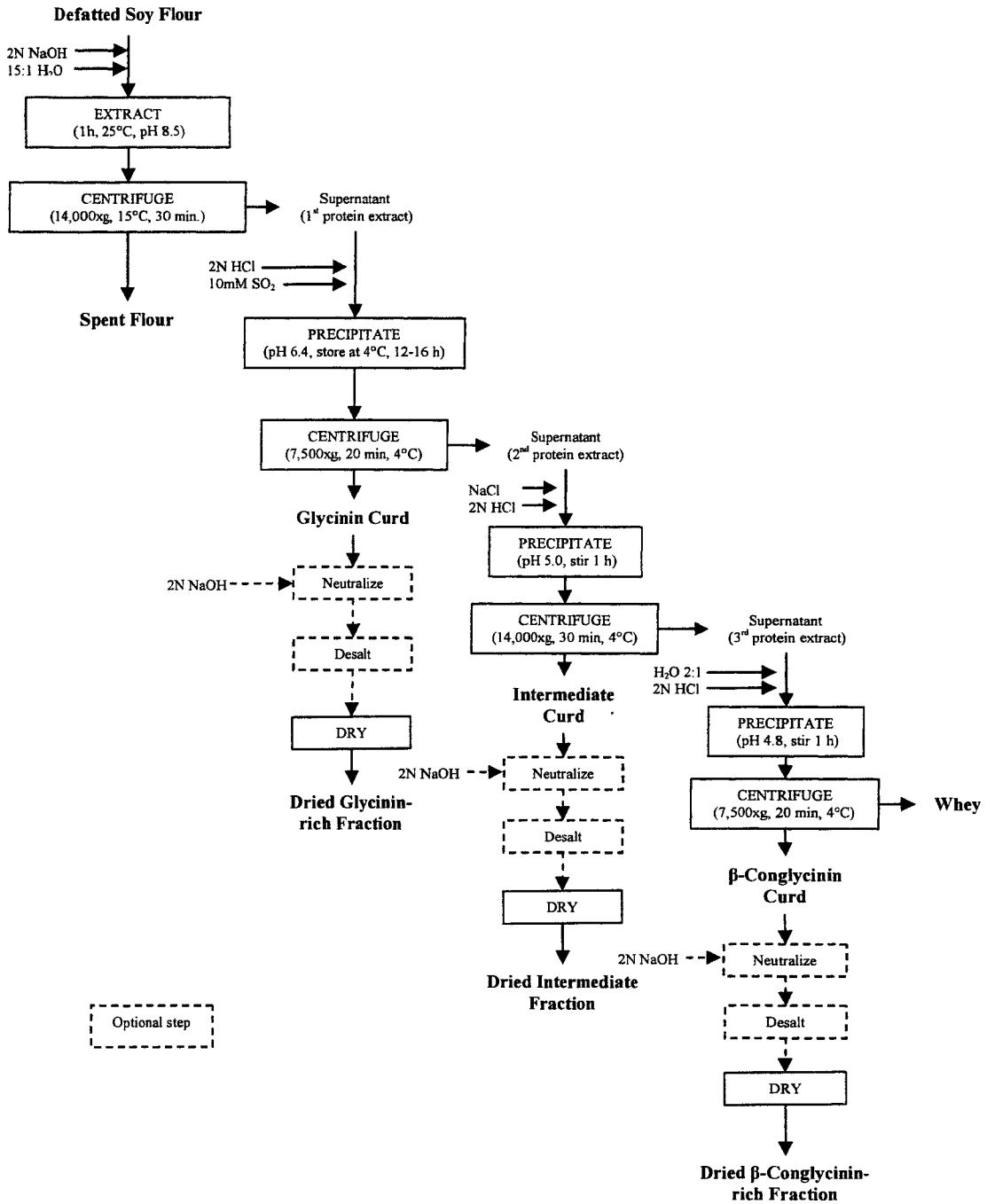

Figure 1-Flow diagram of Wu's fractionation procedure (Wu and others 1999).

2N NaOH, adjusted to pH 7 with 2N NaOH, sampled, and stored in sealed containers at -80°C until freeze-dried. The supernatant, protein extract enriched in β-conglycinin, was adjusted to 250 mM NaCl and pH 5 with 2N HCl, and the slurry was stirred for 1 h. The slurry was then centrifuged at 14,000 x g and 4°C for 30 min. An intermediate fraction (a mixture of glycinin and β-conglycinin) was obtained as the precipitated curd, and this fraction was treated as described for the glycinin-rich fraction. The supernatant was diluted with de-ionized water in a ratio of 2 times its volume and the pH was adjusted to 4.8. The resulting slurry was centrifuged at 7,500 x g and 4°C for 20 min. A β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as described for the glycinin-rich fraction, and the amount of supernatant (whey) was determined and sampled for proximate composition.

Effects of Calcium on the Wu soy protein fractionation procedure

To study the effects of replacing NaCl with calcium salts, the control fractionation scheme described above was followed with modifications. The supernatant after precipitating the glycinin-rich fraction, the starting point for this study, was divided into nine aliquots of ~150 g each. To each aliquot, we added sufficient $CaSO_4$ or $CaCl_2$ to replace the NaCl and obtain 5, 10, 20, 50, 100, 200, 500, and 1000 mM Ca^{2+} and the previously described fractionation procedure was carried out. All procedures for both calcium salts were duplicated and means reported.

Modified Wu soy protein fractionation procedure

The control soy protein fractionation procedure described above was modified by introducing changes after obtaining the glycinin-rich fraction and starting with the resulting supernatant. The protein extract obtained after precipitating the glycinin-rich fraction was divided into nine aliquots of ~150 g each. One aliquot had no salt and the control fractionation procedure was followed as described before with the exception that no dilution step was employed to precipitate the β-conglycinin-rich fraction. The other eight aliquots were divided into two groups. $CaCl_2$ was added to obtain concentrations of 5, 10, 20, and 50 mM Ca^{2+} in one group and the control fractionation procedure was followed as described

above with the exception that no dilution step was employed to obtain the β-conglycinin-rich fraction, In the second group, $CaCl_2$ was added to obtain concentrations of 5, 10, 20, and 50 mM Ca^{2+} and the pH was adjusted to 6.4 and the slurry was stirred for 1 h. The slurries were centrifuged at 14,000 x g and 4°C for 30 min. An intermediate fraction (mixture of glycinin and β-conglycinin) was obtained as the precipitated curd. The supernatant, protein extract resulting from the intermediate fraction precipitation, was adjusted to pH 4.8 with 2N HCl and without the addition of extra water. The resulting slurry was centrifuged at 7,500 x g and 4°C for 20 min. A β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as described for the glycinin-rich fraction of the control procedure, and the amount of supernatant (whey) was determined and sampled for proximate composition. Each treatment was duplicated and means reported.

A new simplified soy protein fractionation procedure

Based on the results obtained by using the above described modified Wu fractionation method, we tested a new soy protein fractionation procedure. About 200 g defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 1 h. After centrifuging at 14,300 x g and 15°C for 30 min, the supernatant enriched in β-conglycinin was decanted, and the mass of insoluble fiber residue was determined and sampled for proximate composition. This extract was divided into eight aliquots of about 250 g each. A different treatment was applied to each of these extracts.

One aliquot was treated by using the control Wu fractionation procedure described above (Wu and others 1999). The second extract was treated with no salt and the pH was adjusted to 6.4 with 2N HCl. The resulting slurry was stored at 4°C for 12-16 h and centrifuged at 14,000 x g and 4°C for 30 min. A glycinin-rich fraction was obtained as the precipitated curd, neutralized, and treated as described above. The supernatant was adjusted to pH 4.8 with HCl, stirred for 1 h, and then centrifuged at 14,000 x g and 4°C for 30 min. A β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as

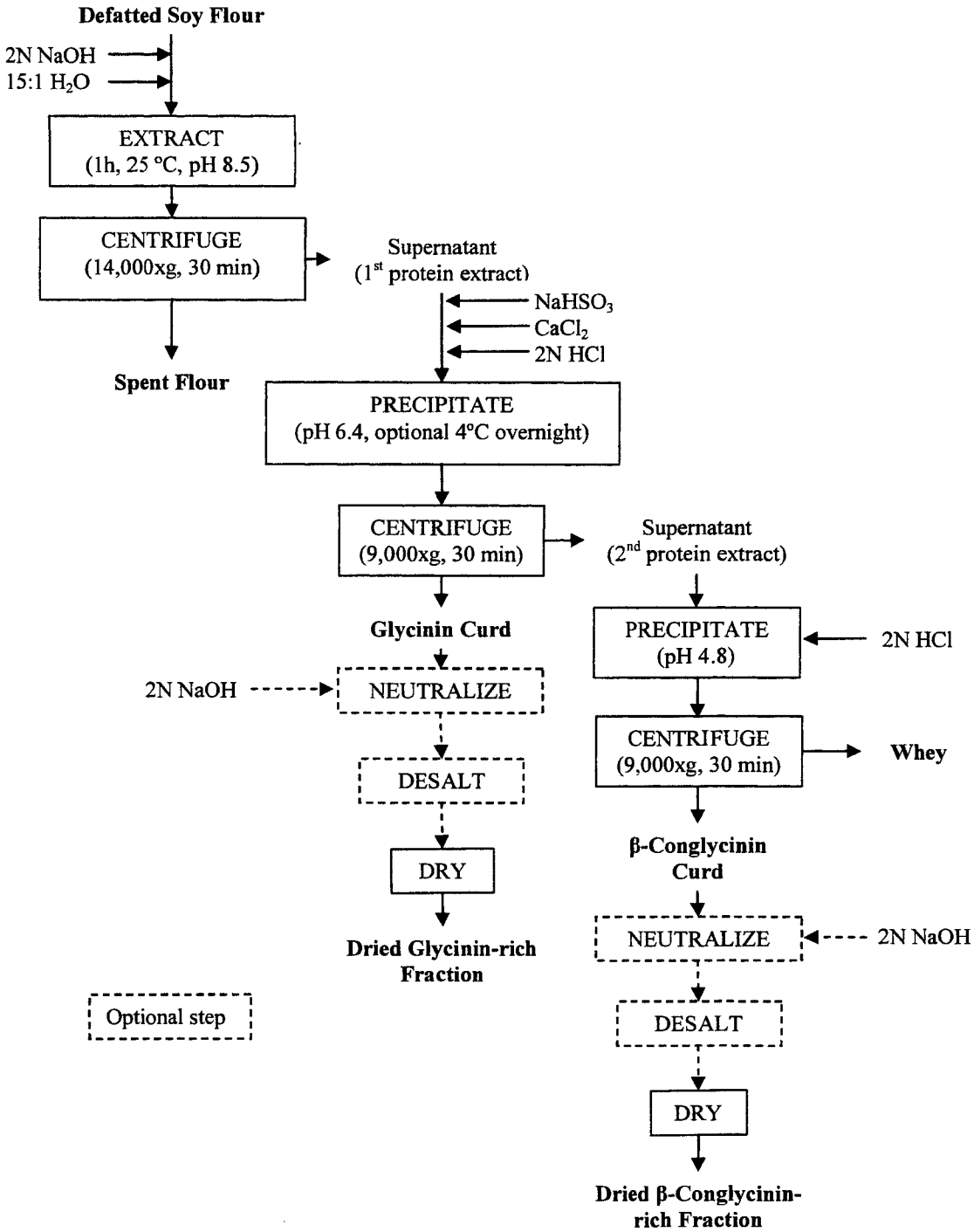

Figure-2. Flow diagram of the new simplified soy protein fractionation procedure.

described above and the amount of supernatant (whey) was determined and sampled for proximate composition. This procedure was identified as the 00 control.

The remaining six aliquots were treated as described above for the simplified fractionation procedure (00 control) but with the following modifications introduced before adjusting to pH 6.4. A third aliquot was treated with no $NaHSO_3$ and sufficient $CaCl_2$ to obtain 5 mM Ca^{2+}. A fourth aliquot was treated with no $NaHSO_3$ and sufficient $CaCl_2$ to obtain 10 mM Ca^{2+}. A fifth aliquot was treated with $NaHSO_3$ to achieve 5 mM SO_2 and $CaCl_2$ to obtain 5 mM Ca^{2+}. A sixth aliquot was treated with sufficient $NaHSO_3$ to achieve 5 mM SO_2 and $CaCl_2$ to obtain 10 mM Ca^{2+}. A seventh aliquot was treated with sufficient $NaHSO_3$ to achieve 10mM SO_2 and $CaCl_2$ to obtain 5 mM Ca^{2+}. An eighth aliquot was treated with sufficient $NaHSO_3$ to achieve 10 mM SO_2 concentration and $CaCl_2$ to obtain 10 mM Ca^{2+}. All fractions obtained were adjusted to pH 7.0 with 2N NaOH and stored at -80°C until freeze-dried. All treatments were duplicated and means reported.

Freeze-drying

All samples were kept at -80°C until freeze-dried in a Vartis Ultra 35 (Gardnier, NY) freeze-dryer. The shelves cooled at -20°C and vacuum was then applied while the temperature was held constant until the vacuum dropped to 100 mTorr. Secondary drying was achieved by heating the freeze-dryer shelves to 26°C at high vacuum. The complete freeze-drying cycle lasted 120 h. Samples were placed into sealed containers until analyzed.

Proximate analysis

Nitrogen contents of the soy flour, isolated products, and byproduct streams were measured by using the combustion or Dumas method (AOAC 1995a) with a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). Nitrogen values were converted to Kjeldahl nitrogen by using the conversion formula of Jung and others (2003). The conversion factor used to convert percentage nitrogen to protein content was 6.25. Moisture was determined by oven drying for 3 h at 130°C (AOAC 1995b). Mass balances of solids and protein were determined for all treatments. All measurements were determined at least three times and means reported.

Urea-SDS-PAGE

Urea-sodium dodecylsulfate-polyacrylamide gel electrophoresis (urea-SDS-PAGE) was performed by using the methods of Rickert and others (2004) to quantify the protein composition profiles of the fractions. Lanes were loaded with 45 μg/well of protein. The proteins were identified by using a pre-stained SDS-PAGE molecular-weight standard, low range (Bio-Rad Laboratories, Hercules, CA). Glycinin and β-conglycinin subunit bands were confirmed by using purified standards produced according to methods of O'Keefe and others (1991). Densitometry was carried out by using the Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). SDS-PAGE results were calculated as % composition; total storage protein in a given fraction = [(sum of storage protein subunit bands)/(sum of all bands)] x 100, fraction purity/composition = [(sum of subunit bands)/(sum of storage protein bands)]; and subunit composition of a specific protein = [(subunit band)/(sum of subunits for the specific protein)]. All measurements were replicated at least four times and means reported.

Statistical analysis

The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM), and the Least Significant Differences (LSD) were calculated at the 5% level to compare treatment means using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

Results and Discussion

Effects of calcium on the Wu fractionation procedure

The yields of solids for the glycinin-rich fractions were 13.1% ± 0.6 and 12.9% ± 0.3 when using $CaSO_4$ and $CaCl_2$, respectively, and the total protein yields for this fraction were 23.5% ± 2.1 and 24.8% ± 1.3, respectively. These results were similar to those reported by Wu and others (1999) and Rickert and others (2004) for the control Wu procedure and much higher than those reported by Nagano and others (1992). The starting point for both studies

was the supernatant obtained after the glycinin-rich fraction precipitation, which had solids yields of 55.9% ± 2.1 and 56.2% ± 2.8, and protein yields of 53.1% ± 1.2 and 53.0% ± 2.0 when using $CaSO_4$ and $CaCl_2$, respectively.

Intermediate fraction. The yields of solids in the intermediate fraction increased as $CaSO_4$ concentration increased (Table 1). This increase in yield of solids was attributed to salt because of the low solubility of $CaSO_4$. The yields of protein, on the other hand, remained approximately constant and were not affected by $CaSO_4$ concentration. No salting-in was observed, most of the protein present in the slurry was recovered in this fraction.

Solids yields for the intermediate fraction decreased with $CaCl_2$ concentration increased probably due to the higher solubility of this salt (Table 1). At high salt levels, the salt composed a significant proportion of the solids. The yields of protein remained approximately constant, did not depend upon $CaCl_2$ concentration, and were slightly higher than the yields obtained with $CaSO_4$ (significant at $p<0.1$, but not significant at $p<0.05$). Both the yields of solids and protein were higher than those reported by Wu and others (1999) and by Rickert and others (2004) for all calcium levels and even for the 0 addition. The results for the treatment without calcium salt were similar to those we reported previously (Deak and others 2004).

β-conglycinin-rich fraction. The yields of solids and protein in the β-conglycinin-rich fraction for both calcium treatments were very low (Table 1). This was a logical consequence of the high yields of solids and protein recovered in the intermediate fraction. Neither calcium salt was effective in fractionating soy proteins by merely adding these salts to the fractionation procedure developed by Wu and others (1999). The yields of solids and proteins for all treatments were much lower than those reported earlier by Wu and others (1999) and Rickert and others (2004), where NaCl was used as the salting-in agent. The 0 salt addition treatment gave similar yields of solids as those that we reported in an earlier study (Deak and others 2004). The addition of only 5 mM Ca^{2+} at pH 5.0 was sufficient to precipitate most of the remaining protein into the intermediate fraction. The low protein recovery, even after diluting the slurry, remained very low also suggesting that Ca^{2+} salts

Table 1-Yields of solids and total protein for the Wu soy protein fractionation procedure when replacing NaCl with $CaSO_4$ and $CaCl_2$[a]

Fraction/Treatment	$CaSO_4$		$CaCl_2$	
	Solids (%)	Protein (%)	Solids (%)	Protein (%)
Intermediate				
0 mM Ca^{2+}	25.9^{e}	41.8^{a,b}	26.7^{a,b}	42.2^{b}
5 mM Ca^{2+}	27.8^{e}	42.4^{a}	23.9^{b,c}	46.6^{a}
10 mM Ca^{2+}	28.5^{e}	42.3^{a}	27.7^{a}	46.0^{a,b}
20 mM Ca^{2+}	31.9^{d,e}	41.4^{a,b}	24.2^{b,c}	44.6^{a,b}
50 mM Ca^{2+}	32.1^{d}	42.6^{a}	25.0^{a,b,c}	44.8^{a,b}
100 mM Ca^{2+}	35.7^{c}	42.1^{a}	25.1^{a,b,c}	45.3^{a,b}
200 mM Ca^{2+}	37.2^{b,c}	42.3^{a}	23.3^{c}	43.9^{a,b}
500 mM Ca^{2+}	40.2^{b}	40.1^{b}	19.4^{d}	42.7^{b}
1000 mM Ca^{2+}	45.4^{a}	42.7^{a}	15.5^{e}	42.8^{b}
LSD	3.6	2.0	2.9	3.5
β-Conglycinin-rich				
0 mM Ca^{2+}	3.1^{a}	3.3^{a}	4.5^{a}	2.9^{a}
5 mM Ca^{2+}	2.0^{b}	0.3^{b}	1.9^{b}	0.4^{b}
10 mM Ca^{2+}	1.6^{b,c}	0.4^{b}	1.6^{b}	0.4^{b}
20 mM Ca^{2+}	1.1^{b,c}	1.1^{b}	1.4^{b}	0.5^{b}
50 mM Ca^{2+}	1.0^{c}	0.1^{b}	0.9^{b}	0.5^{b}
100 mM Ca^{2+}	0.8^{c}	0.2^{b}	1.1^{b}	0.7^{b}
200 mM Ca^{2+}	0.9^{c}	0.2^{b}	0.7^{b}	0.6^{b}
500 mM Ca^{2+}	1.6^{b,c}	1.2^{b}	1.7^{b}	1.2^{b}
1000 mM Ca^{2+}	1.7^{b,c}	1.1^{b}	2.0^{b}	1.1^{b}
LSD	1.0	1.1	1.4	0.9
Whey				
0 mM Ca^{2+}	29.7^{a}	9.4^{e}	28.1^{f}	9.9^{b}
5 mM Ca^{2+}	27.9^{a}	11.2^{b}	30.7^{e}	10.5^{a,b}
10 mM Ca^{2+}	29.5^{a}	11.1^{b}	31.2^{e}	11.6^{a,b}
20 mM Ca^{2+}	25.1^{b}	11.2^{b}	32.0^{d,e}	11.6^{a,b}
50 mM Ca^{2+}	24.8^{b}	11.2^{b}	31.9^{e}	12.1^{a}
100 mM Ca^{2+}	23.3^{b}	12.0^{a,b}	33.3^{d}	11.9^{a}
200 mM Ca^{2+}	20.9^{c}	11.2^{b}	34.8^{c}	10.5^{a,b}
500 mM Ca^{2+}	17.9^{d}	12.2^{a,b}	39.3^{b}	12.0^{a}
1000 mM Ca^{2+}	13.1^{e}	13.5^{a}	42.6^{a}	11.9^{a}
LSD	2.4	1.9	1.4	2.0

[a] n=2. Means within a column for each fraction followed by different superscripts are significantly different at $p<0.05$. LSD denotes least significant difference at $p<0.05$.

do not salt-in the protein, but rather act through site-specific binding, a phenomena reported by Kroll (1984).

Kroll (1984) studied the effects of pH on Ca^{2+} binding by soy proteins and concluded that pH strongly affects the extent of Ca^{2+} binding because hydrogen ions compete with calcium ions for the same binding sites on the protein molecule. These binding sites where identified as being the side-chain carboxyl groups of aspartic and glutamic acid residues and with the imidazole group of histidine residues. The affinity of the binding sites for calcium ions increased as pH increased over the range of 4 to 9, since the binding constant increased from $0.07x10^3$ at pH 4 to $6.38x10^3$ at pH 9. A small change in pH resulted in a large change in the amount of Ca^{2+} bound. At low pH (4-5), calcium ions are weakly bound because they are in direct competition with H^+ for the binding sites in the protein molecule. At high pH (8-9), calcium ions are strongly bound and the binding sites have high affinity for calcium ions; at this pH, the carboxyl and imidazole groups are completely deprotonated.

Whey fraction. The solids loss into the whey fraction decreased as $CaSO_4$ concentration increased (Table 1). This was because most of the salt was already precipitated during recovery of the intermediate fraction and because the yield of solids in the whey deceased. On the other hand, the amount of protein lost to the whey remained relativelt constant. At 1000 mM $CaSO_4$, this loss was slightly higher (significant at $p<0.1$ LSD=1.1%), but much lower than for the same NaCl concentration (Deak and others 2004). Similar results were observed for protein recovery when $CaCl_2$ was used. The solids lost to the whey increased as $CaCl_2$ concentration increased since this salt is more soluble than $CaSO_4$ (Table 1).

Modified Wu soy protein fractionation procedure

To further understand how Ca^{2+} can be used as a soy-protein-fractionating agent we introduced several changes to the method of Wu and others (1999). Because of its higher solubility, we continued using $CaCl_2$. Two different pHs were used (5.0 and 6.4) to precipitate the intermediate fraction. pH strongly affects Ca^{2+} binding to soy proteins (Kroll 1984) and protein solubility behavior can be related to the charge density (charges per unit of surface area) (Yuan and others 2002). Yuan and others 2002 showed that the amount of Ca^{2+}

necessary to precipitate a mole of β-conglycinin was much greater (164 number of calcium ions/mole of protein) than the amount required to precipitate the glycinin fraction (79 number of calcium ions/mole of protein), and related these findings to an amino acid composition based calculation of the charge density per surface area of the proteins with -0.47 e^-/nm^2 for β-conglycinin and -0.17 e^-/nm^2 for glycinin. The amount of calcium ions required to precipitate these proteins increased to 1000 and 435 for β-conglycinin and glycinin, respectively, with the addition of 0.1M NaCl. This work suggested that differential precipitation and complete partitioning of these two storage proteins could be achieved by changing the pH of the medium in the presence of Ca^{2+} ions.

Table 2-Yields of solids and protein and protein composition of fractions obtained with the modified Wu fractionation procedure[a].

Fraction	Flour	First Extract	Glycinin-rich	Second Extract
Yields (%)				
Solids	100.0	67.6 ± 1.2	12.5 ± 0.5	56.3 ± 1.8
Protein	100.0	75.0 ± 1.4	22.7 ± 0.4	52.9 ± 0.9
Storage Protein Content and Protein Composition (%)				
Storage Protein	72.9 ± 0.4	77.8 ± 0.6	91.8 ± 0.2	79.8 ± 1.3
Glycinin	61.6 ± 0.4	57.3 ± 4.2	83.5 ± 0.2	31.7 ± 1.4
Acidic	60.1 ± 1.4	61.0 ± 0.7	52.4 ± 1.6	43.2 ± 0.6
Basic	39.9 ± 1.4	39.0 ± 0.7	47.6 ± 1.6	56.8 ± 0.6
β-Conglycinin	38.4 ± 0.4	42.7 ± 4.2	16.5 ± 0.2	68.3 ± 1.4
α'	33.6 ± 1.5	30.1 ± 0.3	0.0 ± 0.0	29.1 ± 1.9
α	33.5 ± 2.2	34.5 ± 1.4	48.7 ± 1.0	33.6 ± 0.8
β	32.9 ± 0.7	35.4 ± 1.1	51.2 ± 1.0	37.3 ± 0.7

[a] n=2. Means ± one standard deviation

We chose to not use the dilution step prior to precipitating the β-conglycinin-rich fraction since protein precipitation does not depend on salting-in and salting-out behavior, but rather on site-specific binding (Kroll 1984). Yields of solids and protein for the fractions and storage protein composition in the upstream steps are shown in Table 2. The yields of solids and protein for the glycinin-rich fraction were similar to those reported previously (calcium effects on the control Wu procedure). As in the study on calcium effects, the point at which we added Ca^{2+} was the supernatant obtained after precipitating the glycinin-rich

fraction. As shown in Table 2, this extract contained about 80% of storage proteins with a 1:2.15 ratio of glycinin-to-β-conglycinin and contained about one-half of the solids and protein present in the starting flour.

Intermediate fraction. The objective to precipitating the intermediate fraction is to eliminate the remaining glycinin from the extract solution in order to obtain a more pure β-conglycinin-rich fraction in the next step. The yields of solids and protein along with storage protein yields and composition for the intermediate fraction are shown in Table 3. The yields of solids and protein for this fraction when precipitated at pH 5.0 were not affected by calcium concentration. There were no significant differences for 5 to 50 mM $CaCl_2$ concentrations with a mean of solids yield of ~28% and a mean total protein yield of ~45%. The concentration of storage protein in the fraction precipitated at pH 5.0 remained constant for all Ca^{2+} concentrations tested (about 76.5%). The storage protein composition also remained approximately the same for all Ca^{2+} concentrations when precipitating at pH 5.0 (~63% β-conglycinin and ~37% glycinin). The glycinin and β-conglycinin subunit compositions remained approximately the same when the intermediate fraction was precipitated at pH 5.0 (Table 4).

On the other hand, when this fraction was precipitated at pH 6.4, significant differences were observed in both yields of solids and protein (Table 3). The yields of solids and protein in the intermediate fraction increased as $CaCl_2$ concentration increased. The amount of storage protein in this fraction was significantly higher than the same treatment precipitated at pH 5.0. The storage protein composition was also highly dependent on Ca^{2+} concentration. At 5 mM $CaCl_2$, the amount of glycinin exceeded that β-conglycinin and proportionally decreased as $CaCl_2$ concentration increased (Table 3). Subunit compositions are shown in Table 4.

Our results, compared to those reported by Wu and others (1999) and Rickert and others (2004) had two differences. When calcium was added at pH 5.0, we obtained much higher yields of solids and proteins in the intermediate fraction. When the calcium was added at pH 6.4, however, our results were only slightly higher for the 5 mM treatment than those of Wu and Rickert. As calcium concentration increased, our results were much higher than those of earlier reports. Interestingly, the 5 mM treatment gave the highest amount of

glycinin. This amount was higher than that reported by Rickert and others (2004) (47.6%). The objective of precipitating the intermediate fraction is to precipitate as much of the remaining contaminant glycinin present in the supernatant after the glycinin-rich fraction is precipitated (almost 32% of the storage protein present in this extract was glycinin) with the goal of obtaining higher purity in the β-conglycinin-rich fraction.

β-conglycinin-rich fraction. The yields of solids and protein for the β-conglycinin-rich fraction obtained after precipitating the intermediate fraction at pH 5.0 were very low as were their purities (Table 3) and were similar to those obtained in our previous experiment. The low yields were probably obtained because almost all of the precipitable protein was lost to the intermediate fraction.

The yields of solids and protein were much higher after the intermediate fraction was precipitated at pH 6.4. These yields were significantly affected by $CaCl_2$ concentration. The highest yields for both solids and total protein were obtained at 5 mM $CaCl_2$, followed by 10 mM $CaCl_2$, and were significantly lower at higher $CaCl_2$ concentrations. All of the storage protein precipitated in this fraction was β-conglycinin as determined by SDS-PAGE. After adding $CaCl_2$ and centrifuging at pH 6.4, all the remaining precipitable storage protein was β-conglycinin. The subunit composition of this protein was also affected by $CaCl_2$ concentration (Table 4). The content of α' subunits was low at 10 and 20 mM $CaCl_2$, followed by 5 mM and 50 mM $CaCl_2$. The proportion of α subunits increased while the proportion of β subunit decreased as $CaCl_2$ concentration increased. Each treatment produced unique subunit compositions for β-conglycinin. Apparently, the α component needed more calcium to be precipitated, while the β component exhibited salting-in behavior. The α' component was affected to a lesser extent by calcium concentration.

Adding calcium at pH 5.0 failed to effectively fractionate the storage proteins, and our yields were much lower than those earlier reported by Wu and others (1999) and Rickert and others (2004). In contrast, our results were much better when adding calcium at pH 6.4,. Comparing our yields of solids and proteins to those of Wu and Rickert, we had higher amounts of solids and protein recovered in this fraction. In addition, we could not detect glycinin contamination in any of the samples when calcium was added at pH 6.4. We achieved 100% purity and this is in good agreement with the model described by Yuan and

Table 3-Yields of solids and protein and protein composition (%) of fractions obtained by using the modified Wu soy protein fractionation procedure[a].

Fraction/Treatment ($CaCl_2$, pH of int. fr. ppt)	Fraction Yields (%)		Storage Proteins Content and Composition (%)		
	Solids	Protein	Total	β-Conglycinin	Glycinin
Intermediate					
0 mM $CaCl_2$, pH 5.0	25.8^{c}	41.8^{c}	$79.5^{a,b}$	$64.1^{c,d}$	$35.9^{c,d}$
5 mM $CaCl_2$, pH 5.0	$28.0^{a,b,c}$	46.2^{a}	76.7^{b}	$61.4^{d,e}$	$38.6^{b,c}$
10 mM $CaCl_2$, pH 5.0	$28.4^{a,b}$	46.0^{a}	76.3^{b}	$65.2^{b,c}$	$34.8^{d,e}$
20 mM $CaCl_2$, pH 5.0	$27.5^{a,b,c}$	$44.5^{a,b}$	75.8^{b}	$63.6^{c,d}$	$36.4^{c,d}$
50 mM $CaCl_2$, pH 5.0	29.1^{a}	44.0^{b}	77.2^{b}	$63.4^{c,d}$	$36.6^{c,d}$
5 mM $CaCl_2$, pH 6.4	15.1^{e}	22.8^{f}	82.5^{a}	37.7^{f}	62.3^{a}
10 mM $CaCl_2$, pH 6.4	19.3^{d}	28.1^{e}	80.9^{a}	59.5^{e}	40.5^{b}
20 mM $CaCl_2$, pH 6.4	$26.5^{b,c}$	38.6^{d}	81.3^{a}	$68.7^{a,b}$	$31.3^{e,f}$
50 mM $CaCl_2$, pH 6.4	$27.4^{a,b,c}$	40.8^{c}	82.2^{a}	70.2^{a}	29.8^{f}
LSD	2.3	2.0	3.3	3.7	3.7
β-Conglycinin-rich					
0 mM $CaCl_2$, pH 5.0	$3.2^{d,e}$	2.9^{d}	82.2^{b}	89.9^{b}	10.1^{d}
5 mM $CaCl_2$, pH 5.0	$2.2^{e,f}$	0.6^{f}	58.8^{g}	72.5^{c}	27.5^{c}
10 mM $CaCl_2$, pH 5.0	$1.8^{e,f}$	0.6^{f}	59.6^{g}	70.1^{d}	29.9^{b}
20 mM $CaCl_2$, pH 5.0	$1.6^{e,f}$	$1.6^{e,f}$	69.4^{f}	62.7^{e}	37.3^{a}
50 mM $CaCl_2$, pH 5.0	1.0^{f}	0.5^{f}	85.1^{a}	89.2^{b}	10.8^{d}
5 mM $CaCl_2$, pH 6.4	15.2^{a}	24.7^{a}	75.8^{d}	100.0^{a}	0.0^{e}
10 mM $CaCl_2$, pH 6.4	11.1^{b}	18.0^{b}	78.1^{c}	100.0^{a}	0.0^{e}
20 mM $CaCl_2$, pH 6.4	5.1^{c}	7.2^{c}	$75.1^{d,e}$	100.0^{a}	0.0^{e}
50 mM $CaCl_2$, pH 6.4	$4.2^{c,d}$	5.5^{c}	73.0^{e}	100.0^{a}	0.0^{e}
LSD	1.9	2.1	2.2	1.6	1.6
Whey					
0 mM $CaCl_2$, pH 5.0	28.3^{c}	9.2^{c}	60.0^{f}	23.4^{b}	76.6^{c}
5 mM $CaCl_2$, pH 5.0	$30.2^{a,b,c}$	$10.1^{b,c}$	73.1^{d}	11.3^{d}	88.7^{a}
10 mM $CaCl_2$, pH 5.0	$31.1^{a,b}$	$11.6^{a,b}$	$74.1^{c,d}$	10.4^{d}	89.6^{a}
20 mM $CaCl_2$, pH 5.0	$31.0^{a,b}$	12.1^{a}	$74.4^{c,d}$	11.4^{d}	88.6^{a}
50 mM $CaCl_2$, pH 5.0	$31.1^{a,b}$	12.1^{a}	88.4^{a}	19.7^{c}	80.3^{b}
5 mM $CaCl_2$, pH 6.4	$29.1^{b,c}$	9.7^{c}	69.5^{e}	10.7^{d}	89.3^{a}
10 mM $CaCl_2$, pH 6.4	$29.4^{a,b,c}$	9.4^{c}	69.5^{e}	11.2^{d}	88.8^{a}
20 mM $CaCl_2$, pH 6.4	$31.2^{a,b}$	$9.8^{b,c}$	75.0^{c}	9.0^{d}	91.0^{a}
50 mM $CaCl_2$, pH 6.4	31.8^{a}	$9.9^{b,c}$	81.8^{b}	30.4^{a}	69.6^{d}
LSD	2.5	1.9	1.9	3.1	3.1

[a] n=2. Means within a column for each fraction followed by different superscripts are significantly different at $p<0.05$. LSD denotes least significant difference at $p<0.05$; int. fr. ppt. denotes intermediate fraction precipitation pH.

Table 4-Glycinin and β-conglycinin subunit composition of fractions obtained when using the modified Wu soy protein fractionation procedure[a].

Fraction/Treatment ($CaCl_2$; pH int. fr. ppt)	β-Conglycinin Subunit Composition (%)			Glycinin Subunit Composition (%)	
	α'	α	β	Acidic	Basic
Intermediate					
0 mM $CaCl_2$, pH 5.0	28.0^{d}	34.8^{a}	$37.2^{b,c}$	$45.9^{b,c}$	$54.1^{c,d}$
5 mM $CaCl_2$, pH 5.0	$29.5^{c,d}$	$32.8^{a,b}$	$37.7^{b,c}$	50.5^{a}	49.5^{e}
10 mM $CaCl_2$, pH 5.0	$30.2^{c,d}$	$33.7^{a,b}$	36.1^{c}	$49.7^{a,b}$	$50.3^{d,e}$
20 mM $CaCl_2$, pH 5.0	$30.6^{c,d}$	$32.5^{b,c}$	36.9^{c}	48.7^{a}	51.3^{e}
50 mM $CaCl_2$, pH 5.0	$29.9^{c,d}$	30.7^{c}	39.3^{b}	45.2^{c}	54.8^{c}
5 mM $CaCl_2$, pH 6.4	$32.1^{b,c}$	26.2^{d}	41.7^{a}	52.0^{a}	48.0^{e}
10 mM $CaCl_2$, pH 6.4	$33.9^{a,b}$	26.8^{d}	39.3^{b}	39.3^{d}	60.7^{b}
20 mM $CaCl_2$, pH 6.4	$34.9^{a,b}$	29.6^{c}	35.5^{c}	$35.8^{d,e}$	$64.2^{a,b}$
50 mM $CaCl_2$, pH 6.4	36.0^{a}	27.2^{d}	36.8^{c}	35.3^{e}	64.7^{a}
LSD	3.0	2.2	2.3	3.9	3.9
β-Conglycinin-rich					
0 mM $CaCl_2$, pH 5.0	$19.2^{e,f}$	64.3^{a}	16.5^{g}	100.0^{a}	0.0^{d}
5 mM $CaCl_2$, pH 5.0	$20.3^{d,e}$	52.6^{b}	$27.1^{c,d}$	32.9^{c}	67.1^{b}
10 mM $CaCl_2$, pH 5.0	20.9^{d}	53.2^{b}	25.9^{e}	26.2^{d}	73.8^{a}
20 mM $CaCl_2$, pH 5.0	18.2^{f}	50.0^{c}	31.8^{b}	45.1^{b}	54.9^{c}
50 mM $CaCl_2$, pH 5.0	32.1^{c}	42.4^{e}	25.5^{e}	44.8^{b}	55.2^{c}
5 mM $CaCl_2$, pH 6.4	34.7^{b}	29.1^{g}	36.2^{a}	0.0^{e}	0.0^{d}
10 mM $CaCl_2$, pH 6.4	37.4^{a}	33.6^{f}	29.0^{c}	0.0^{e}	0.0^{d}
20 mM $CaCl_2$, pH 6.4	38.7^{a}	34.9^{f}	$26.4^{d,e}$	0.0^{e}	0.0^{d}
50 mM $CaCl_2$, pH 6.4	32.3^{c}	48.0^{d}	19.7^{f}	0.0^{e}	0.0^{d}
LSD	1.7	2.0	2.0	2.7	2.7
Whey					
0 mM $CaCl_2$, pH 5.0	0.0^{b}	0.0^{c}	100.0^{a}	60.8^{a}	$39.2^{d,e}$
5 mM $CaCl_2$, pH 5.0	0.0^{b}	0.0^{c}	100.0^{a}	45.5^{e}	54.5^{a}
10 mM $CaCl_2$, pH 5.0	0.0^{b}	0.0^{c}	100.0^{a}	44.1^{e}	55.9^{a}
20 mM $CaCl_2$, pH 5.0	0.0^{b}	0.0^{c}	100.0^{a}	$54.7^{b,c}$	$45.3^{c,d}$
50 mM $CaCl_2$, pH 5.0	0.0^{b}	51.6^{a}	48.4^{b}	$55.0^{b,c}$	$45.0^{c,d}$
5 mM $CaCl_2$, pH 6.4	0.0^{b}	0.0^{c}	100.0^{a}	$51.3^{c,d}$	$48.7^{b,c}$
10 mM $CaCl_2$, pH 6.4	0.0^{b}	0.0^{c}	100.0^{a}	$51.4^{c,d}$	$48.6^{b,c}$
20 mM $CaCl_2$, pH 6.4	0.0^{b}	0.0^{c}	100.0^{a}	$47.2^{d,e}$	$52.8^{a,b}$
50 mM $CaCl_2$, pH 6.4	25.8^{a}	45.0^{b}	29.2^{c}	56.0^{b}	44.0^{d}
LSD	1.0	1.4	1.5	4.4	4.4

[a] n=2. Means within a column for each fraction followed by different superscripts are significantly different at $p<0.05$. LSD denotes least significant difference at $p<0.05$; int. fr. ppt. denotes intermediate fraction precipitation pH.

others (2002) suggesting that calcium can be used as an effective fractionating agent and theorizing 100% separation in diluted samples. Our purity was much better than that reported by Wu and others (~73%) and Rickert and others (68-79%). The storage protein content of our β-conglycinin-rich fractions, however, ranged between 73 and 78%, indicating substantial contamination with non-storage protein components.

Whey fraction. The solids losses in the form of whey were approximately the same for both precipitation pHs and all $CaCl_2$ concentrations (Table 3). The protein lost to the whey fraction was slightly higher when the intermediate fraction was precipitated at pH 5.0. Significantly higher amounts of storage proteins were lost at 50 mM $CaCl_2$ for both pHs, and this loss was mainly β-conglycinin (Table 3). This suggests that there was some salting-in of storage proteins at 50 mM $CaCl_2$. This increased loss was mostly α subunits at 50 mM $CaCl_2$ and pH 5.0, and α and α' subunits at 50 mM $CaCl_2$ and pH 6.4 (Table 4)

New simplified soy protein fractionation procedure

Based on our previous work, we developed a new simplified fractionation method using mM amounts of $CaCl_2$ and sulfites. The principal advantage of this new procedure is that it does not produce an intermediate fraction (mixture glycinin and β-conglycinin). A general flow chart for this procedure is shown in Figure 2 and our proposed mechanistic model is shown in Figure 3.

Glycinin-rich fraction. The yields of solids and protein and the storage protein compositions for the glycinin-rich fractions are shown in Table 5. All four new procedures yielded more solids than did the control (0 mM SO_2, mM $CaCl_2$.) and the procedure of Wu and others (1999). Yields of glycinin protein in the glycinin-rich fractions of the new procedures were either equal or higher than the control or the procedure of Wu and others (1999). Of the four new procedures, 10 mM $CaCl_2$ gave the highest yields of solids and protein for the glycinin-rich fraction, but produced slightly lower purities than obtained by using the method of Wu and others (1999). The highest purity for the glycinin-rich fraction was obtained at 10 mM SO_2 and 5 mM $CaCl_2$. Total storage protein in the glycinin-rich fraction was approximately the same for all treatments and similar to that of the Wu procedure. The fractions obtained without reducing agent had lower purities than did the new

procedure and the Wu procedure. Subunit compositions of the storage proteins in this fraction are shown in Table 6. The principal contaminant of the glycinin-rich fraction was the β subunit of β-conglycinin, which was not surprising and has been reported in earlier studies (Damodaran and others 1982, Utsumi and others 1984, Deak and others 2004a, 2004b). Interestingly, the acidic polypeptides were the principal components of glycinin for all four of our new procedures, and this component increased for the treatments where 10 mM calcium ions were added. As reported earlier by Wolf and others (1993) and our earlier reports, the presence of a reducing agent greatly improved purity of the glycinin-rich fraction.

Our results for yields and purities for procedures where calcium was used as a fractionating agent with no reducing agent were similar to those of Saio and others (1973). On the other hand, our yields were lower and purities were more than 20% better for treatments where as little as 5 mM sulfites were added. This was probably because Saio and others used 10 mM calcium, but they did not add reducing agents to their procedure. When comparing our results to those of Wu and others (1999) and Rickert and others (2004), our yields of solids and protein were significantly better over the entire range of calcium and sulfite concentrations tested, and our purities were similar to those, where sulfites were added.

β-conglycinin-rich fraction. All four new procedures yielded at least twice as much solids as a β-conglycinin-rich fraction than did the Wu procedure of Wu. Protein yields were also much higher (Table 5). Of the four new procedures, 10 mM SO_2 and 5 mM $CaCl_2$ gave the highest yields of solids and protein, but also yielded slightly lower purity compared to the same fraction of the procedure of Wu and others. The highest purities for the β-conglycinin-rich fraction were obtained by using the Wu procedure, and the new processes with 5 and 5, and 10 and 10 mM of SO_2, and $CaCl_2$, respectively. Total storage protein in the β-conglycinin-rich fraction was slightly lower for the new procedures. The β-conglycinin-rich fractions obtained by using procedures not employing reducing agent had low purities, similar to the control treatment. Subunit compositions of the storage proteins in the β-conglycinin-rich fraction are shown in Table 6. The subunit compositions of β-conglycinin were approximately the same for all of the new procedures and the Wu procedure. Subunit

Table 5-Yields of solids and protein and storage protein compositions of fractions obtained when using the new simplified fractionation procedures[a].

Fraction/Treatment	Fraction Yields (%)		Storage Proteins Content and Composition (%)		
	Solids	Protein	Total	β-Conglycinin	Glycinin
Flour	100.0	100.0	73.2±0.2	38.1±0.2	61.9±0.2
Protein Extract	69.9±1.6	78.6±2.0	78.5±0.2	42.8±0.7	57.2±0.7
Glycinin-rich					
Wu	13.0^{d,e}	27.8^{c}	86.4^{a,b}	16.2^{d}	83.8^{b}
Control	12.1^{e}	21.2^{d}	84.8^{b}	26.0^{b}	74.0^{d}
0 mM SO_2, 5 mM $CaCl_2$	15.1^{c,d}	25.1^{c}	84.5^{b}	26.0^{b}	74.0^{d}
0 mM SO_2, 10 mM $CaCl_2$	28.6^{a}	47.1^{a}	83.8^{b}	35.3^{a}	64.7^{e}
5 mM SO_2, 5 mM $CaCl_2$	15.5^{c}	27.8^{c}	88.1^{a}	14.8^{d}	85.2^{b}
5 mM SO_2, 10 mM $CaCl_2$	18.2^{b}	31.2^{b}	86.6^{a,b}	24.4^{b}	75.6^{d}
10 mM SO_2, 5 mM $CaCl_2$	14.0^{c,d,e}	25.5^{c}	85.5^{a,b}	11.9^{e}	88.1^{a}
10 mM SO_2, 10 mM $CaCl_2$	18.5^{b}	31.9^{b}	84.5^{b}	20.5^{c}	79.5^{c}
LSD	2.3	3.1	2.9	1.9	1.9
Intermediate					
Wu	7.8±0.5	26.8±0.2	77.1±1.5	45.7±1.1	54.3± 1.1
β-Conglycinin-rich					
Wu	10.1^{e}	18.5^{f}	85.1^{a,b}	81.9^{a}	18.1^{d}
Control	26.6^{a}	48.4^{a}	81.1^{c}	65.9^{c}	34.1^{b}
0 mM SO_2, 5 mM $CaCl_2$	25.5^{a,b}	43.2^{b}	86.8^{a}	58.9^{d}	41.1^{a}
0 mM SO_2, 10 mM $CaCl_2$	13.3^{d}	21.6^{e}	83.5^{b}	64.3^{c}	35.7^{b}
5 mM SO_2, 5 mM $CaCl_2$	23.6^{b}	39.7^{c}	79.8^{c}	80.9^{a}	19.1^{d}
5 mM SO_2, 10 mM $CaCl_2$	21.5^{c}	35.7^{d}	77.6^{d}	80.4^{a}	19.6^{d}
10 mM SO_2, 5 mM $CaCl_2$	24.9^{a,b}	41.2^{b,c}	80.5^{c}	75.3^{b}	24.7^{c}
10 mM SO_2, 10 mM $CaCl_2$	21.2^{c}	34.8^{d}	77.5^{d}	80.3^{a}	19.7^{d}
LSD	2.0	2.1	2.1	2.9	2.9
Whey					
Wu	36.9^{a}	12.8^{a}	72.5^{b}	30.9^{a}	69.1^{e}
Control	28.2^{d}	9.2^{b}	78.3^{a}	16.0^{e}	84.0^{a}
0 mM SO_2, 5 mM $CaCl_2$	31.2^{b,c}	9.8^{b}	66.9^{c}	24.3^{b,c}	75.7^{c,d}
0 mM SO_2, 10 mM $CaCl_2$	29.9^{c,d}	10.5^{a,b}	62.6^{d}	23.5^{b,c,d}	76.5^{b,c,d}
5 mM SO_2, 5 mM $CaCl_2$	32.7^{b}	10.5^{a,b}	59.3^{e}	25.8^{b}	74.2^{d}
5 mM SO_2, 10 mM $CaCl_2$	32.0^{b,c}	11.8^{a,b}	61.7^{d}	21.0^{d}	79.0^{b}
10 mM SO_2, 5 mM $CaCl_2$	32.8^{b}	11.6^{a,b}	61.8^{d}	24.0^{b,c}	76.0^{c,d}
10 mM SO_2, 10 mM $CaCl_2$	32.1^{b,c}	11.8^{a,b}	61.8^{d}	22.8^{c,d}	77.2^{b,c}
LSD	2.8	2.8	1.9	3.0	3.0

[a] n=2. Means within a column for each fraction followed by different superscripts are significantly different at $p<0.05$. LSD denotes least significant difference at $p<0.05$. LSD denotes least significant difference at $p<0.05$; Wu, Wu process; Control, 0 mM SO_2 and 0 mM $CaCl_2$.

compositions for the control treatment and for the procedures not employing reducing agent were significantly different. The control treatment had an even distribution of α', α, and β subunits, while the α subunit predominated in the other two treatments.

Comparing our results to those reported earlier by Saio and others (1973), our β-conglycinin-rich fractions yielded more solids and proteins, throughout the whole range of calcium and sulfites concentrations. The best results were obtained with the addition of 5 and 5, 5 and 10, an 10 and 10 mM of sulfites and calcium, respectively. We identified the 5 and 5 treatment to be the best since simultaneously was able to yield good amounts of solids and proteins for both the glycinin and the β-conglycinin-rich fractions, and had the purities for both fractions. When comparing our results for β-conglycinin-rich fraction to those reported by Wu and others (1999) and Rickert and others, our identified best procedures yielded more than two times the solids and proteins than theirs did, and at the same time had comparable purities. One of the reasons for this great discrepancy in yields is in that the procedure reported by Wu and others (1999) does need to precipitate an intermediate fraction, which in some cases is a substantial amount of the precipitable protein that enters the procedure. This new procedure is more efficient in those terms and does not need dilution steps, since works by calcium specific binding, rather than by salting-in and salting out mechanisms.

Whey fraction. Losses of solids and protein to the whey fraction were highest for the procedure of Wu and others, probably due to the NaCl used. The other treatments lost approximately the same amounts of solids and protein to the whey fraction (Table 5). The amount of storage protein lost was also lower for the new procedures than for the Wu procedure and the control procedure. The Wu procedure lost higher amounts of β-conglycinin. Sulfite addition did not increase the protein loss in the whey fraction. All procedures lost β-conglycinin in the form of β subunits. The procedure of Wu and others also lost significant amounts of α subunits, while procedures employing calcium also lost α' subunits.

Table 6-Glycinin and β-conglycinin subunit compositions (%) of fractions obtained using the new simplified fractionation procedure[a].

Fraction/Treatment	β-Conglycinin			Glycinin	
	α'	α	β	Acidic	Basic
Flour	33.5±0.3	35.0±0.2	31.5±0.1	58.9±0.3	41.1±0.3
First Extract	30.1±0.1	34.7±0.3	35.2±0.2	58.0±1.6	42.0±1.6
Glycinin-rich					
Wu	0.0^{d}	49.3^{a}	50.7^{a}	56.0^{d}	44.0^{a}
Control	$28.6^{a,b}$	32.0^{c}	39.4^{d}	$60.3^{c,d}$	$39.7^{a,b}$
0 mM SO_2, 5 mM $CaCl_2$	$27.7^{b,c}$	32.2^{c}	40.1^{d}	$60.8^{b,c}$	$39.2^{b,c}$
0 mM SO_2, 10 mM $CaCl_2$	30.1^{a}	34.7^{b}	35.2^{e}	$58.7^{c,d}$	$41.3^{a,b}$
5 mM SO_2, 5 mM $CaCl_2$	$28.7^{a,b}$	27.3^{d}	44.0^{c}	$62.6^{b,c}$	$37.4^{b,c}$
5 mM SO_2, 10 mM $CaCl_2$	$29.3^{a,b}$	$26.6^{d,e}$	44.1^{c}	$65.0^{a,b}$	$35.0^{c,d}$
10 mM SO_2, 5 mM $CaCl_2$	26.1^{c}	27.7^{d}	46.2^{b}	68.6^{a}	31.4^{d}
10 mM SO_2, 10 mM $CaCl_2$	$28.3^{a,b}$	25.3^{e}	46.4^{b}	68.3^{a}	31.7^{d}
LSD	1.9	2.1	1.8	4.4	4.4
Intermediate					
Wu	26.0 ±1.0	32.2±0.8	41.8±1.2	44.4± 0.9	55.6± 0.9
β-Conglycinin-rich					
Wu	28.7^{d}	37.1^{b}	34.1^{a}	45.5^{c}	54.5^{b}
Control	33.6^{a}	33.8^{c}	32.6^{a}	33.6^{d}	66.4^{a}
0 mM SO_2, 5 mM $CaCl_2$	$30.2^{c,d}$	39.5^{a}	30.3^{b}	43.5^{c}	56.5^{b}
0 mM SO_2, 10 mM $CaCl_2$	$32.5^{a,b}$	40.1^{a}	27.4^{c}	41.9^{c}	58.1^{b}
5 mM SO_2, 5 mM $CaCl_2$	$30.9^{b,c}$	35.8^{b}	33.3^{a}	$54.2^{a,b}$	$45.8^{c,d}$
5 mM SO_2, 10 mM $CaCl_2$	$29.9^{c,d}$	37.5^{b}	32.6^{a}	52.2^{b}	47.8^{c}
10 mM SO_2, 5 mM $CaCl_2$	$31.1^{b,c}$	35.7^{b}	33.2^{a}	45.0^{c}	55.0^{b}
10 mM SO_2, 10 mM $CaCl_2$	$32.4^{a,b}$	37.1^{b}	30.5^{b}	57.0^{a}	43.0^{d}
LSD	2.0	2.0	1.9	4.8	4.8
Whey					
Wu	0.0^{d}	46.8^{a}	53.2^{e}	57.8^{a}	42.2^{d}
Control	0.0^{d}	0.0^{b}	100.0^{a}	49.2^{b}	50.8^{c}
0 mM SO_2, 5 mM $CaCl_2$	45.8^{a}	0.0^{b}	$54.2^{d,e}$	38.6^{d}	61.4^{a}
0 mM SO_2, 10 mM $CaCl_2$	42.1^{b}	0.0^{b}	57.9^{c}	38.6^{d}	61.4^{a}
5 mM SO_2, 5 mM $CaCl_2$	43.3^{b}	0.0^{b}	$56.7^{c,d}$	44.2^{c}	55.8^{b}
5 mM SO_2, 10 mM $CaCl_2$	42.8^{b}	0.0^{b}	57.2^{c}	39.9^{d}	60.1^{a}
10 mM SO_2, 5 mM $CaCl_2$	41.3^{b}	0.0^{b}	$58.7^{b,c}$	39.6^{d}	60.4^{a}
10 mM SO_2, 10 mM $CaCl_2$	38.7^{c}	0.0^{b}	61.3^{b}	37.7^{d}	62.3^{a}
LSD	2.1	0.4	2.6	3.3	3.3

[a] n=2. Means within a column for each fraction followed by different superscripts are significantly different at $p<0.05$. LSD denotes least significant difference at $p<0.05$; Wu, Wu process; Control, 0 mM SO_2 and 0 mM $CaCl_2$.

Proposed mechanism for SO_2 action in combination with Ca^{2+} during soy protein fractionation

In an earlier study, we have proposed a mechanism for the action sulfites during soy protein fractionation. This mechanism was based on the assumption that sulfites preferentially break the disulfide bonds between acidic and basic polypeptides of glycinin subunits (Deak and others 2004b). Based on this mechanism, we further propose a complementary mechanism for the action of calcium ions in combination with sulfites for an effective soy protein fractionation.

Protein-phytate interaction has been widely reported in the literature and was the basis for our previously proposed mechanism for soy protein fractionation. Saito and others (2000) claim successfully fractionating soy protein by treating soybean flour with phytase enzymes and breaking the protein-phytate interaction facilitating separation of glycinin and β-conglycinin. Omosaiye and Cheryan suggested that fairly strong protein-phytate interaction occurs in aqueous soy protein extracts at alkaline pHs and that calcium ions bridge between phytate and protein. Furthermore, several studies report that it is difficult to separate phytate from soy proteins at alkaline pHs where phytate is usually insoluble. Kroll (1984) reported that about 30% of the calcium and 20% of the phosphorus present in soy protein isolates are bound to the protein. Brooks and Morr (1985) reported that both glycinin and β-conglycinin co-eluted in a gel-filtration procedure along with significant amounts of calcium and phosphorus, and suggested that these salt-mediated linkages interfere with soy protein fractionation and characterization, especially for the β-conglycinin component.

Yuan and others (2002) have reported on protein-calcium interactions, and suggested that glycinin could be completely separated from β-conglycinin by utilizing the differences in calcium-mediated precipitation behavior between these proteins over a range of pHs. Yuan worked on pure protein systems diluted to 1 mg of protein/mL of slurry, which is much lower than the concentrations normally used in industrial manufacturing of soy protein ingredients.

Based on our observations and the current understanding of glycinin (Nielsen 1985) and β-conglycinin structures (Thanh and Shibasaki 1978, 1979), we propose the following mechanism for action of reducing agent in combination with calcium ions for effective soy protein fractionation. Phytic acid binds to the different protein components of a protein slurry

(Fig. 3A). Sulfites preferentially break the disulfide bond between the acidic and basic polypeptides which are participating in a calcium bridge with phytate (Fig. 3B). Furthermore we reported that these bridges are more likely to occur with the acidic polypeptide (since they are exposed to the glycinin surface) and the α or α' subunits of β-conglycinin (they possess extensive regions that are prone to electrostatic interactions since they have more polar amino acids as reported by Maruyama and others 2002 and Mills and others). We believe that the electrostatic forces involved in this calcium bridge preferentially expose these particular glycinin subunits for action of SO_2.

Once the disulfide bridge is broken, the acidic polypeptide remains in solution along with β-conglycinin, and the basic polypeptide are liberated to the medium where they can interact with the β subunits of β-conglycinin and, depending on the degree of aggregation, precipitate or remain in solution (Damodaran and Kinsella 1982) (Fig. 3C). The addition of calcium, which follows the addition of sulfites, disrupts calcium-mediated phytate-protein bridges (Ford and others 1978). Since calcium ions are added in excess, free calcium is able to complex to the protein. Once the pH is adjusted to 6.4, which falls in the pH range where Yuan reported that the amount of calcium ions needed to precipitate glycinin is much smaller than the amount needed to precipitate β-conglycinin. As a consequence, there is a preferential formation of calcium-mediated glycinin-rich aggregates (Fig 3D) and the glycinin-rich fraction precipitates along with completely calcium-complexed phytate, which is insoluble (Graf 1983). β-Conglycinin also binds calcium ions, but needs more ions to form insoluble aggregates, because it is a smaller protein and has higher surface charge density, which prevents it from aggregating by electrostatic repulsive forces (Yuan 2002). After centrifuging and decanting, the resulting supernatant is adjusted to pH 4.8 and the β-conglycinin-rich fraction is isoelectrically precipitated. This change in surface charge also liberates some of the calcium that was attached to this protein. The surface charge neutralization also favors aggregation and precipitation. A substantial amount of phytate is also expected to precipitate in this fraction, because of the calcium liberated by the protein at this pH. The remaining supernatant also contains some form of phytate, some storage proteins (as evidenced by our data) and probably some free subunits and polypeptides that were liberated during the fractionation procedure and were not able to precipitate. The higher efficiency of calcium

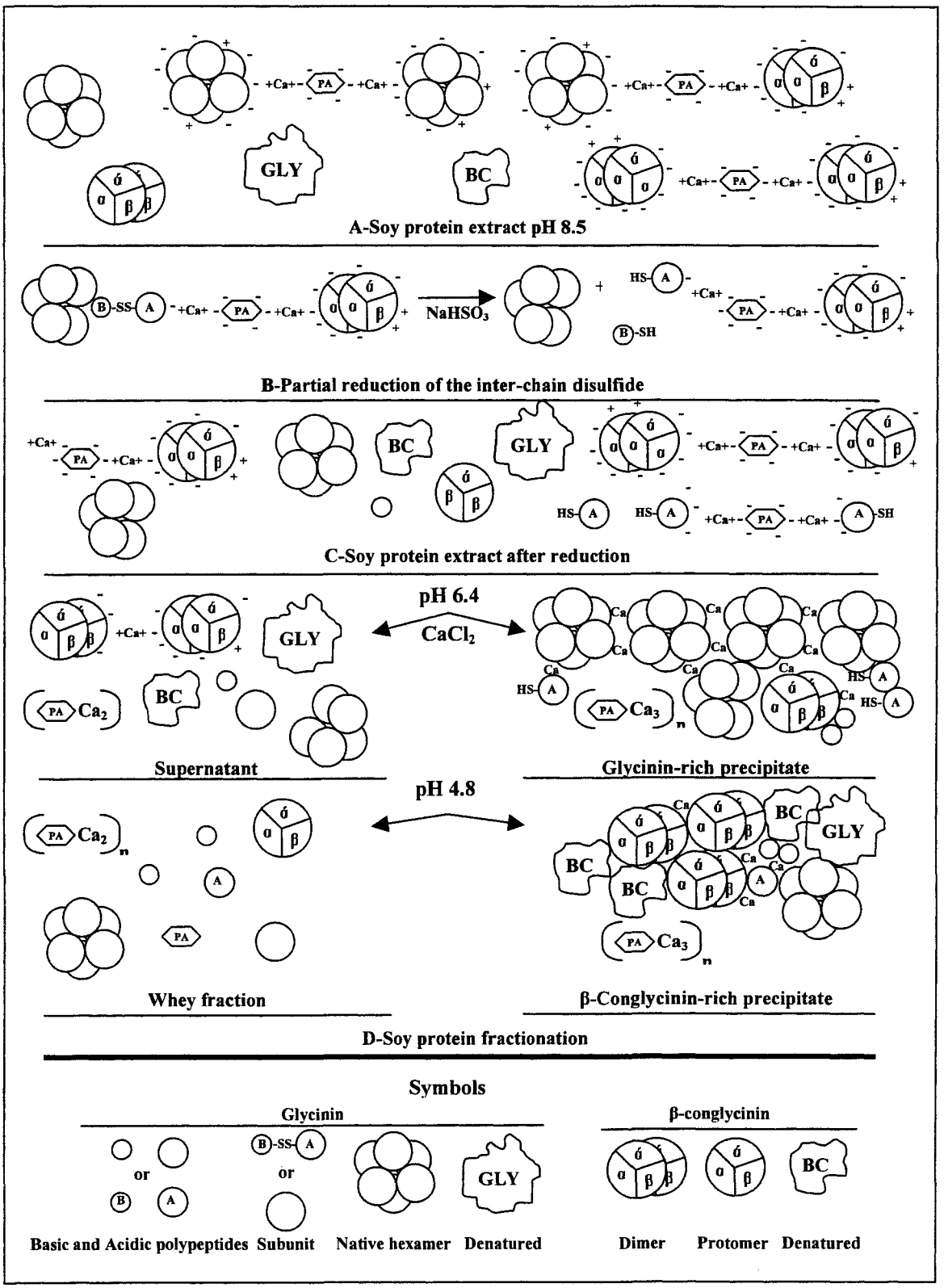

Figure 3. Schematic representation of soy protein fractionation.

addition to the modified Wu procedure can be explained principally by the lower protein concentration in the supernatant to which calcium was added. As a consequence, less co-precipitation and protein-protein interactions occured.

Conclusions

Calcium can be effectively used to fractionate soy proteins. $CaCl_2$ is much more effective than $CaSO_4$, probably because the later has low solubility. The fractionation procedure employing $CaCl_2$ is highly pH dependent. $CaCl_2$ can be effectively used to precipitate the remaining glycinin from solution in a three-step fraction procedure when precipitation is carried out at pH 6.4. No additional dilution is needed to obtain the β-conglycinin-rich fraction. The addition of sulfites plays an important role in the effectiveness of Ca^{2+} as a fractionating agent. A new two-step soy-protein fractionation procedure was developed, avoiding an intermediate fraction and producing significantly higher amounts of glycinin-rich and β-conglycinin-rich fractions with similar purities to those obtained by previous soy-protein fractionation procedures. When employing 5 mM SO_2 and 5 mM $CaCl_2$, about 28% of the protein is recovered as a glycinin-rich fraction containing 96% protein with 85.2% purity and about 40% of the protein is recovered as a β-conglycinin-rich fraction containing 90% protein with 80.9% purity.

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CHAPTER 5. CHARACTERIZING FRACTIONATED SOY PROTEINS PRODUCED BY A SIMPLIFIED PROCEDURE

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ABSTRACT

By using a new simplified soy protein fractionation procedure utilizing $CaCl_2$ and $NaHSO_3$, it was possible to fractionate soy protein into two soy protein isolates (>90% protein) that were enriched in either glycinin or β-conglycinin. The new procedure produced fractions with improved yields of solids, protein, and isoflavone and similar purities to an established, more complex fractionation procedure. The glycinin-rich fraction produced by using the new procedure contained 15.5% of the solids, 24.4% of the protein, and 20.5% of the isoflavones in the starting soy flour, whereas the established procedure contained only 11.6% of the solids, 22.3% of the protein, and 9.6% of the isoflavones. The β-conglycinin-rich fraction produced by using the new procedure contained 23.1% of the solids, 37.1% of the protein, and 37.5% of the isoflavones in the starting soy flour, whereas this fraction produced by using the established procedure contained only 11.5% of the solids, 18.5% of the protein, and 3.3% of the isoflavones. Fraction purities were similar for the two procedures and were > 80% for both fractions. The new procedure also gave protein fractions with improved functional properties. The glycinin-rich fraction of the new process had significantly more total denaturation enthalpy (19.8 mJ/mg of protein) than did the same fraction produced by using the established procedure (16.1 mJ/mg of protein). The established procedure gave protein fractions with slightly higher solubilities and similar surface hydrophobicities; whereas, the fractions produced by using the new procedure had superior emulsification and foaming properties and similar dynamic viscosity behaviors than did the fractions produced by the established procedure.

INTRODUCTION

Glycinin and β-conglycinin are the two major storage proteins in soybeans. They have been erroneously classified by their sedimentation coefficients 11S (glycinin) and 7S

(β-conglycinin). Not all the 7S protein present in soy is β-conglycinin but this classification is commonly used. Although several procedures have been developed with mixed success to fractionate these two proteins into fractions rich in these individual proteins, there is no commercial process used to obtain industrial amounts of these individual storage proteins in a cost-effective way.

Researcher have attempted to scale up some laboratory procedures and to adapt them to pilot-plant production to produces large quantities of these proteins so that they can be evaluated in clinical trials for their health-promoting benefits. Saio and Watanabe (1) developed a laboratory method in which defatted soybean meal was extracted with buffer containing 10 mM $CaCl_2$ to obtain a β-conglycinin-rich extract and a glycinin-rich fiber precipitate that was further extracted to obtain a glycinin-rich extract. The purities of the protein fractions obtained by using this procedure were about 60-65% when analyzed by an ultra-centrifugation procedure. This fractionation procedure has a serious drawback in that it utilized several costly fiber extraction and dilution steps, and fraction purities were poor.

Wu et al. (2) successfully scaled-up a method developed by Nagano et al. (3) to obtain Kg quantities of the individual storage soy proteins. This procedure utilized a reducing agent (SO_2), NaCl for protein salting-in, and diluting with water for salting-out. This procedure used high salt concentrations and large amounts of water for diluting the salt concentration. The fraction yields were low and the procedure was extremely costly and complicated for industrial production. This procedure was improved (4) by obtaining three protein fractions, a β-conglycinin-rich, a glycinin-rich, and an intermediate mixture of the two storage proteins and a significant amount of lipoxygenase. Fraction yields were improved but at the expense of purity and the procedure remained complex.

Preferential binding of calcium ions to glycinin has been reported (5, 6) and this binding is surface charge dependent (6). Yuan et al. (7) reported that the number of calcium ions required to precipitate a mole of β-conglycinin is much greater than that required to precipitate a mole of glycinin (164 and 79, respectively). These studies led us to consider a new simplified procedure to fractionate soy proteins using $CaCl_2$ as the salt and sulfites as the reducing agent (8). This simplified two-step procedure yields two protein products, a glycinin-rich fraction and a β-conglycinin-rich fraction. In the present study, we evaluated

several combinations of different concentrations of Ca^{2+} in the form of $CaCl_2$ and SO_2 in the form of $NaHSO_3$. We previously identified the ideal combination to be 5 mM $CaCl_2$ and 5 mM SO_2 since these concentrations gave at least 80% purities for both the glycinin-rich and β-conglycinin-rich fractions and high yields of solids and protein (8). The objective of the present study was to characterize and compare the fractions produced by this new process to those produced by the Wu procedure. We also evaluated eliminating the chilling step prior to precipitating the glycinin-rich fraction on the fractions produced.

EXPERIMENTAL PROCEDURES

Materials. Protein fractions were prepared from air-desolventized, hexane-defatted white flakes (soybean variety IA2020, 1999 harvest) produced in the extraction pilot plant of the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). These flakes contained 57.3% protein and 1922 μg/g total isoflavones as determined in our laboratory and 93.8 protein dispersibility index (PDI) as determined by Silliker Laboratories (Minnetonka, MN). The flakes were milled until 100% of the material obtained passed through a 50-mesh screen by using a Krups grinder (Distrito Federal, Mexico) in small quantities (~ 10 g) to preserve the native protein state. The soy flours were stored in sealed containers at 4°C until used.

Modified Nagano's (Wu) soy protein fractionation procedure. The soy protein fractionation procedure utilized as the control in this study has been reported by Wu et al. (2) and is a modification of the procedure of Nagano et al. (3). The flow diagram is shown in Figure 1. About 100 g of defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 1 h. After centrifuging at 14,300 x g and 15°C for 30 min, the protein extract (first extract) was decanted and the amount of insoluble fiber residue was determined and sampled for proximate composition. Sufficient $NaHSO_3$ was added to the resulting protein extract to achieve 10 mM SO_2 and the pH was adjusted to 6.4 with 2N HCl. The resulting slurry was stored at 4°C for 12-16 h and then centrifuged at 7,500 x g and 4°C for 20 min. A glycinin-

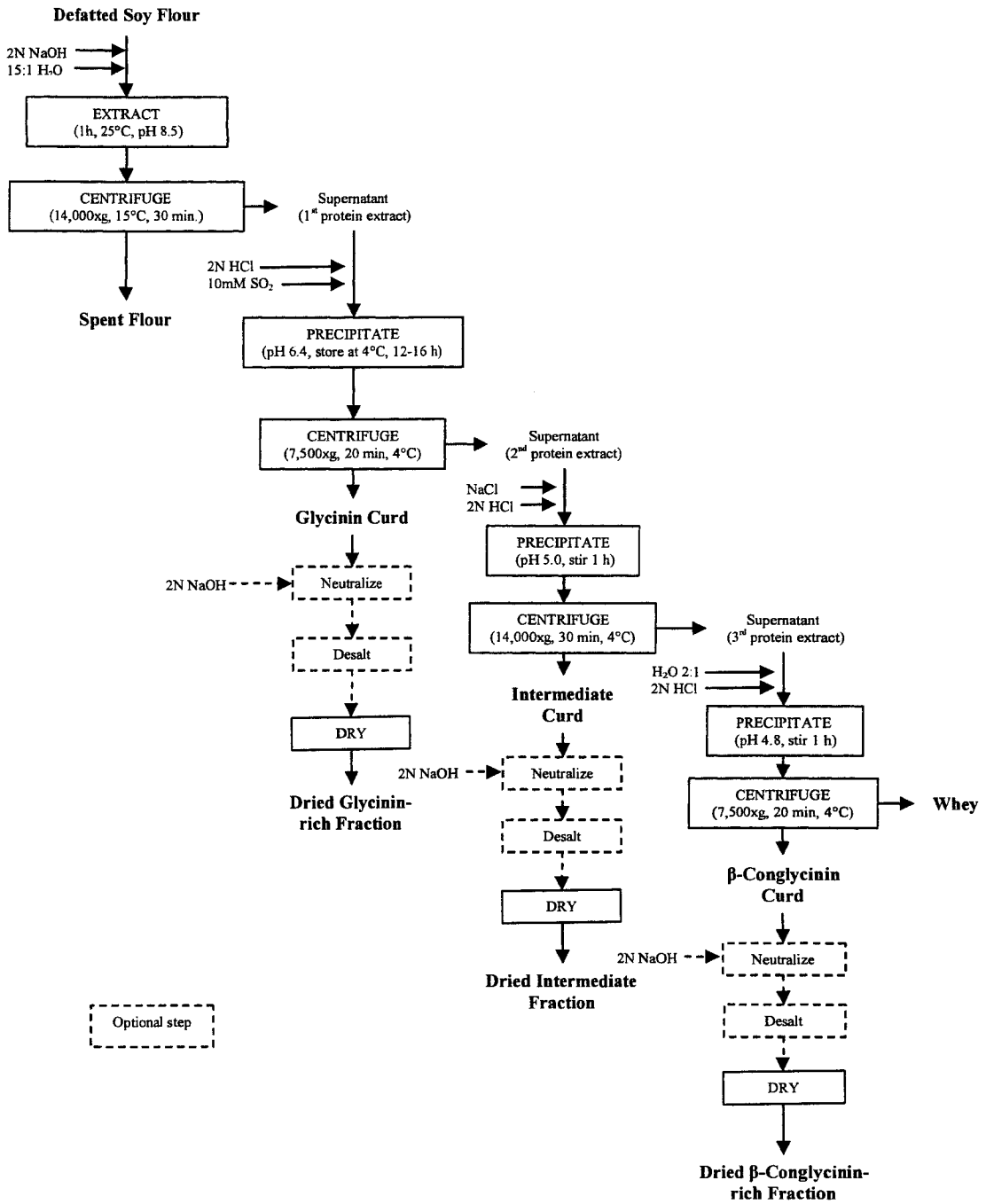

FIG. 1. Flow diagram of the Wu fractionation procedure (Wu et al. 1999).

rich fraction was obtained as the precipitated curd. This fraction was redisolved with de-ionized water and adjusted to pH 7 with 2N NaOH, sampled, and stored in sealed containers at -80°C until freeze-drying. To the supernatant, second protein extract, sufficient NaCl was added to obtain 250 mM, the pH was adjusted to 5 with 2N HCl and the resulting slurry was stirred for 1 h. The slurry was centrifuged at 14,000 x g and 4°C for 30 min. An intermediate fraction (mixture of glycinin and β-conglycinin) was obtained as the precipitated curd and was treated as described above. The supernatant, third protein extract, was diluted with de-ionized water at the ratio of two times the volume of the extract and the pH was adjusted to 4.8. The resulting slurry was centrifuged at 7,500 x g and 4°C for 20 min. A β-conglycinin-rich fraction was obtained as the precipitated curd and was treated as described above. The amount of supernatant (whey) was determined and sampled for proximate composition. This procedure was replicated two times and means reported.

New simplified soy protein fractionation procedure. The flow diagram for the new simplified procedure is shown in Figure 2. About 100 g of defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 1 h. After centrifuging at 14,300 x g and 15°C for 30 min, the protein extract (first extract) was decanted and the amount of insoluble fiber residue was determined and sampled for proximate composition. This extract was combined with sufficient $NaHSO_3$ and $CaCl_2$ to obtain 5 mM SO_2 and 5 mM Ca^{2+}, and the pH was adjusted to 6.4 with 2N HCl. The resulting slurry was either stored at 4°C for 12-16 h (this treatment is identified as New 4C) or stirred for 1 h at ~25°C (this treatment is identified as New RT). In both cases, protein fractionation was continued by centrifuging the slurry at 14,000 x g and 4°C for 30 min. A glycinin-rich fraction was obtained as the precipitated curd, which was neutralized and treated as described above. The supernatant, second protein extract, was adjusted to pH 4.8 with HCl and the slurry was stirred for 1 h. The slurry was centrifuged at 14,000 x g and 4°C for 30 min. A β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as described.

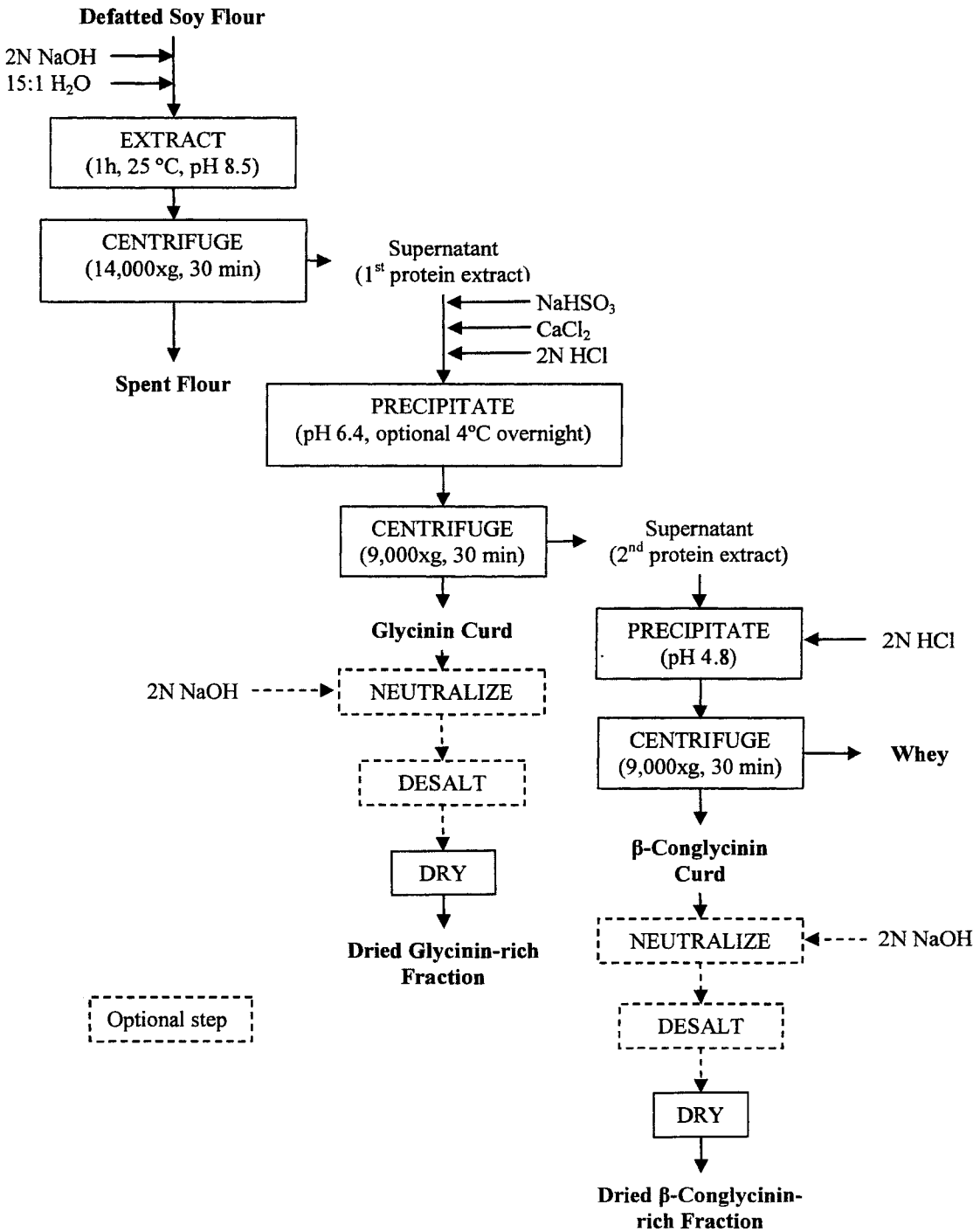

FIG. 2. Flow diagram of the new simplified soy protein fractionation procedure.

above, and the amount of supernatant (whey) was determined and sampled for proximate composition. Both treatments (New 4C and New RT) were replicated twice and means reported.

Freeze-drying. All samples were kept at –80°C and placed in a Virtis Ultra 35 (Gardnier, NY) freeze-dryer with shelves cooled to -20°C. High vacuum was applied while the temperature was held constant until the vacuum dropped to 100 mTorr. Secondary drying was achieved by heating the freeze-dryer shelves to 26°C at high vacuum. The complete freeze-drying cycle lasted for 120 h. Samples were placed in sealed containers until analyzed.

Proximate analysis and mass balances. Nitrogen contents were measured using the combustion or Dumas method (9) with a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). These values were converted to Kjeldahl nitrogen by using the conversion formula of Jung et al. (10). The factor used to convert percentage of nitrogen to protein content was 6.25. Moisture content was determined by oven-drying for 3 h at 130°C (11). Ash content was measured by using AACC methods (12). Mass balances for solids and protein were determined for all fractions. All measurements were replicated at least three times and means reported.

Protein profile analysis. Urea-sodium dodecylsulfate-polyacrylamide gel electrophoresis (urea-SDS-PAGE) was performed by using methods of Rickert et al. (4) to quantify the protein composition profiles of the fractions. Storage proteins were identified by using a pre-stained SDS-PAGE molecular-weight standard, low range (Bio-Rad Laboratories, Hercules, CA). Glycinin and β-conglycinin subunit bands were confirmed by using purified standards produced according to methods of O'Keefe et al. (13). Densitometry was carried out by using the Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). SDS-PAGE results were calculated as % composition; total storage protein in a given fraction = [(sum of storage protein subunit bands)/(sum of all bands)] x 100, fraction purity/composition = [(sum of subunit bands)/(sum of storage protein bands)], and

subunit composition of a specific protein = [(subunit band)/(sum of subunits for the specific protein)]. All measurements for each fraction were replicated at least four times and means reported.

Isoflavone yield and composition. Isoflavone extractions and HPLC analyses were conducted by using methods of Murphy et al. (*14*). About 2.5 g of each freeze-dried fraction was extracted with 10 mL acetonitrile, 2 mL 0.1 N HCl, and sufficient water, and this slurry was stirred for 2 h at 25°C. After filtering, the samples were rotary evaporated at <30°C. The residue was redisolved in 80% HPLC-grade methanol. Aliquots of these extracts were filtered and analyzed by HPLC within 10 h of extraction. Total isoflavone contents were adjusted for the molecular weight differences and expressed as aglucon contents of the individual isoforms (μ/g), this adjusted contents were also used for yield calculation, where yield % in a given fraction = [(total isoflavone concentration in a given fraction * mass of the given fraction)/ (total isoflavone concentration in the starting flour * initial mass of flour)] *100. For isoflavone profile analysis we used molar concentrations. Samples were run in duplicate and means reported.

Thermal behavior. Thermal behavior of the protein fractions was assessed by using differential scanning calorimetry (DSC). Samples (15-20 mg) of 10% (w/w, dry basis) dispersion were hermetically sealed in aluminum pans. A sealed empty pan was used as reference. The samples were heated from 25 to 120°C at 10°C/min using an SII Exstar 6000 (Seiko Instrument, Inc., Tokyo, Japan). All samples were analyzed at least three times and means reported.

Solubility. Solubility was evaluated according to the method of Rickert et al. (4) by preparing 1% (w/w dry basis) sample dispersions in de-ionized water. The pH was adjusted to 7.0 using 2N HCl or NaOH. The dispersions were stirred for 1.0 h. Aliquots (25 mL) of the dispersions were transferred to 50-mL centrifuge tubes and centrifuged at 10,000 x g and 20°C for 10 min. The protein content of the supernatant was measured by using the Biuret method with bovine serum albumen (Sigma, St. Louis, MO) as the reference standard.

Solubility was calculated as % Solubility = (protein in supernatant/initial protein content) x 100.

Surface hydrophobicity. Surface hydrophobicity was measured by using the method of Wu et al. (2) with 1-anilino-8-naphthalene sulfonic acid magnesium salt monohydrate (ANS, ICN Biomedicals, Inc., Aurora, OH). Protein dispersions prepared as in the solubility test were stirred, adjusted to pH 7.0, and centrifuged as described above. An aliquot of soluble protein (supernatant) was serially diluted to obtain 6.25 to 100 μg/mL protein with 0.1 M phosphate buffer (pH 7.0) as diluent. 40 μL ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was dispersed in 3-mL aliquots of each dilution. Fluorescence intensity (FI) was measured by using a Turner Quantech® spectrophotometer (Barnstead Thermolyne, Dubuque, IA) and 440 nm (excitation) and 535 (emission) filters. FI were standardized using a solution of 40 μL ANS in 3 mL of phosphate buffer as the zero point and 15 μL of ANS in 3 mL of methanol assigned an arbitrary value of 80 FI. FIs were plotted versus percentage protein concentration. The slope of the regression line was reported as surface hydrophobicity. Samples were run in triplicate and means reported.

Emulsification properties. Emulsification capacity was measured according to the method of Bian et al. (15) with modifications. Twenty-five mL of 2% (w/w, dry basis) sample dispersions was adjusted to pH 7.0 with 2 N HCl or NaOH and transferred to a 400-mL plastic beaker. Soybean oil, dyed with approximately 4 ppm Sudan Red 7B (Sigma, St. Louis, MO), was continuously blended into the protein dispersion at 37 mL/min flow rate by using a Bamix wand mixer (ESGE AG Model 120, Mettlen, Switzerland) at the low setting until phase inversion was observed. Emulsification capacity (g oil/g sample) was calculated as g of oil used to cause inversion multiplied by 2. Samples were run at least in triplicate and means reported.

Emulsification activity and emulsification stability index were determined by using methods of Rickert et al. (4). Twenty-one mL of 2 % (w/w, dry basis) sample dispersions adjusted to pH 7.0 were blended with 7 mL of refined soybean oil (Bakers and Chefs Vegetable Oil, North Arkansas Wholesale Company Inc., Bentonville, AK) in a 250-mL

glass beaker for 1.0 min by using the Bamix wand mixer at low speed. Immediately after mixing, the emulsion was diluted 1:1000 with 0.1% sodium dodecyl sulfate. The absorbance was measured at 500 nm and recorded as emulsification activity. After 15 min, the absorbance was measured again. These two absorbance readings were used to calculate emulsification stability index (ESI):

$$\text{ESI (min)} = (A_0/A_0\text{-}A_{15})t$$

where A_0 and A_{15} are absorbances at time 0 and 15 min, respectively, and t is the time interval. Samples were run in triplicate and means reported.

Foaming properties. Foaming properties were determined by using methods of Sorgentini et al. (16) with modifications (4). A 0.5% (w/w, dry basis) sample dispersion was prepared and the pH adjusted to 7.0. A 95-mL aliquot was loaded into a custom-designed glass column (58.5 cm x 2 cm) with a coarse glass frit at the bottom, and N_2 was purged through the sample at 100 mL/min flow rate. Time for the foam to reach the 300-mL mark, time for one-half of the liquid incorporated into the foam to drain back, and volume of liquid incorporated into foam were measured. Three parameters were calculated:

$$\text{Foaming capacity (FC)} = V_f/(f_r \times t_f)$$

$$\text{Specific rate constant of drainage (K)} = 1/(V_{max} \times t_{1/2})$$

$$\text{Rate of liquid conversion to foam } (V_i) = V_{max}/t_f$$

where V_f = a fixed volume of 300 mL, f_r = the flow rate of the gas, t_f = time to reach V_f, V_{max} is the volume of liquid incorporated into foam, and $t_{1/2}$ is the time to drain one-half of the liquid incorporated into the foam. Samples were run in triplicate and means reported.

Dynamic viscosity. A 10% (w/w, dry basis) sample dispersion was prepared at pH 7.0 (4). The sample was applied to the plate of a RS-150 Rheo Stress (Haake, Karlsruhe, Germany) and shear was applied with a 60-mm 2° titanium cone (C60/2 Ti) over the 10-500/s shear rate range at constant temperature (23°C). Shear rate (γ) and shear stress (τ) over the course of the analysis, in combination with the power law formula application, were used to determine the consistency coefficient (k) and flow behavior index (n), where $\tau = k\gamma^n$.

Using k, n, and γ, apparent viscosity (η) was estimated by the formula $\eta = k\gamma^{n-1}$. Samples were run in triplicate and means reported.

Statistical analysis. The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM). Least significant differences (LSD) were calculated at $p<0.05$ to compare treatment means by using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Yields and proximate compositions. The fractionation procedure of Wu et al (2) (designated as Wu) yielded slightly more total solids (41.3%) for the three protein fractions added than did the new fractionation procedure with chilling at 4°C (N4C) (38.6%) and the new fractionation process at 25°C (NRT) (39.0%) (Table 1). The total protein yields were also higher for the Wu procedure (67.6%) than for the new procedure with chilling (N4C, 61.5%) and the new procedure without chilling (NRT, 62.3%), but almost 40% of the precipitated protein was recovered in the intermediate fraction (mixture of β-conglycinin and glycinin), which was not produced in the new simplified procedure.

Both new procedures yielded protein fractions with significantly higher amounts of isoflavones (58.0 and 50.7% for N4C and NRT procedures, respectively) compared to the fractions of the Wu procedure (33.8%). Two-thirds of the total isoflavones recovered by the Wu procedure were recovered in the intermediate fraction (Table1).

When comparing the glycinin-rich fractions for all treatments, both new procedures yielded significantly higher amounts of solids, proteins and isoflavones than did the Wo procedure. The protein contents of the glycinin-rich fractions for all treatments were >90%. The ash content for the glycinin-rich fraction of the Wu procedure was significantly higher than for either of the new procedures. The glycinin-rich fraction obtained by using the new procedure with chilling had the highest isoflavone content, probably because isoflavones are less soluble at low temperatures (17).

When comparing the β-conglycinin-rich fractions for all treatments, both new procedures yielded more than twice as much solids as did the Wu procedure (Table 1).

Protein yields for the new procedures were also significantly higher. The new procedures yielded more than 10 times the amount of isoflavones in the β-conglycinin-rich fractions, as did the Wu procedure. The total protein contents of the β-conglycinin-rich fractions for all treatments were also >90%, (but in all cases, less than the protein contents of the glycinin-rich fractions). The Wu procedure gave fractions containing very high ash and very low isoflavone contents in the β-conglycinin-rich fraction compared to these fractions produced by new procedures due to the higher salt concentrations used in the Wu procedure.

The Wu procedure also produced an intermediate fraction, whose protein content was 10% lower than the β-conglycinin-rich fractions and about 15% lower than the glycinin-rich fractions. This fraction does not meet the critical protein content required to be a protein isolate (>90%). The ash content of this fraction was also the highest among all fractions. Considerable amounts of solids, protein, and isoflavones were recovered in this less useful fraction (Table 1).

The yields of solids and protein in the glycinin-rich and β-conglycinin-rich fractions for the Wu procedure compared well with those reported in the literature (2, 4), but we obtained almost twice as much yield of solids in our intermediate fraction. One possible explanation for these results is that all three studies used different varieties of soybeans and our soy flour had higher PDI than that of Rickert et al (4). This later observation is also in agreement with our thermal behavior results. In our experiments, we had significant amounts of non-denatured protein in our intermediate fraction while both Wu et al (2) and Rickert et al (4) found very little thermal activity remaining indicating substantial protein denaturation in this fraction. We were able to extract more solids and protein from our flour obtaining higher total yields of solids (glycnin-rich + β-conglycinin-rich + intermediate fractions), 41.3 versus 32.8 (2) and 30.6% (4), but most of the difference was due to the intermediate fraction. The yields of protein and solids for the glycinin-rich and β-conglycinin-rich fractions when using the Wu et al (2), Rickert et al (4), and our procedures were much higher than those reported by Nagano et al (3).

We obtained higher protein yields in our β-conglycinin-rich fractions and lower protein yields in our glycinin-rich fraction when using the N4C and NRT procedures than did Saio et al (1). Apart from the fact that both studies used different soybeans, Saio's procedure

to fractionate soy proteins differed from ours in that they started by preferentially extracting the β-conglycinin from the flour while we started with alkali extraction. These differences were apparent in their low purities (62 and 68% for glycinin-rich and β-conglycinin-rich fractions, respectively) that also resulted in their glycinin-rich fraction yielding a high amount of protein.

TABLE 1
Yields and Compositions (dry basis) of Soy Protein Fractions Prepared by Using the Wu and New Fractionation Procedures [a].

Fraction/Treatment	Solids Yield (%)	Protein Yield (%)	Isoflavone Yield (%)	Protein Content (%)	Ash (%)	Isoflavone Content (μg/g)
Wu glycinin	11.6[b]	22.3[c]	9.6[c]	96.7[b]	3.9[a]	1591[c]
N4C glycinin	15.5[a]	24.4[b]	20.5[a]	98.9[a]	3.2[b]	2547[a]
NRT glycinin	15.7[a]	29.9[a]	15.9[b]	96.6[b]	3.0[c]	1942[b]
LSD	1.2	1.8	2.0	0.9	0.2	155
Wu intermediate	18.2±1.0	26.8±1.3	20.9±1.2	80.3±1.2	14.3±0.2	2213±130
Wu β-conglycinin	11.5[b]	18.5[c]	3.3[c]	92.2[a]	10.1[a]	548[c]
N4C β-conglycinin	23.1[a]	37.1[a]	37.5[a]	90.0[b]	6.0[b]	3120[a]
NRT β-conglycinin	23.3[a]	32.4[b]	34.8[b]	91.2[a]	5.3[c]	2868[b]
LSD	2.4	1.7	2.5	1.2	0.3	184
LSD[b]	2.2	1.9	2.6	1.4	0.2	192

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference for comparing all fractions within a column.

Protein composition. The total storage protein contents of the glycinin-rich fractions were about 90% for all treatments (Table 2) but were higher in glycinin-rich fractions produced with the new procedures. The purities of the glycinin-rich fractions were approximately the same for the Wu and the new procedure with chilling (>80%), but about 10% lower for the glycinin-rich fraction recovered at room temperature. The principal subunit of the contaminant β-conglycinin in the glycinin-rich fraction was the β subunit, but unlike the new procedures, the glycinin-rich fraction of the Wu procedure contained no α'

subunits. For all three procedures, there were more acidic subunits than basic subunits in the glycinin recovered in the glycinin-rich fractions. The new procedure with chilling yielded glycinin with more acidic subunits than did the same procedure without chilling.

The β-conglycinin-rich fractions recovered by all procedures contained more than 80% storage proteins (Table 2). The highest purity was achieved by using the new procedure with chilling (85% β-conglycinin). The subunit compositions produced by all procedures were approximately the same for β-conglycinin but significantly different for the contaminant glycinin. The β-conglycinin subunits were nearly evenly distributed among the three subunit types. The glycinin contamination was comprised of more basic subunits when using the new procedures than using the Wu procedure.

The intermediate fraction produced by using the Wu procedure contained about 10% less storage protein than did the glycinin-rich fraction and about 5% less than did the β-conglycinin-rich fraction. About 45% of the storage protein present in the intermediate fraction produced by using the Wu procedure was β-conglycinin and 55% glycinin. The subunit distribution of the β-conglycinin component in the intermediate fraction was unique in that the principal subunit was β. Approximately the same amounts of acidic and basic subunits were recovered in the glycinin component of the intermediate fraction.

The purities of our glycinin-rich fractions (83.7%) were lower than those reported by Nagano et al (3) (>90%), Wu et al (2) (84.2-90.5%), and Rickert et al (4) (85-90%). On the other hand, the purity of our β-conglycinin-rich fraction (83.8%) was higher than those reported by Wu et al (73%) and Rickert et al (68-79%) but lower than reported by Nagano et al (>90%),. This variation in purity data among studies for similar procedures was partially attributed to the differences in soy flour and to the fact that some of the results were achieved at significantly larger scale.

When comparing the purities of our fractions produced by the N4C and NRT procedures with those reported by Saio et al (1), we obtained higher purities for both protein fractions and for both procedures. The purity differences were greater for the N4C procedure, which were probably due to the absence a reducing agent in their procedure (8).

TABLE 2
Protein Compositions and Subunit Profiles of the Protein Fractions Prepared by Using the Wu and New Fractionation Procedures [a].

Fraction/ Treatment	Storage Protein in Fraction (%)	β-Conglycinin				Glycinin		
		%	Subunit Composition (%)			%	Subunit Composition (%)	
			α'	α	β		A	B
Wu glycinin	89.0[a]	16.3[b]	0.0[b]	49.5[a]	50.5[a]	83.7[a]	54.1[b]	45.9[a]
N4C glycinin	94.2[a]	19.0[b]	26.9[a]	25.0[c]	48.1[b]	81.0[a]	64.1[a]	35.9[b]
NRT glycinin	93.8[a]	28.6[a]	28.0[a]	30.7[b]	41.3[c]	71.4[b]	57.1[b]	42.9[a]
LSD	7.2	5.2	1.5	2.2	2.3	5.2	5.6	5.6
Wu intermediate	79.1±2.0	45.3±2.3	23.7±1.2	31.7±2.1	44.6±1.0	54.7±2.3	46.3±4.0	53.7±4.0
Wu β-conglycinin	85.2[a]	83.8[b]	28.7[a]	36.7[a]	34.6[a]	16.2[b]	43.5[a]	56.5[c]
N4C β-conglycinin	81.9[b]	85.6[a]	27.3[a]	38.0[a]	34.7[a]	14.4[c]	39.8[b]	60.2[b]
NRT β-conglycinn	84.3[a]	78.6[c]	29.4[a]	38.5[a]	32.0[b]	21.4[a]	31.9[c]	68.1[a]
LSD	2.2	0.4	2.8	3.6	2.2	0.4	1.8	1.8
LSD[b]	4.5	2.6	3.4	2.1	3.8	2.6	3.7	3.7

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; A, acidic subunits of glycinin; B, basic subunits of glycinin; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference for comparing all fractions within a column.

Isoflavone composition. The isoflavones commonly found in soybeans are genistein, daidzein, and glycitein, which occur in four forms, the aglycon, the glucoside, the malonylglucoside, and the acetylglucoside isoforms. The glucoside and malonylglucoside predominate in soybeans and soy protein products (14). The isoflavone profile and isoform distribution are altered during processing (18, 19).

The isoflavone contents of the soy flour and the protein fractions for all procedures are shown in Table 3. The soy flour contained 42.9% daidzein, 50.4% genistein, and 6.5% glycitein. The isoform distribution was 3.2% aglucons, 1.8% acetylglucosides, 27.2 glucosides, and 67.6% malonylglucosides. The fractionation procedure significantly affected the isoflavone distribution of the glycinin-rich fraction. The glycinin-rich fraction obtained by using the Wu procedure contained 26.3% daidzein, 61.9% genistein, and 11.8% glycitein. The isoform distribution was also significantly affected. The glycinin-rich fraction obtained by using the Wu procedure contained 30.5% aglycons, about 10 times the amount in the initial flour. At the same time, both the glucosides and malonylglucosides decreased (to 16.7 and 44.5%, respectively). This conversion to glucosides from malonylglucosides and the following conversion to aglycons have been reported before (17, 18). Alkali extraction and the action of β-glucosidases cause this conversion. The glycinin-rich fraction obtained by using the new procedure with the chilling step contained 22.0% daidzein, 64.1% genistein, and 3.9% glycitein. The isoform distribution of this fraction was also different, containing 24.1% aglycons, 10.8% glucosides, 63.7% malonylglucosides, and 1.41% acetylglucosides. The profile for the glycinin-rich fraction obtained by using the new procedure without chilling was 31.5% daidzein, 49.5% genistein, and 3.9% glycitein. Apparently, chilling to 4°C favored the recovery of genistein, since the total isoflavone content of the glycinin-rich fraction was higher when precipitated at 4°C. The isoform distribution was similar to the glycinin-rich fraction obtained by using the new procedure with a chilling step.

Choice of fractionation procedure also significantly affected the isoflavone profile and distribution in the β-conglycinin-rich fraction (Table 3). The total isoflavone content of the β-conglycinin-rich fraction obtained by using the Wu procedure was about one-fifth that of the same fraction obtained by using the new fractionation procedures. The isoflavone

TABLE 3.Isoflavone Profiles of Protein Fractions Prepared by Using the Wu and New Fractionation Procedures, μmol/g[a].

Fraction/Treatment	Din	MDin	AcDin	Dein	Glyin	MGly	Glyein	Gin	MGin	AcGin	Gein	Total
Flour	0.73	2.18	0.05	0.13	0.22	0.25	0.00	1.01	2.44	0.08	0.10	7.20
Wu glycinin	0.20[a]	0.73[b]	0.03[a]	0.59[b]	0.13[a]	0.22[a]	0.25[a]	0.66[a]	1.69[c]	0.36[a]	0.96[c]	5.92[c]
N4C glycinin	0.24[a]	1.42[b]	0.05[a]	0.84[a]	0.09[b]	0.17[b]	0.11[b]	0.69[a]	3.99[a]	0.09[c]	1.34[a]	9.52[a]
NRT glycinin	0.22[a]	1.91[a]	0.05[a]	0.60[b]	0.07[b]	0.13[c]	0.08[c]	0.57[a]	2.85[b]	0.17[b]	1.09[b]	7.26[b]
LSD	0.08	0.10	0.13	0.02	0.03	0.01	0.01	0.19	0.05	0.02	0.10	0.28
Wu intermediate	0.44	0.90	0.05	1.09	0.25	0.25	0.31	1.31	1.82	0.15	1.60	8.26
Wu β-conglycinin	0.07[b]	0.20[c]	0.03[c]	0.33[c]	0.05[b]	0.07[b]	0.09[b]	0.17[b]	0.36[b]	0.07[b]	0.58[c]	2.05[b]
N4C β-conglycinin	0.40[a]	2.48[a]	0.05[b]	1.13[a]	0.11[a]	0.22[a]	0.11[a]	1.11[a]	4.20[a]	0.08[b]	1.79[a]	11.68[a]
NRT β-conglycinin	0.42[a]	2.25[b]	0.09[a]	0.88[b]	0.12[a]	0.21[a]	0.09[b]	1.13[a]	4.02[a]	0.18[a]	1.33[b]	10.73[a]
LSD	0.06	0.21	0.01	0.19	0.01	0.01	0.02	0.26	0.58	0.04	0.38	1.39
LSD[b]	0.15	0.13	0.07	0.10	0.06	0.02	0.02	0.37	0.30	0.02	0.19	0.82

[a] n=2. Means within a column followed by different superscripts are significant different at $P<0.05$. Din denotes daidzin; MDin, malonyldaidzin; AcDin, acetyldaidzin; Dein, daidzein; Gly, glycitin; MGly, malonylglycitin; Glyein, glycitein; Gin, genistin; MGin, malonylgenistin; AcGin, acetylgenistin; and Gein, genistein. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.

[b] Least significant difference for comparing all fractions within a column.

distribution of the β-conglycinin-rich fraction obtained by using the Wu procedure was 30.1% daidzein, 57.8% genistein, and 10.7% glycitein. This isoform profile was also unique in that this fraction had the highest aglycon (48.7%) and lowest malonylglucoside (30.8%) contents of all fractions recovered, and its glucoside contents were also low (14.1%). The isoflavone profile and isoform distribution for the β-conglycinin-rich fractions produced by using both new fractionation procedures were similar. Daidzein contents were 34.7 and 33.9%, genistein content 61.6 and 62.2%, and glycitein content 2.8 and 3.9%, for the new fractionation procedures (N4C and NRT, respectively). The aglycons were 25.9 and 21.4%, glucosides 13.8 and 15.6%, malonylglucosides 59.1 and 60.5% for the new fractionation procedures (N4C and NRT, respectively). The intermediate fraction produced by using the Wu procedure, which contained about 60% of the original isoflavones in the soy flour, had a similar isoflavone distribution as did the β-conglycinin-rich fraction obtained by using the Wu procedure, but was significantly different in isoform distribution (Table 3).

Thermal behavior. The thermal behaviors of the glycinin-rich fractions for all treatments are shown in Table 4. The peak denaturation temperature remained approximately the same for all treatments and for both the glycinin portions and for the β-conglycinin contaminant. The contaminant β-conglycinin in the glycinin-rich fractions comprised 2.0 to 4.0% of the total denaturation enthalpy. The glycinin-rich fraction had the highest total denaturation enthalpy in all three procedures. While containing only slightly more glycinin, the glycinin-rich fractions produced by using the new procedures had significantly higher denaturation enthalpies. These trends were also observed by Scilingo and Anon (20, 21) and were attributed to calcium ions stabilizing the structure of glycinin through specific ion-protein binding. This later observation is consistent with our proposed mechanism for soy protein fractionation with calcium ions (8). The denaturation temperature of the β-conglycinin contaminant in the glycinin-rich fraction was lower than that of β-conglycinin in the β-conglycinin-rich fraction, probably due to the low concentration of native β-conglycinin present in the glycinin-rich fraction.

TABLE 4.
Thermal Behaviors of Protein Fractions Prepared by Using the Wu and New Fractionation Procedures[a].

Fraction/Treatment	β-Conglycinin Td (°C)	Glycinin Td (°C)	β-Conglycinin Enthalpy (mJ/mg)	Glycinin Enthalpy (mJ/mg)
Wu glycinin	74.7[a]	89.1[a]	0.32[a]	15.65[b]
N4C glycinin	73.3[a]	91.0[a]	0.61[a]	19.23[a]
NRT glycinin	72.8[a]	91.3[a]	0.81[a]	19.33[a]
LSD	2.0	2.1	0.55	2.31
Wu intermediate	74.8±1.1	93.1±0.5	1.48±0.37	2.91±0.64
Wu β-conglycinin	75.1[a]	88.9[c]	10.64[a]	0.06[b]
N4C β-conglycinin	75.1[a]	89.8[b]	6.47[b]	0.55[a,b]
NRT β-conglycinin	74.7[a]	90.8[a]	4.96[c]	1.19[a]
LSD	1.0	0.9	1.12	0.92
LSD[b]	1.6	1.5	0.93	1.06

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; β-conglycinin, β-conglycinin-rich fraction; Intermediate, intermediate fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference for comparing all fractions within a column.

The peak denaturation temperature for the β-conglycinin component of the β-conglycinin-rich fraction remained approximately the same for all treatments. The peak denaturation temperature for the contaminant glycinin in the β-conglycinin-rich fraction was slightly different for each treatment. The glycinin contaminant of the β-conglycinin-rich fraction comprised 0.6 to 19.3% of the total denaturation enthalpy in this fraction. The Wu procedure produced a β-conglycinin-rich fraction with the highest denaturation enthalpy even though the β-conglycinin contents were similar among all procedures. This was probably due the new procedures not having intermediate fractions, which is the fraction that yields most of the denatured proteins produced when using the Wu procedure. Apparently, β-conglycinin structure was less affected by calcium ions than was glycinin (20, 21).

The intermediate fraction obtained by using the Wu procedure had the lowest total denaturation enthalpy, indicating that some of protein present in this fraction was denatured. This later observation is in good agreement with reports of Wu et al (2) and Rickert et al (4), although they found lower denaturation enthalpies for their intermediate fractions, especially for the β-conglycinin component. We attribute these differences to the higher PDI of our defatted soy flour. The intermediate fraction also had the highest denaturation temperature for its glycinin component, which we attribute to the high salt content in this fraction (20).

Solubility. The fractionation procedure used significantly affected protein solubilities of the different fractions (Table 5). The glycinin-rich fraction obtained by the Wu procedure had slightly higher solubility (88%) although the new procedures also gave good solubilities (80-85%).

The β-conglycinin-rich fraction obtained by using the Wu procedure also had higher solubility (93%) than those produced by using the new procedures (70-80%). The differences in solubility among treatments were significantly greater for this fraction than for the glycinin-rich fraction. The higher solubilities observed for the fractions obtained by using the Wu procedure were attributed the new procedures producing only two fractions while the Wu procedure produces an intermediate fraction, which also had much lower solubility (40%). These lower solubilities can be explained taking in account their thermal behaviors, the products with lower total enthalpy were also less soluble, probably due to more denaturation.

Rickert et al (4) found no differences in solubility behavior for their glycinin-rich and β-conglycinin-rich fractions at pH 7.0. In contrast, our results for the Wu procedure and those reported for a similar procedure (15) showed that β-conglycinin-rich fractions were more soluble than glycinin-rich fractions at pH 7.0. Differences in thermal histories of the soy flours used may account for this discrepancy.

Surface hydrophobicity. The presence of calcium does not prevent the ANS probe from interacting with the proteins (20). The surface hydrophobicities of the fractions were affected to a lesser extent than were solubilities (Table 5). Apparently, the amount of calcium present in the system did not cause structural changes to the proteins structure. In contrast,

Scilingo and Anon (20) found that calcium-treated soy protein isolates had lower surface hydrophobicities than untreated ones and attributed this phenomenon to the formation of soluble aggregates promoted by the presence of calcium.

TABLE 5.
Solubilities and Surface Hydrophobicities of Protein Fractions Prepared by Using the Wu and New Fractionation Procedures[a].

Fraction/Treatment	Solubility (%)	Surface Hydrophobicity (dimensionless)
Wu glycinin	88.1[a]	160[a]
N4C glycinin	85.2[b]	161[a]
NRT glycinin	80.5[c]	153[a]
LSD	2.5	39
Wu intermediate	39.7 ± 2.1	156 ± 22
Wu β-conglycinin	93.8[a]	178[b]
N4C β-conglycinin	71.8[c]	226[a]
NRT β-conglycinin	80.5[b]	187[b]
LSD	5.1	35
LSD[b]	3.9	39

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference for comparing all fractions within a column.

There were no significant differences in surface hydrophobicity for the glycinin-rich fraction among fractionation procedures. The β-conglycinin-rich fraction obtained by using the new procedure with chilling had the highest surface hydrophobicity. This observation was consistent with the thermal behavior and solubility for this fraction. The protein precipitated in this fraction had low denaturation enthalpies and solubility. In general, the β-conglycinin-rich fractions had high surface hydrophobicities, which is in agreement with Wu et al (2) and in contrast with observations of Rickert et al (4) for similar fractionation procedures.

Emulsification properties. The emulsification properties of the protein fractions are shown in Table 6. The emulsification capacities of the fractions produced by using the Wu procedure were similar to those previously reported for similar procedures (4, 15) with the β-conglycinin-rich fraction having the best emulsification capacity among all fractions collected.

TABLE 6.
Emulsification Properties of Protein Fractions Prepared by Using the Wu and New Fractionation Procedures[a].

Fraction/Treatment	Emulsification Capacity (g of oil emulsified/g of product)	Emulsification Activity (absorbance at 500 nm)	Emulsification Stability Index (dimensionless)
Wu glycinin	351[c]	0.152[a]	84[a]
N4C glycinin	876[a]	0.140[a]	73[a]
NRT glycinin	684[b]	0.149[a]	68[a]
LSD	28	0.015	22
Wu intermediate	232 ± 29	0.168 ± 0.026	62 ± 26
Wu β-conglycinin	586[b]	0.306[a]	194[a]
N4C β-conglycinin	678[a]	0.276[b]	192[a]
NRT β-conglycinin	647[a]	0.244[c]	151[b]
LSD	35	0.028	38
LSD[b]	30	0.022	32

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference for comparing all fractions within a column.

The glycinin-rich fractions produced by using either new procedure had significantly higher emulsification capacities (2.5 and 1.9 times as much oil emulsified, respectively) compared to the same fraction produced by using the Wu procedure. This higher emulsification capacity may be due to less protein denaturation, or more likely, due to

calcium-mediated associations between glycinin molecules (7) that introduce structural changes (20). Emulsification activities and emulsification stability indexes were approximately the same for all glycinin-rich fractions, regardless of procedure used to produce them. Probably the structural changes introduced to the glycinin in the presence of calcium improved its ability to reach the water/oil interface, but did not allow for the flexibility needed for emulsion stabilization. One possible explication consistent with our previous observations (8) is that soy protein forms soluble aggregates in the presence of low calcium concentrations (20).

The β-conglycinin-rich fractions obtained by using the new procedures had significantly higher emulsification capacities, than did the same fraction obtained by using the Wu procedure, although this difference was less dramatic with the glycinin-rich fraction. For the β-conglycinin-rich fraction, the Wu procedure gave the highest emulsification activity but was closely followed by those of the new procedures. Emulsification stability index was also significantly affected by the procedure used to fractionate soy protein. These observations agree with our previous ones for solubility. Apparently, the presence of calcium ions preferentially affects glycinin (21). The intermediate fraction obtained by using the Wu procedure had the poorest emulsification properties. The β-conglycinin fractions formed more stable emulsions with quite high emulsification capacities. These results could not be correlated to solubility nor surface hydrophobicity data. The new procedures gave fractions with better overall emulsification properties.

Foaming properties. Foaming capacities, stabilities and foaming rates of the fractions are shown in Table 7. Foaming capacity is expressed in mL of foam formed per mL of a 0.5% solids dispersion. Foam stability is expressed by k, which is the time for one-half of the liquid to drain from the foam. The smaller that k is, the more stable the foam. Rate of foaming is a measure of speed of foam formation.

For the glycinin-rich fraction, the new procedure without chilling gave the best foaming properties. This fraction had about 70% higher foaming capacity, its foam was significantly more stable, and foam was produced five times faster than for the same fraction obtained by using the Wu procedure. This fraction was also a significantly better foaming agent,

compared to the same fraction obtained by using the new procedure with chilling. The significantly improved foaming properties of the glycinin-rich fractions produced by using the new procedures can partially be attributed to these fractions containing high amounts of acidic polypeptides from glycinin, which are good foaming agents (22). In addition, calcium-mediated associations among the different components of this fraction improved film formation. The improved foaming properties of the glycinin-rich fraction produced by using the new procedure without chilling were probably due, in part, to the fact that this fraction had a significant amount of β-conglycinin contamination. The interaction between glycinin and β-conglycinin components present in this fraction were likely responsible for the improved foaming properties (4). Our results for the glycinin-rich fraction produced by using the Wu procedure were different from those of Bian et al (15) and Rickert et al (4). Our glycinin-rich fractions had lower foaming capacities, foam stabilities and foaming rates but similar solubilitis, thermal behaviors, and surface hydrophobicities.

For the β-conglycinin-rich fraction, the new procedure without chilling also gave the best foaming properties. This fraction had about 50% greater foaming capacity, its foam was twice as stable, and it formed foam about three times faster than did the same fraction made by using the Wu procedure. The β-conglycinin-rich fraction produced by using the new procedure with chilling step also had good foaming capacity, rate of foaming, and foam stability. We attributed these differences in foaming properties to more denatured protein that is recovered in the intermediate fraction of the Wu procedure as evidenced by our thermal analysis. The intermediate fraction has the best foaming stabilities and rates of foaming among all fractions recovered by using the Wu procedure (4, 15). Our β-conglycinin-rich fraction produced by using the Wu procedure had similar foaming stabilities to those reported in the literature (4, 15). The intermediate fraction obtained by using the Wu procedure had low foaming capacity, the highest foaming stability of all fractions produced, and the highest foaming rate compared to the other two fractions made with this procedure. In general, the β-conglycinin-rich fractions had better foaming properties than did the glycinin-rich fractions (Table 7).

TABLE 7.
Foaming Properties of the Protein Fractions Prepared by Using the Wu and New Fractionation Procedures[a].

Fraction/Treatment	Foaming Capacity (mL/mL)	Foaming Stability (k=1/(mL*min))	Rate of foaming (Vi=mL/min)
Wu glycinin	0.964[c]	0.089[c]	2.0[c]
N4C glycinin	1.428[b]	0.075[b]	8.4[b]
NRT glycinin	1.654[a]	0.068[a]	10.3[a]
LSD	0.159	0.006	1.9
Wu intermediate	0.958±0.059	0.004 ± 0.001	17.2 ± 3.1
Wu β-conglycinin	1.069[c]	0.018[b]	12.4[c]
N4C β-conglycinin	1.597[b]	0.008[a]	32.0[b]
NRT β-conglycinin	1.648[a]	0.007[a]	34.5[a]
LSD	0.124	0.008	2.0
LSD[b]	0.130	0.009	2.3

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference for comparing all fractions within a column.

Dynamic viscosity. Dynamic viscosity is characterized by two factors: the flow consistency index (K), which is a measure of how much energy the system is taking up to flow; and the flow behavior index (n), which is a measure of how far the system behaves from an ideal Newtonian fluid. There were no significant differences among treatments for the glycinin-rich fraction for any of these two variables (Table 8). There were, however, significant differences among treatments for the β-conglycinin-rich fractions. The β-conglycinin-rich fraction obtained by using the Wu procedure had the highest consistency index, was the most viscous, and behaved furthest from an ideal fluid. Between the new treatments, the β-conglycinin-rich fraction produced by using the new procedure with chilling had higher viscosity than did the same fraction obtained by using the new procedure without chilling. The β-conglycinin-rich fraction obtained by using the new fractionation

procedure without chilling was also the fraction with highest glycinin contamination, which had the lowest viscosity of all fractions tested.

TABLE 8.
Dynamic Viscosities of Protein Fractions Prepared by Using the Wu and New Fractionation Procedures[a].

Fraction/Treatment	Flow Consistency Index (K=mPa*s)	Flow Behavior Index (n, dimensionless)
Wu glycinin	0.010[a]	0.925[a]
N4C glycinin	0.011[a]	0.867[a]
NRT glycinin	0.010[a]	0.917[a]
LSD	0.008	0.079
Wu intermediate	0.167 ± 0.027	0.739 ± 0.051
Wu β-conglycinin	0.617[a]	0.471[c]
N4C β-conglycinin	0.521[b]	0.585[b]
NRT β-conglycinin	0.070[c]	0.789[a]
LSD	0.082	0.058
LSD[b]	0.049	0.067

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference for comparing all fractions within a column.

In general, the glycinin-rich fractions were less viscous than were the β-conglycinin-rich fractions and the intermediate fraction obtained by using the Wu procedure gave intermediate dynamic viscosities. This result contrasts with findings of Rickert et al (4), who found the intermediate fraction to be the most viscous fraction for a similar fractionation procedure.

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CHAPTER 6. COMPOSITIONS OF SOY PROTEIN INGREDIENTS PREPARED FROM HIGH-SUCROSE/LOW-STACHYOSE SOYBEANS

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ABSTRACT

By using high-sucrose/low-stachyose (HS/LS) soybeans, it was possible to prepare a low-fiber soybean protein concentrate (LFSPC) by merely extracting defatted soy flour with alkali to remove fiber, neutralizing and drying. These LFSPCs were produced by using two different pHs (7.5 and 8.5) for protein extraction and the protein fractions were compared to traditional ethanol-washed soy protein concentrate (EWSPC) and soy protein isolate (SPI) prepared from normal and HS/LS soybeans. HS/LS soybeans contained lower total concentrations of free sugars, dramatically less stachyose, and significantly more galactinol (galactopyranosyl-myo-inositol). The LFSPCs had slightly lower yields of solids and protein (~70 and ~81%, respectively) than did conventional EWSPC (~77 and ~93%, respectively) but much higher than did conventional SPI (~42 and ~70%, respectively). The LFSPCs prepared from HS/LS soybeans had significantly higher protein contents (~66%) than did the same LFSPCs prepared from normal soybeans (~63%). Total isoflavone contents of these LFSPCs (~12 μmol/g) were significantly higher than for EWSPC (~1.5 μmol/g) or SPI (~10 μmol/g). The LFSPCs prepared from HS/LS soybeans contained higher sugar contents (~15%) than did either traditional EWSPC (~2.5%) or SPI (~1.5%); but, the sums of stachyose and raffinose were only ~1% for the LFSPCs compared to ~1% for EWSPC and 0.5% for SPI prepared from normal soybeans.

INTRODUCTION

Soybeans are an important world commodity because of their wide range of geographical adoption, unique chemical composition (i.e., high protein content), high nutritional value, potential health benefits, and versatile uses. Yet, only a small portion of the annual soybean production is used for food. There are several constraints associated with using soybeans and soy protein ingredients in food, including beany flavor, low oxidative

stability of soybean oil, and presence of protease inhibitors, and flatulence-causing oligosaccharides (1).

Excessive accumulation of intestinal gas, known as flatulence, has been a significant limiting factor to utilizing soybeans and soy protein ingredients in food and feed. Flatulence results from the presence of significant amounts of α-linked oligosaccharides, mainly raffinose and stachyose in soybeans. These two sugars are non-reducing and composed of one or two galactose units linked to sucrose. Humans and other monogastric animals lack α-1,6-galactosidase in their intestinal mucosa. When ingested, these soluble sugars are not absorbed and pass into the lower intestinal tract where they are metabolized by intestinal microflora, which contain the enzyme, leading to gas production (2).

The elimination of these unwanted oligosaccharides from soy protein has been largely accomplished in the past through processing but more recently genetic control offers promise. Soy protein concentrates (SPC) are widely used in the food industry and three processes, differing in the method used to render the protein insoluble in the extracting solvent, have been used commercially to prepare them. While insolubilizing, however, the protein is denatured and functionality and potential applications are compromised. The three traditional processes include washing with aqueous ethanol, washing with acid (at pH 4.5), and water washing after moist heating (1). All of these processes have the objective of extracting the soluble sugars and ash mineral components from the protein fraction of soy meal to obtain a SPC composed of at least 65% protein. The most widely used method is aqueous ethanol extraction because better flavor is achieved. All of these processes produce a by-product of soy molasses, which poses disposal problems. During ethanol washing significant amounts of potentially healthy isoflavones are lost into the molasses and protein is denatured.

There is considerable natural variation in raffinose (0.1-0.9%) and stachyose (1.4-4.1%) contents among commonly grown varieties of soybeans (3). It is now also possible to use molecular biology to genetically modify soybeans so that the sugar composition is shifted to being high in sucrose and low in oligosaccharides (4). Utilizing defatted meal from these high-sucrose/low-stachyose (HS/LS) soybeans has made it possible to consider new methods to prepare soy protein ingredients. The US patents Crank and Kerr (4) and Johnson (5)

disclosed new methods based on only removing the fiber while retaining the sugars to produce new low-fiber soy protein ingredients by merely extracting with alkali, neutralizing and spray-drying the extract. These new products have not been systematically characterized and evaluated, but one expects very different compositions and functionalities than are offered by today's soy protein ingredients. The objective of this study was to characterize and compare these LFSPCs with traditional EWSPC and SPI.

EXPERIMENTAL PROCEDURES

Materials. Air-desolventized, hexane-defatted white flakes from a commonly grown variety of normal soybeans (IA2020 variety, 1999 harvest) and from HS/LS soybeans (2 HS Soybeans, Low Stachyose, Lot-980B0001 OPTIMUM, Pioneer-DuPont, Johnston, IA) were prepared in the pilot plant at the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). The flakes were milled until 100% of the material obtained passed through a 50-mesh screen by using a Krups grinder (Distrito Federat, Mexico) and small quantities (~10 g) to preserve the native protein state. The flours were stored in sealed containers at 4°C until used.

Preparation of LFSPCs. LFSPCs were prepared by simulating the methods in the Crank and Kerr patent (4) in which protein is extracted at 7.5 and in the Johnson patent (5) in which protein is extracted at pH 8.5 (Fig. 1). About 100 g of defatted soy flour was extracted with de-ionized water at 10:1 water-to-flour ratio, the pH was adjusted to 7.5 or 8.5 with 2N NaOH, and the resulting slurry was stirred for 30 min at 60°C. After centrifuging at 14,300 x g for 30 min, a protein extract was obtained and the insoluble fiber residue was re-extracted with additional de-ionized water at 5:1 water-to-insoluble fiber ratio. The pH was adjusted as described before and the slurry was stirred for 30 min at 60°C. After centrifuging at 14,300 x g for 30 min, the second protein extract was combined with the first extract, and the insoluble fiber was discarded. The combined extract was adjusted to pH 7.0 with 2N HCl and freeze-dried. After freeze-drying, the dry fractions were stored in sealed containers until used. These procedures were replicated three times for each flour.

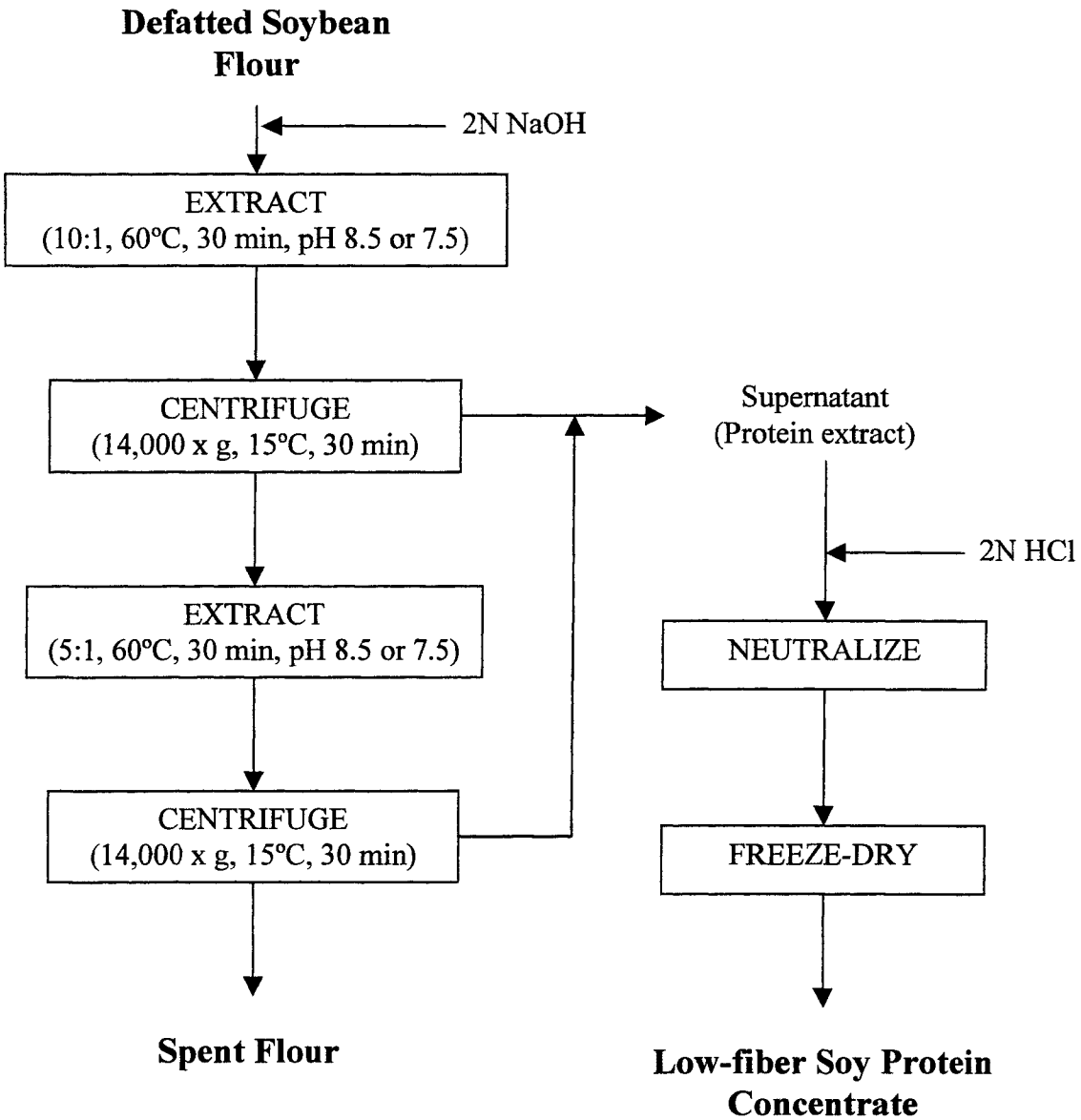

FIG. 1. Procedure for preparing new low-fiber soy protein concentrates.

Preparation of EWSPC. About 100 g of defatted soy flour was extracted with 60% ethanol/40% de-ionized water at 10:1 solvent-to-flour ratio and 40°C, and the resulting slurry was stirred for 30 min in sealed containers to avoid ethanol evaporation. After centrifuging at 14,300 x g for 30 min, SPC was obtained as the residual solids and the extract (supernatant, soy molasses), containing primarily soluble sugars, was discarded. The SPC was air-desolventized in a fume hood at 25°C for 24 h. The samples were then freeze-dried and stored in sealed containers until used. These procedures were replicated three times for each flour.

Preparation of SPI. About 150 g of defatted soy flour was extracted with de-ionized water at 10:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 30 min at 60°C. After centrifuging at 14,300 x g for 30 min, a protein extract was obtained and the insoluble fiber residue was discarded. The protein extract was adjusted to pH 4.5 with 2N HCl and centrifuged as described above. A protein curd was obtained as the precipitate and the supernatant (whey) was discarded. The curd was re-dissolved in de-ionized water and 2N NaOH was added to achieve pH 7 with approximately 10% solids content. The resulting slurry was freeze-dried and stored in sealed containers until used. These procedures were replicated three times for each flour.

Proximate analyses and mass balances. The nitrogen contents of the soy flours and each product and waste stream were determined by using the combustion or Dumas method (6) and a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). These values were converted to Kjeldahl nitrogen concentration using the conversion equation of Jung et al. (7). The conversion factor used to convert percentage of nitrogen to protein content was 6.25. Moisture content was determined by oven drying for 3 h at 130°C (8). Ash and crude fiber contents were determined by using AACC (9) and AOCS standard methods (10), respectively. Protein dispersibility index (PDI) was determined by Silliker Laboratories (Minnetonka, MN). Mass balances of solids and protein were performed for all products. All measurements were replicated at least three times and the means reported.

Protein profile analysis. Urea-sodium dodecylsulfate-polyacrylamide gel electrophoresis (urea-SDS-PAGE) was performed by using methods of Rickert et al. (11) to quantify the protein composition profiles of the protein fractions. Lipoxygenase and soy storage protein bands were identified by using pre-stained SDS-PAGE molecular-weight standard, low range (Bio-Rad Laboratories, Hercules, CA). Glycinin and β-conglycinin subunit bands were confirmed by using purified standards produced according to methods of O'Keefe et al. (12). Amounts of all unidentified bands were summed and reported as "others." Densitometry was carried out by using the Kodak 1D Image Analysis, version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). SDS-PAGE results were calculated as % composition = [(band or sum of subunit bands)/(sum of all bands)] x 100. All measurements were replicated at least four times and means reported.

Sugar composition. Samples (approximately 2 g) were extracted with 50 mL of 1:1 mixture of denatured ethanol:water. The extracts were then filtered through a 0.45-μm PTFE syringe filter (Alltech Associates, Deerfield, IL) and analyzed by HPLC. The HPLC column was an Interaction CHO-620 (Alltech Associates, Deerfield, IL) with water containing a small amount of calcium disodium EDTA as the mobile phase at 0.5 mL/min flow rate. The column was operated at 80°C. The Waters 2410 refractive index detector (Waters Corporation, Milford, MA) was operated at x 64 sensitivity. The injection volume was 20 μL. TurboChrom data system software was used for data collection and report generation. Peaks identified using standards were stachyose, raffinose, sucrose, galactinol, glucose, galactose, and fructose. Samples were run in triplicate and means reported.

Isoflavone composition. Isoflavones were extracted and analyzed by HPLC using methods of Murphy et al. (*13*). About 2.5 g of each freeze-dried sample was extracted with 10 mL of acetonitrile, 2 mL of 0.1 N HCl and about 10 mL of water, and this slurry was stirred for 2 h at 25°C. After filtering, the samples were dried by rotary evaporating at <30°C. The dry residue was redisolved in 80% HPLC-grade methanol. Aliquots of these extracts were filtered and analyzed by HPLC within 10 h of extraction. Total isoflavone contents

were adjusted for the molecular weight differences and expressed as aglucon contents of the individual isoforms (μ/g), this adjusted contents were also used for yield calculation, where yield % in a given fraction = [(total isoflavone concentration in a given fraction * mass of the given fraction)/ (total isoflavone concentration in the starting flour * initial mass of flour)] *100. For isoflavone profile analysis we used molar concentrations. Samples were run in duplicate and means reported.

Statistical analysis. The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM). Least significant differences (LSD) were calculated at $p<0.05$ to compare treatment means using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Compositions of soy flours. The flour prepared from HS/LS soybeans contained 58.3% protein, 13.3% total sugars (0.7% stachyose, 1.0% raffinose, 10.5% sucrose, 0.7% galactinol), and 2657 μg/g total isoflavones, and had 95.0 PDI. The flour prepared from IA2020 soybeans (control normal soybeans) contained 57.3% protein, 14.9% total sugars, 5.1% stachyose, 1.4% raffinose, 7.5% sucrose, 0.2% galactinol, and 1922 μg/g total isoflavones, and had 93.8 PDI.

Yields and proximate compositions of LFSPCs. The LFSPC from HS/LS soy flour extracted at pH 8.5 had higher yields of solids and protein compared to the LFSPC extracted at pH 7.5. On the other hand, the yields of solids and protein for the LFSPCs prepared from the control soy four were not different (Table 1). The yields of isoflavones were not significantly different for the LFSPCs prepared from HS/LS soy flour. In contrast, the isoflavone yield for the LFSPC extracted at pH 8.5 was higher than that for the LFSPC extracted at pH 7.5 made from the control soy flour. The proximate compositions of these products were similar, with the exception of ash contents, which was slightly higher for the LFSPC extracted at pH 8.5 for both soybean varieties. Both LFSPCs prepared from HS/LS

flour had protein contents exceeding critical minimum limit of 65% and very low crude fiber contents, whereas those prepared from normal soybeans did not quite meet the critical minimum protein content. The LFSPCs prepared from the control flour yielded significantly higher amounts of solids and isoflavones, but the LFSPC extracted at pH 8.5 had lower protein yield compared with the same fraction prepared from HS/LS flour. The protein fractions produced from the control flour (IA2020, pH 8.5 and IA2020, pH 7.5) had significantly lower protein and isoflavone contents, significantly higher total sugar contents, and similar ash and crude fiber contents. This was probably due to the control flour having lower protein and isoflavone contents and higher sugar contents.

TABLE 1
Yields and Compositions of Protein Ingredients Prepared from Normal and High-sucrose, Low-stachyose Soybeans (%, dry basis)[a].

Soybeans/ Product	Solids Yield (%)	Protein Yield (%)	Isoflavone Yield (%)	Protein Content (%)	Sugar Content (%)	Ash (%)	Crude Fiber (%)	Isoflavone Content (μg/g)
IA2020 Soybeans								
LFSPC, pH 7.5	70.4^{c}	81.4^{d}	89.6^{a}	62.3^{d}	19.1^{a}	8.0^{c}	0.3^{c}	2992^{a,b}
LFSPC, pH 8.5	71.5^{c}	82.3^{d}	87.1^{b}	62.7^{d}	18.9^{a}	8.8^{a}	0.4^{c}	2880^{b}
EWSPC	76.1^{b}	92.4^{b}	16.3^{f}	70.0^{b}	2.9^{c,d}	5.7^{e}	3.4^{b}	412^{d}
SPI	40.7^{g}	69.7^{g}	54.4^{d}	91.3^{a}	1.8^{d}	4.2^{g}	0.3^{c}	2570^{c}
HS/LS Soybeans								
LFSPC, pH 7.5	67.4^{e}	79.5^{e}	78.4^{c}	66.6^{c}	14.7^{b}	8.4^{b}	0.3^{c}	3092^{a}
LFSPC, pH 8.5	69.1^{d}	84.0^{c}	80.0^{c}	66.3^{c}	14.7^{b}	8.7^{a}	0.2^{c}	3087^{a}
EWSPC	78.4^{a}	94.8^{a}	12.2^{g}	69.4^{b}	2.2^{c}	6.0^{d}	4.4^{a}	416^{d}
SPI	42.4^{f}	71.6^{f}	49.9^{e}	92.1^{a}	1.3^{d}	4.5^{f}	0.3^{c}	3129^{a}
LSD	1.3	1.2	1.8	1.4	0.9	0.2	0.3	176

[a] n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose, low-stachyose soybeans; IA2020, normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and spray-drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference at $p<0.05$.

Significantly higher amounts of solids and protein were recovered in the LFSPCs, compared to traditional SPI, and less solids and protein compared to EWSPC. The isoflavone yields were significantly higher in the LFSPCs compared to traditional soy protein ingredients. This is because most of the isoflavones are washed out during ethanol/water extraction when producing EWSPC and a significant amount of isoflavones are lost to the

whey in producing SPI (14). The LFSPCs also had much higher total sugar contents compared to the SPIs and EWSPCs as would be expected. The LFSPCs had crude fiber contents similar to SPI and were significantly lower in fiber than was EWSPC. The isoflavone contents of the LFSPC products were significantly higher than those of the traditional soy protein ingredients because the total extract is dried in the case of the LFSPCs.

In general, the protein fractions prepared from HS/LS soybeans yielded significantly higher amounts of protein than did those from normal soybeans, probably due to the higher protein content of the soy flour. The products prepared from IA2020 soybean yielded higher amounts of isoflavones, but had lower concentrations of these phytochemicals in the finished protein fractions. This was also probably due to the significantly lower isoflavone content of the IA2020 flour.

Protein composition. The protein component profiles of the two flours were similar (Table 2). There were no differences in the protein component profiles for the two LFSPCs and extraction pH did not affect the protein components of the LFSPCs prepared from HS/LS soybeans. The LFSPCs prepared from IA2020 soybeans had significantly different protein profiles from those prepared from HS/LS soybeans. The LFSPCs prepared from IA2020 soybeans had significantly less β-conglycinin and higher glycinin contents.

Comparing the LFSPCs to traditional soy protein ingredients, there were differences in protein component profile. The LFSPCs contained more β-conglycinin and less glycinin than did either EWSPC or SPI. The EWSPCs prepared from IA2020 soybeans had significantly higher lipoxygenase contents compared to the other protein fractions. The protein component profiles of all protein fractions were different from those of the starting soy flours. This differential partitioning of the proteins was attributed to different extents of solubilizing each protein. This increased concentration in β-conglycinin may impact the functional properties of these ingredients.

The protein fractions prepared from HS/LS soybeans also had different protein component compositions from the same fractions prepared from IA2020 soybeans. The SPI prepared from HS/LS soybeans contained less lipoxygenase than did the SPI prepared from IA2020 soybeans. The EWSPC prepared from HS/LS soybeans contained less lipoxygenase

than did EWSPC prepared from IA2020 soybeans. The proportions of β-conglycinin to glycinin for SPI and EWSPC were the same for both flours.

TABLE 2
Protein Component Profiles of Flours and Protein Ingredients Prepared from Normal and High-sucrose, Low-stachyose Soybeans (% of total protein) [a].

Soybeans/Product	Lipoxygenase	β-conglycinin	Glycinin	Others
IA2020 Soybeans				
Flour	5.37^{a}	30.08^{c}	50.22^{b}	$14.33^{a,b}$
LFSPC, pH 7.5	3.90^{c}	30.49^{c}	$52.22^{a,b}$	13.39^{b}
LFSPC, pH 8.5	3.07^{d}	$32.41^{b,c}$	54.23^{a}	$10.29^{d,e}$
EWSPC	4.63^{b}	$31.42^{b.c}$	52.93^{a}	$11.02^{c,d}$
SPI	3.92^{c}	33.52^{b}	52.73^{a}	9.83^{e}
HS/LS Soybeans				
Flour	5.94^{a}	29.01^{c}	50.51^{b}	14.54^{a}
LFSPC, pH 7.5	3.84^{c}	36.80^{a}	48.60^{b}	$10.76^{c,d,e}$
LFSPC, pH 8.5	3.72^{c}	36.85^{a}	48.73^{b}	$10.69^{c,d,e}$
EWSPC	$3.37^{c,d}$	$31.38^{b,c}$	54.99^{a}	$10.26^{d,e}$
SPI	2.86^{d}	$31.81^{b,c}$	53.79^{a}	11.54^{c}
LSD	0.61	2.29	3.66	1.12

[a] n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose, low-stachyose soybeans; IA2020, normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and spray-drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference at $p<0.05$.

Sugar compositions. The sugar profile of the LFSPC extracted at pH 8.5 prepared from HS/LS soybeans was not significantly different from that of the LFSPC extracted at pH 7.5 (Table 3). The sugar profiles of the same protein fractions prepared from IA2020 soybeans were different, with much higher contents of stachyose (over 13 times more) and about 16% higher raffinose content than those of fractions prepared from HS/LS soybeans. The LFSPCs prepared from IA2020 soybeans had slightly lower sucrose contents, about one seventh as much galactinol, and higher glucose and lower fructose contents.

The LFSPCs had much higher sugar contents did the traditional soybean protein ingredients; however, the stachyose contents of the LFSPCs were similar to that of SPI and less than that of EWSPC. The raffinose contents of the LFSPCs were slightly higher than

those of the traditional soybean protein ingredients. The LFSPCs were about 10-fold higher in sucrose and 30-fold higher in galactinol contents than were the traditional SPI and EWSPC.

The LFSPCs prepared from HS/LS soybeans had very different sugar profiles due to compositional differences of the soy flours. The protein fractions prepared from IA2020 soybeans had higher sugar contents than those of the same fractions prepared from HS/LS soybeans. The SPI prepared from HS/LS soybeans had one-tenth of the amount of stachyose as in SPI prepared from normal soybeans and six times as much galactinol and similar amounts of the other sugars.

TABLE 3
Sugar Compositions of Defatted Flours and Protein Ingredients Prepared from Normal and High-sucrose, Low-stachyose Soybeans (% dry basis)[a].

Soybeans/Product	Stachyose	Raffinose	Sucrose	Galactinol	Glucose	Galactose	Fructose
IA2020 Soybeans							
Flour	5.07^{b}	1.38^{a}	7.48^{d}	0.16^{c}	0.58^{a}	0.09^{a}	0.11^{c}
LFSPC, pH 7.5	6.17^{a}	0.77^{c}	11.56^{b}	0.09^{d,e}	0.49^{b}	0.00^{d}	0.08^{d}
LFSPC, pH 8.5	6.08^{a}	0.75^{c}	11.45^{b}	0.10^{d}	0.46^{c}	0.00^{d}	0.07^{d,e}
EWSPC	0.90^{c}	0.22^{e}	1.55^{e,f}	0.02^{f}	0.08^{f}	0.07^{b}	0.04^{e}
SPI	0.47^{d}	0.05^{f}	1.16^{f}	0.01^{f}	0.05^{g}	0.00^{d}	0.05^{c,d}
HS/LS Soybeans							
Flour	0.71^{c,d}	0.98^{b}	10.54^{c}	0.71^{a}	0.23^{d}	0.00^{d}	0.08^{d}
LFSPC, pH 7.5	0.44^{d,e}	0.62^{d}	12.65^{a}	0.62^{b}	0.11^{e}	0.00^{d}	0.29^{a}
LFSPC, pH 8.5	0.45^{d}	0.66^{d}	12.60^{a}	0.61^{b}	0.09^{e,f}	0.00^{d}	0.25^{b}
EWSPC	0.07^{e,f}	0.10^{f}	1.81^{e}	0.09^{d,e}	0.03^{g,h}	0.05^{c}	0.05^{e}
SPI	0.04^{f}	0.04^{f}	1.07^{f}	0.06^{e}	0.01^{h}	0.00^{d}	0.07^{d,e}
LSD	0.38	0.07	0.54	0.03	0.03	0.01	0.03

[a] n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose, low-stachyose soybeans; IA2020, a line of normal soybeans; LFSPC, low fiber soy protein concentrate prepared by alkali extraction, neutralizing and spray-drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference at $p<0.05$.

In October 1999 the FDA approved a health claim for soy protein and soy protein containing products. To meet the requirements for this health claim foods must contain 6.25 grams of soy protein per serving (14). Parsons et al (15) compared the total metabolisable energy of three conventional soybean meals and five low-oligosaccharide soybean meals fed to roosters, and concluded that the total metabolisable energy of low-oligosaccharide soybean

meals was significantly higher. Suarez et al (16) compared the gas production and gaseous symptoms in healthy human subjects fed either normal or HS/LS soybeans, and concluded that those subjects fed soy flour low in oligosaccharides had less gas production than those fed with conventional soy flour. Both studies (15, 16) used soybean materials that had similar sugar profiles to our soybean flours. Based on these studies and the health claim on soy protein we calculated the amounts of ingredients that would be needed per serving to meet FDA's requirements to be 10.7 g for HS/LS soy flour, 9.4 g for LFSPC made from HS/LS soy flour, 8.9 g for EWSPC made from normal soybeans, and 6.85 g for SPI made from normal soybeans. Based on the sugar profile, we calculated the amount of indigestible sugars (stachyose + raffinose + galactinol, we assumed galactinol is indigestible) that each of these servings would contain (0.25 g for HS/LS soy flour, 0.16 g for LFSPC made from HS/LS soybeans, 0.11 g for EWSPC, and 0.04 for SPI of indigestible sugar/serving). When these same calculations were made for normal soy flour the amount of indigestible sugar increased to 0.72 g/serving. LFSPC made from HS/LS soybeans contained higher amounts of indigestible sugars compared to traditional soy protein ingredients (45% more than EWSPC and 4 times more than SPI), but these amounts were significantly lower than for normal soy flour (about 78% less). These LFSPC ingredients have reduced amounts of indigestible sugars and can to replace some traditional soy protein ingredients without causing intestinal gas.

Isoflavone composition. The isoflavone profiles of the soy flours and protein fractions are shown in Table 4. The isoflavones commonly found in soybeans and soy protein products are genistein, daidzein, and glycitein, which occur in four forms, the aglucon, the β-glucoside, the malonyl-β-glucoside, and the acetyl-β-glucoside. Of these four isoforms, the β-glucosides and the malonyl-β-glucosides predominant in soybeans (13) and the isoflavone profile and isoforms distribution are altered during processing (17, 18).

The isoflavone contents and profiles of the soy flours were significantly different between the two types of soybeans. Soy flour prepared from HS/LS soybeans contained about 38% more total isoflavones than did soy flour prepared from IA2020 soybeans. Because of this difference, we converted data in Table 4 to percentages of the total isoflavone

contents to be able to compare isoflavone isoforms conversion and partitioning. The defatted flour prepared from HS/LS soybeans contained 47.5% daidzein, 45.0% genistein, and 7.7% glycitein; whereas, the flour prepared from IA2020 soybeans contained 42.9% daidzein, 50.4% genistein, and 6.5% glycitein. Both flours contained about 95% glucosides plus malonylglucosides and only 5% of the other two isoforms. The aglycon isoflavone contents for both flours were about 3%.

The extraction pH used to prepare the LFSPCs did not significantly affect isoflavone extraction and the LFSPCs prepared from both soybean types contained about 40% daidzein, 55% genistein, and 5% glycitein, with similar total yields and concentrations. The distribution of isoforms, however, was significantly affected. The LFSPC extracted at pH 8.5 had significantly less malonylglucosides (43.2%) and acetylglucosides (1.5%), and higher amounts of glucosides (49.1%) than did same fractions extracted at pH 7.5 (63.2% malonylglucosides, 2.3% acetylglucosides, and 28.0% glucosides). The conversion from malonylglucoside to glucoside isoform has been previously reported (17,18). Apparently, alkali extraction significantly favors conversion. The aglucon isoform contents for both extraction pHs significantly increased, from 3.5% in the flour prepared from HS/LS soybeans to 6.4% in the LFSPCs. This result can be partly attributed to the action of native soybean β-glucosidases during the extraction step (18).

When comparing the LFSPCs prepared from HS/LS soybeans to those prepared from IA2020 soybeans, we observed different isoflavone profiles. The LFSPCs prepared from IA2020 soybeans had similar total daidzein and genistein contents (~46%), and about 8% glycitein. Their isoform distribution followed the same trend as was observed for the HS/LS protein fractions, but with higher aglucon isoform production (10.1 and 12.4% for the products extracted at pH 8.5 and 7.5, respectively).

The LFSPCs had significantly different isoflavone profiles compared to those of traditional soy protein ingredients. The LFSPCs prepared from IA2020 soybeans had similar contents of daidzein, genistein and glycitein as did the LFSPCs prepared from HS/LS soybeans. Apparently, the isoflavones present in the soy flour of normal soybeans were more completely solubilized during extraction than those in HS/LS soy flour. The EWSPC had significantly lower total isoflavone content than did the LFSPCs (10:1) and the distribution

TABLE 4.
Isoflavone Profiles of Defatted Flours and Protein Ingredients Prepared from Normal and High-sucrose, Low-stachyose Soybeans (μmol/g) [a].

Soybeans/Product	Din	MDin	AcDin	Dein	Glyin	MGly	Glyein	Gin	MGin	AcGin	Gein	Total
IA2020 Soybeans												
Flour	0.73^{f}	2.18^{c}	0.05^{c}	0.13^{d}	0.22^{d}	$0.25^{b,c}$	0.00^{e}	1.01^{f}	2.44^{c}	0.08^{f}	0.10^{c}	7.20^{d}
LFSPC, pH 7.5	$1.58^{c,d}$	3.08^{a}	0.09^{b}	0.61^{a}	0.35^{b}	0.40^{a}	0.11^{a}	1.47^{e}	2.76^{c}	0.11^{d}	0.67^{a}	$11.24^{a,b}$
LFSPC, pH 8.5	2.60^{a}	1.87^{d}	0.06^{c}	0.44^{b}	0.50^{a}	0.27^{b}	0.09^{b}	2.57^{c}	1.79^{e}	0.09^{e}	$0.57^{a,b}$	10.85^{b}
EWSPC	0.19^{g}	0.36^{e}	0.04^{d}	0.09^{d}	0.06^{e}	0.06^{e}	0.03^{d}	0.25^{g}	0.34^{f}	0.07^{g}	0.07^{c}	1.54^{e}
SPI	1.70^{c}	1.71^{d}	0.08^{b}	$0.40^{b,c}$	0.31^{c}	0.23^{c}	$0.07^{b,c}$	2.44^{c}	2.05^{d}	0.15^{c}	$0.51^{a,b}$	9.65^{c}
HS/LS Soybeans												
Flour	$1.51^{c,d}$	2.95^{b}	0.07^{c}	0.18^{d}	$0.34^{b,c}$	0.39^{a}	0.04^{d}	$1.62^{d,e}$	2.65^{c}	0.09^{e}	0.13^{c}	9.97^{c}
LFSPC, pH 7.5	1.24^{e}	3.17^{a}	0.11^{a}	0.27^{c}	0.22^{d}	$0.26^{b,c}$	0.06^{c}	1.79^{d}	3.89^{a}	0.16^{b}	0.40^{b}	11.59^{a}
LFSPC, pH 8.5	2.18^{b}	2.12^{c}	0.06^{c}	0.28^{c}	0.31^{c}	0.18^{d}	$0.05^{c,d}$	3.21^{a}	2.70^{c}	0.11^{d}	0.41^{b}	11.60^{a}
EWSPC	0.12^{g}	0.39^{e}	0.04^{d}	0.06^{d}	0.04^{e}	0.05^{e}	0.00^{e}	0.22^{g}	0.48^{f}	0.08^{f}	0.06^{c}	1.56^{e}
SPI	1.45^{d}	2.26^{c}	0.10^{a}	0.30^{c}	0.21^{d}	$0.24^{b,c}$	0.06^{c}	2.93^{b}	3.47^{b}	0.21^{a}	0.49^{b}	11.72^{a}
LSD	0.20	0.20	0.01	0.13	0.03	0.04	0.02	0.27	0.21	0.01	0.18	0.67

[a] n=3. Means within a column followed by different superscripts are significantly different at $P<0.05$. Din denotes daidzin; MDin, malonyldaidzin; AcDin, acetyldaidzin; Dein, daidzein; Glyin, glycitin; MGly, malonylglycitin; Glyein, glycitein; Gin, genistin; MGin, malonylgenistin; AcGin, acetylgenistin; and Gein, genistein. HS/LS denotes high-sucrose, low-stachyose soybeans; IA2020, a specific line of normal soybeans; LFSPC, low fiber soy protein concentrate prepared by alkali extraction, neutralizing and spray-drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference at $p<0.05$.

was also significantly different. This was not surprising since isoflavones are known to be lost during ethanol washing of soy flour. The LFSPC prepared from IA2020 soybeans had an isoflavone distribution of 44.1% daidzein, 47.4% genistein, and 9.7% glycitein. When comparing the isoform distributions, the LFSPC prepared from IA2020 soybeans had similar glucoside and malonylglucoside contents as did the LFSPC prepared from HS/LS soybeans when extracted at pH 8.5 (46.1 and 41.4%, respectively), but had significantly higher acetylglucoside and aglucon contents (3.3 and 10.2%, respectively). The EWSPC prepared from IA2020 soybeans had a unique isoform distribution, 49.3% malonylglucosides, 32.5% glucosides, 7.1% acetylglucosides, and 12.3% aglycons. These data indicated significant conversion of malonylglucosides to acetylglucosides and aglycons occurred or the ethanol extraction redistributed the native isoflavone profile. The later reason is more likely since only about 10% of the original soy flour isoflavones were recovered in EWSPC and the processing temperatures of 40°C, ethanol concentration of about 60%, and extraction pH of about 6.8 should have limited the activity of native β-glucosidases, heat conversion and alkaline hydrolysis.

In general, the protein fractions prepared from HS/LS soybeans had significantly different isoflavone profiles than did the same fractions prepared from IA2020 soybeans. The protein fractions prepared from IA2020 soybeans had consistently higher aglucon isoform contents (3 to 4 times higher than in the starting soy flour). The protein fractions prepared from HS/LS soybeans had consistently higher genistein and lower daidzein contents than did the same fractions prepared from IA2020 soybeans, which is not surprising since the ingredients made from HS/LS soy flour had higher levels of isoflavones.

Integration of yield and compositional data. The LFSPCs were low in crude fiber and indigestible sugars, and high in minerals, and isoflavones. This new procedure yielded significantly higher amounts of solids and protein compared to alternative processes, and the protein fraction has unique sugar, protein, and isoflavone profiles, and exceeds the critical industrial standard of having at least 65% protein. The LFSPCs had unique protein profile, enriched in the β-conglycinin component, which should influence their functional properties.

The LFSPCs were not exposed to acid or aqueous ethanol, which denature protein. Therefore, these LFSPCs should have unique applications as food ingredients.

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CHAPTER 7. FUNCTIONAL PROPERTIES OF SOY PROTEIN INGREDIENTS PREPARED FROM HIGH-SUCROSE/LOW-STACHYOSE SOYBEANS

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ABSTRACT

High-sucrose/low-stachyose (HS/LS) soybeans were used to prepare ethanol-washed soy protein concentrate (EWSPC), soy protein isolate (SPI) and a new low-fiber soy protein concentrate (LFSPC) in which the protein was extracted with alkali to remove fiber and the protein extract was neutralized and freeze-dried. The LFSPCs were high in soluble sugars and low in fiber compared to traditional EWSPC and SPI. For both normal and HS/LS soybean varieties, the LFSPCs had higher denaturation enthalpies than did EWSPC and the SPI prepared from, indicating that the LFSPC procedure denatured less protein. Water solubilities, surface hydrophobicities and emulsification properties were highest for the LFSPCs and lowest for EWSPC. The LFSPCs also had good foaming properties and low viscosities. These desirable functional properties of the LFSPCs make them unique among alternative soy protein ingredients and highly suitable for industrial applications as food additives and ingredients.

INTRODUCTION

Consumer demand for soy-based foods and soy-protein food ingredients is rapidly increasing. U.S. retail sales of foods containing soy protein have grown by more than 10% per year for the last seven years reaching an estimated annual retail market of $3.65 billion in 2002 (1). The current driving force in the soy food industry is increasing recognition of the health properties of soy protein ingredients. This growth in consumer demand, however, is limited by concerns for poor flavor, presence of antinutritional factors and flatus-causing sugars, and limited functionality (2). Producing new products with enhanced health benefits and superior functional properties is key to further increasing consumption of soy products.

There are a number of ways in which biotechnology can improve soy-based foods to increase their consumption. Reducing contents of indigestible and flatus-causing sugars is one example (1) and this genetic modification was recently achieved by Pioneer-DuPont (Johnston, IA). In our previous work (3), we reported on the compositional characteristics of a new low-fiber soy protein concentrate (LFSPC) based on a new soybean variety that has been genetically modified to contain high sucrose and low stachyose contents (3) and a different processing approach in which defatted soy flour is merely extracted with alkali, and the protein extract is neutralized and freeze-dried (5, 6). Defatted soy flour prepared from high-sucrose/low-stachyose (HS/LS) typically contains ~0.7% stachyose and ~10.5% sucrose (3) compared to ~4.7 and ~5.7%, respectively, for defatted soy flour prepared from normal soybeans (4). Even though the soluble sugars are present in the protein extracts and dried protein fractions, the modified sugar contents do not need to be removed because they are digestible and do not cause flatulence and contribute sweetness. In all other soy protein ingredients, soluble sugars are removed.

Although most of the soy protein in the United States is used as toasted meal for feeding livestock, a growing proportion of this inexpensive protein is used to produce refined food ingredients (2). The utilization of soy protein as food ingredients is based upon their functional properties. Functional properties are the physico-chemical characteristics of proteins that determine their behavior and performance in food systems during processing, storage, food preparation, and consumption (7). The desired functional properties, and as a consequence the applications that they are useful in, vary from product to product (8). Soy flours, protein concentrates, and protein isolates have distinctly different applications in the food products.

Several factors influence the functional properties of protein ingredients, including intrinsic, environmental and processing (7). The development of any new soy protein ingredient requires functional characterization to identify food applications where it has competitive advantages. The functional properties of a new protein ingredient will determine its value and applicability to different food systems. In the present work, our central hypothesis was that the difference in the composition HS/LS soybeans and the method of producing a new SPC will result in functional properties that are different from those of

traditional SPC and soy protein isolates (SPI). The objectives of this study were to characterize the functional behavior of this LFSPC, compare two different extraction pH's used in their preparation, and compare the functional properties of LFSPCs to traditional ethanol-washed soy protein concentrate (EWSPC) and isoelectric-precipitated SPI.

EXPERIMENTAL PROCEDURES

Preparation of soy flours and protein ingredients. All protein ingredients were prepared from air-desolventized, hexane-defatted white flakes of control normal soybeans (IA 2020 variety, 1999 harvest) and HS/LS white flakes (2 HS Soybeans, Low Stachyose, Lot-980B0001 OPTIMUM, Pioneer-DuPont, Johnston, IA, 1999 harvest). Both white flake samples were produced from soybeans extracted in the Crops Products Pilot Plant of the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). Triplicate runs from each flour type and for each of the four procedures (new SPC prepared at two different extraction pHs (8.5 and 7.5), EWSPC, and SPI) were prepared according to procedures described in our previous work (8). The freeze-dried products were stored in sealed containers at 4°C until used.

Thermal behavior. Thermal behaviors of the protein fractions were determined by using differential scanning calorimetry (DSC). Samples (15-20 mg) of 10% (w/w, dry basis) dispersions were hermetically sealed in aluminum pans. Sealed, empty pans were used as references. The samples were heated from 25 to 120°C at 10°C/min using an SII Exstar 6000 DSC (Seiko Instrument, Inc., Tokyo, Japan). All samples were analyzed at least three times and means reported.

Solubility. Solubility was evaluated according to methods of Rickert et al. (8) by preparing 1% (w/w dry basis) sample dispersions in de-ionized water. The pH was adjusted over the range 2.0 to 11.0 by using 2N HCl or NaOH. The dispersions were stirred for 1.0 h. Aliquots (25 mL) of the dispersions were transferred to 50-mL centrifuge tubes and centrifuged at 10000 x g and 20°C for 10 min. The protein content of the supernatant was measured by using the Biuret method with bovine serum albumen (Sigma, St. Louis, MO) as

the standard of reference. Solubility was calculated as % Solubility = (protein in supernatant/initial protein content) x 100.

Surface hydrophobicity. Surface hydrophobicity was measured by using the methods of Wu et al. (9) with 1-anilino-8-naphthalene sulfonic acid magnesium salt monohydrate (ANS, ICN Biomedicals, Inc., Aurora, OH). Protein dispersions (prepared as in the solubility test) were stirred, adjusted to pH 7.0, and centrifuged at 10000 x g and 20°C for 10 min. Aliquots of soluble protein (supernatant) were serially diluted with 0.1 M phosphate buffer (pH 7.0) to obtain 6.25 to 100 μg/mL protein and 40 μL of ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was dispersed in 3-mL aliquots of each dilution. Fluorescence intensity units (FIU) were measured with a Turner Quantech® spectrophotometer (Barnstead Thermolyne, Dubuque, IA) using 440 nm (excitation) and 535 (emission) filters. FIUs were standardized by using a solution of 40 μL of ANS in 3 mL of phosphate buffer as the zero point and 15 μL of ANS in 3 mL of methanol assigned an arbitrary value of 80 FIU. FIUs were plotted against protein concentration. The slope of the regression line was reported as surface hydrophobicity. Samples were run in triplicate and means reported.

Emulsification properties. Emulsification capacity (EC) was measured according to the method of Bian et al. (10) with modifications. Dispersions (25 mL) of 2 % (w/w, dry basis) sample were adjusted to pH 3.0, 4.0, 5.0, or 7.0 with 2 N HCl or NaOH as needed and transferred to 400-mL plastic beakers. Soybean oil, dyed with approximately 4 ppm Sudan Red 7B (Sigma, St. Louis, MO), was continuously blended into the protein dispersions at 37 mL/min flow rate by using a Bamix wand mixer (ESGE AG Model 120, Mettlen, Switzerland) at the low setting until phase inversion was observed. EC (g oil/g sample) was calculated as g of oil needed to cause inversion multiplied by 2. Samples were run at least in triplicate and means reported.

Emulsification activity (EA) and emulsification stability index (ESI) were measured according to methods of Rickert et al. (6). Dispersions of 21 mL of 2 % (w/w, dry basis) samples adjusted to pH 3.0, 4.0, 5.0 or 7.0 were blended with 7 mL of refined soybean oil (Bakers' and Chefs' Vegetable Oil, North Arkansas Wholesale Company Inc., Bentonville,

AK) in a 250-mL glass beaker for 1.0 min by using the Bamix wand mixer at low speed. Immediately after mixing, the emulsion was diluted 1:1000 with 0.1% sodium dodecil sulfate. The absorbance was measured at 500 nm and recorded as EA. After 15 min, the absorbance was measured again. These two absorbance readings were used to calculate ESI as:

$$ESI\ (min) = (A_0/A_0\text{-}A_{15})t$$

where A_0 and A_{15} are absorbance at time 0 and 15 min, respectively, and t is the time interval. Samples were run in triplicate and means reported.

Foaming properties. Foaming capacity (FC), foaming stability (K), and rate of foaming (Vi) were measured according to methods of Sorgentini et al. (11) with modifications (8). A 0.5% (w/w, dry basis) sample dispersion was prepared and the pH adjusted to 7.0. A 95-mL aliquot was loaded into a custom-designed glass column (58.5 cm x 2 cm) fitted with a coarse glass frit at the bottom, and N_2 was purged through the sample at 100 mL/min flow rate. The time for the foam to reach the 300-mL mark, the time for one-half of the liquid incorporated into the foam to drain back, and the volume of the liquid incorporated into the foam were measured. Three parameters were calculated as:

$$FC = V_f/(f_r \times t_f)$$

$$K\ (\text{specific rate constant of drainage}) = 1/(V_{max} \times t_{1/2})$$

$$V_i\ (\text{rate of liquid conversion to foam}) = V_{max}/t_f$$

where V_f = the fixed volume of 300 mL, f_r = the flow rate of the gas, t_f = time to reach V_f, V_{max} is the volume of liquid incorporated into foam, and $t_{1/2}$ is the time to drain one-half of the liquid incorporated into the foam. Samples were run in triplicate and means reported.

Dynamic viscosity. A 10% (w/w, dry basis) sample dispersion was prepared at pH 7.0 (8). The sample was applied to the plate of a RS-150 Rheo Stress (Haake, Karlsruhe, Germany) and shear was applied with a 60-mm 2° titanium cone (C60/2 Ti) from 10 to 500/s shear rate, at constant temperature (23°C). Shear rate (γ) and shear stress (τ) over the course of the analysis, in combination with the power-law formula application, were used to determine the consistency coefficient (k) and flow behavior index (n), where $\tau = k\gamma^n$. Using

k, n, and γ, apparent viscosity (η) was estimated by the equation $\eta = k\gamma^{n-1}$. Samples were run in triplicate and means reported.

Statistical analyses. The data were analyzed by using Analysis of Variance (ANOVA) and General Linear Model (GLM), and least significant differences (LSD) were calculated at the 5% level to compare treatment means using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Thermal behavior. The LFSPC extracted at pH 8.5 had lower denaturation enthalpies for both the glycinin and β-conglycinin components than did the LFSPC extracted at pH 7.5 (Table 1), probably due to more denaturation because of the higher pH used for extraction (12). The peak denaturation temperature for β-conglycinin was slightly lower for the LFSPC extracted from HS/LS soy flour at pH 8.5 than for the LFSPC extracted HS/LS soy flour at pH 7.5. This was probably due to more denaturation of β-conglycinin during alkali extraction. The β-conglycinin component of LFSPC prepared from HS/LS soybeans was less thermally active than the same products made from IA2020 soybeans and for both extraction pHs. Interestingly, the LFSPCs made from HS/LS soybeans had significantly more β-conglycinin (3) than did the same ingredients made from IA2020 soybeans, but this component was more readily denatured. The thermal behaviors of the glycinin components were similar for both varieties.

Comparing the LFSPCs to the traditional soy protein ingredients, the LFSPC extracted at pH 8.5 had similar denaturation enthalpies to those of SPI and significantly higher than those of EWSPC. The LFSPC extracted at pH 7.5, however, had significantly higher denaturation enthalpies than did the SPI and EWSPC. It is interesting to note that for both flour types, EWSPC had substantial thermal activities. This may be due, in part, to an incomplete desolventization of the ethanol adsorbed to the protein fractions. The denaturation temperatures shifted to significantly lower temperatures for EWSPC and SPI, probably caused by partial denaturation of the proteins by either ethanol or acid that reduces the activation energy required for denaturation (12). In general, the protein fractions prepared

from HS/LS soybeans had lower denaturation enthalpies for β-conglycinin and higher enthalpies for glycinin, when comparing the protein fractions from the two different flours. One possible explanation for this phenomenon is that the subunit make-up of the β-conglycinin component was different among soybean varieties and affected the thermal behavior of this protein (13).

TABLE 1
Thermal Properties of Protein Ingredients Prepared from Normal and High-sucrose/Low-stachyose Soybeans [a].

Soybeans/Protein Fraction	β-Conglycinin Peak Denaturation Temperature (°C)	Glycinin Peak Denaturation Temperature (°C)	β-Conglycinin Enthalpy (mJ/mg of protein)	Glycinin Enthalpy (mJ/mg of protein)
IA2020 Soybeans				
LFSPC, pH 7.5	74.2[a]	94.4[a]	2.17[a]	8.35[a]
LFSPC, pH 8.5	73.9[a,b]	93.8[a]	1.14[c]	7.27[c]
SPI	73.4[b,c]	91.9[b]	1.18[b,c]	7.25[c]
EWSPC	71.7[d]	89.7[c]	0.83[d]	6.89[d]
HS/LS Soybeans				
LFSPC, pH 7.5	73.9[a,b]	94.0[a]	1.40[b]	8.66[a]
LFSPC, pH 8.5	72.9[c]	94.4[a]	0.78[d]	7.71[b]
SPI	72.9[c]	92.5[b]	0.67[d]	7.77[b]
EWSPC	71.3[d]	89.1[c]	0.64[d]	6.41[e]
LSD	0.6	0.7	0.22	0.33

[a] n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose/low-stachyose soybeans; IA2020, a line of normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference.

Solubility. The protein solubilities over the pH range 2 to 11 for all protein fractions are shown in Table 2. Both LFSPCs were generally much more soluble than were the two traditional protein fractions and exhibited the characteristic U shaped solubility curves when solubility was plotted against pH. Although the LFSPC extracted at pH 7.5 had a higher proportion of thermally active protein remaining, it was consistently less soluble than was the LFSPC extracted at pH 8.5.

TABLE 2

Protein Solubilities of Protein Ingredients Prepared from Normal and High-sucrose/Low-stachyose Soybeans(%) [a].

Soybeans/Protein Fraction	pH									
	2	3	4	5	6	7	8	9	10	11
IA2020 Soybeans										
LFSPC, pH 7.5	96.9^{a}	94.8^{a,b}	21.8^{a}	19.8^{a}	91.8^{a}	98.2^{a}	99.3^{a}	98.8^{a}	98.9^{a}	99.2^{a}
LFSPC, pH 8.5	98.6^{a}	96.6^{a}	20.3^{a}	17.8^{b}	92.9^{a}	98.7^{a}	98.7^{a}	97.7^{a}	98.5^{a}	98.2^{a}
SPI	89.9^{a}	87.9^{c}	24.4^{a}	7.3^{d}	87.0^{b}	91.0^{b}	90.7^{b}	90.2^{b}	90.7^{b}	90.3^{b,c}
EWSPC	41.0^{f}	16.3^{e}	4.3^{c}	4.8^{e}	15.0^{d}	20.3^{d}	26.6^{d}	34.8^{c}	44.5^{d}	78.9^{d}
HS/LS Soybeans										
LFSPC, pH 7.5	85.2^{d}	84.5^{d}	10.9^{b}	7.6^{d}	66.0^{c}	85.9^{c}	86.1^{c}	88.5^{b}	87.3^{c}	88.1^{c}
LFSPC, pH 8.5	96.7^{a}	92.3^{b}	18.7^{a}	14.1^{c}	84.9^{b}	96.8^{a}	99.6^{a}	100.7^{a}	100.7^{a}	100.4^{a}
SPI	87.6^{c}	87.1^{c,d}	12.0^{b}	0.9^{f}	85.7^{b}	88.3^{c}	88.6^{b,c}	89.9^{b}	91.0^{b}	90.7^{b}
EWSPC	44.4^{e}	15.1^{e}	6.0^{b,c}	4.8^{e}	8.7^{e}	12.7^{e}	18.4^{e}	22.3^{d}	39.1^{e}	75.2^{e}
LSD	2.1	3.0	6.1	1.5	2.9	2.5	3.5	3.4	3.4	2.3

[a] n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose/low-stachyose soybeans; IA2020, a line of normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference.

The LFSPCs had significantly higher solubilities than did EWSPC and similar solubilities to those of SPI. The solubility of EWSPC is quite poor unless jet cooked or alkaline homogenized to break up denatured protein aggregates (14). The solubilities in the high pH range (7-11) and the low pH range (2-3) for the LFSPC extracted at pH 8.5 were significantly higher than were the solubilities for SPI, while the LFSPC extracted at pH 7.5 had less solubility.

Both LFSPCs and SPI prepared from IA2020 soybeans had significantly higher solubilities than did the same products produced from HS/LS soybeans. This same trend was also observed for the EWSPC prepared from IA2020 soybeans at pH >6, but no differences for pHs <5. The thermal behavior data partially explain this phenomenon. It seems that the protein present in HS/LS soybeans was more readily solubilized from the soy flour matrix (3), especially the β-conglycinin component, but at the same time this protein was more readily denatured and lost solubility.

Surface hydrophobicity. Surface hydrophobicity depends on two main factors: the combination of denaturation processes (heat, alkali, acid, ethanol, etc), which tend to increase surface hydrophobicity by unfolding the protein structure and exposing hydrophobic regions; and the aggregation phenomena that tends to decrease surface hydrophobicity by means of protein-protein interactions and consequential reduction of exposed hydrophobic region to the probe (12).

For both soybean lines, the LFSPCs extracted at pH 8.5 had significantly higher surface hydrophobicity than did the LFSPCs extracted at pH 7.5 (Table 3). We believe this effect was due to the greater extent of protein denaturation of the LFSPC extracted at pH 8.5. The loss of native state causes unfolding of globular proteins with the consequential exposure of hydrophobic regions and increased surface hydrophobicity. In addition, the LFSPCs extracted at pH 8.5 had higher ash contents than did the LFSPCs extracted at pH 7.5 (3) that might have caused the higher surface hydrophobicities of these protein products.

The LFSPC had significantly higher surface hydrophobicities than did EWSPC. In general, the EWSPC had very low solubilities and surface hydrophobicities probably due to the formation of large protein aggregates during ethanol extraction. In addition, the ANS

probe only measures the hydrophobicity of soluble proteins making data interpretation for EWSPCs difficult. When comparing the two LFSPCs to SPI, the LFSPC extracted at pH 8.5 had similar surface hydrophobicity and the LFSPC extracted at pH 7.5 had lower surface hydrophobicities. Apparently, the acid treatment used to precipitate the isolate curd did not significantly affect surface hydrophobicity of SPI, but rather the higher pH of extraction (common to both the LFSPC pH 8.5 and SPI procedures). This later observation is in good agreement with data reported by Petrucelli and Anon (12).

TABLE 3
Surface Hydrophobicities of Protein Ingredients Prepared from Normal and High-sucrose/Low-stachyose Soybeans at pH 7.0 (dimensionless) [a].

Soybeans/Protein Fraction	Surface Hydrophobicity
IA2020 Soybeans	
LFSPC, pH 7.5	267[d]
LFSPC, pH 8.5	379[a]
SPI	351[b]
EWSPC	56[e]
HS/LS Soybeans	
LFSPC, pH 7.5	297[c]
LFSPC, pH 8.5	353[b]
SPI	383[a]
EWSPC	63[e]
LSD	13

[a] n=3. Means followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose/low-stachyose soybeans; IA2020, a line of normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference.

Emulsification properties. The emulsifying properties of a protein depend on two factors, the ability to reduce interfacial tension because of its adsorption to the interface and the ability to form a film, which would act as an electrostatic, structural, and mechanical barrier. In order to achieve these properties, protein molecules must have both hydrophilic and hydrophobic regions and retain flexibility in order to unfold. Emulsions are thermodynamically unstable and once formed, an emulsion can undergo a number of

changes. It is of interest to know not only how efficient a protein dispersion is in emulsifying but also how stable the resulting emulsion is. Emulsion formation depends on rapid desorption, unfolding at the interface, and reorientation; whereas, stability is determined by the decrease in interface free energy and rheological properties of the film (15).

EC, EA, and ESI data on dry-weight basis are shown in Table 4. The LFSPC extracted at pH 8.5 had significantly higher EC at pH 7 and significantly lower EC at pH 4 than did the LFSPC extracted at pH 7.5. Both LFSPCs had similar ECs at pH 3 and 5. The LFSPC extracted at pH 8.5 had higher EAs and ESIs at pH 4, 5, and 7 compared to the LFSPC extracted at pH 7.5. Both LFSPCs had poor emulsification properties at pH 4 and 5 due to the close proximity of the isoelectric point for soy protein. The superior emulsification properties of the LFSPC extracted at pH 8.5 compared to the LFSPC extracted at pH 7.5 was attributed to the former's higher solubility and surface hydrophobicity.

The LFSPCs had superior emulsification properties to those of EWSPC, which was supported by both solubility and surface hydrophobicity data. EWSPCs have very low emulsification capacities (14). The LFSPCs had similar emulsification properties to those of SPI. When the emulsification properties were expressed on a protein basis (by converting data in Table 4), the LFSPCs had superior emulsification properties to SPI. On a protein basis, the ECs of the LFSPC extracted at pH 8.5 were 715, 528, 514, and 811 g of oil emulsified/g of protein at pHs 3, 4, 5, and 7, respectively. The LFSPC extracted at pH 7.5, had ECs of 691, 565, 523, and 753 g of oil emulsified/g of protein at pHs 3, 4, 5, and 7, respectively. The SPI prepared from the IA2020 soybeans had ECs of 593, 374, 394, and 638 g of oil emulsified/g of protein at pHs 3, 4, 5, and 7, respectively. Similarly, after converting EA data to a protein basis, the LFSPC extracted at pH 8.5 had ECs of 0.326, 0.145, 0.152, and 0.371 g of oil emulsified/g of protein; the LFSPC extracted at pH 7.5 had ECs of 0.225, 0.159, 0.123, and 0.339 g of oil emulsified/g of protein; whereas the SPI had ECs of 0.196, 0.151, 0.080, and 0.310 g of oil emulsified/g of protein, for pH 3, 4, 5, and 7, respectively. On a protein basis, the ESIs for the LFSPC extracted at pH 8.5 were 179, 51, 57, and 237 at pHs 3, 4, 5, and 7, respectively; the ESIs for the LFSPC extracted at pHs 7.5 were 107, 54, 47, and 194; and the ESIs for SPI were 117, 42, 50, and 192 for pH 3, 4, 5, and 7, respectively. The processing differences among LFSPCs and SPIs were mainly that SPI was

TABLE 4-Emulsification Properties of Protein Ingredients Prepared from Normal and High-sucrose/Low-stachyose Soybeans at Different pHs[a].

Emulsification Property/Soybeans/ Protein Fraction	PH 3	PH 4	PH 5	PH 7
Emulsification Capacity (g of oil emulsified/g of product)				
IA2020 Soybeans				
LFSPC, pH 7.5	437[c]	314[c]	338[d]	486[d]
LFSPC, pH 8.5	447[c]	343[b]	392[a]	527[c]
SPI	541[a]	341[b]	360[b,c]	583[b]
EWSPC	223[d]	125[d]	95[e]	334[f]
HS/LS Soybeans				
LFSPC, pH 7.5	460[b,c]	376[a]	348[c,d]	501[d]
LFSPC, pH 8.5	474[b]	350[b]	341[c,d]	538[c]
SPI	524[a]	315[c]	369[b]	617[a]
EWSPC	209[d]	125[d]	93[e]	360[e]
LSD	26	16	20	20
Emulsification Activity (absorbance at 500 nm)				
IA2020 Soybeans				
LFSPC, pH 7.5	0.160[c]	0.075[e]	0.074[c]	0.233[b]
LFSPC, pH 8.5	0.216[a]	0.093[d]	0.096[a,b]	0.245[b]
SPI	0.179[b]	0.138[a]	0.073[c]	0.283[a]
EWSPC	0.060[d]	0.025[f]	0.031[d]	0.089[c]
HS/LS Soybeans				
LFSPC, pH 7.5	0.150[c]	0.106[c]	0.082[b,c]	0.226[b]
LFSPC, pH 8.5	0.216[a]	0.096[d]	0.101[a]	0.246[b]
SPI	0.160[c]	0.125[b]	0.072[c]	0.280[a]
EWSPC	0.031[e]	0.020[f]	0.020[d]	0.078[c]
LSD	0.017	0.010	0.015	0.027
Emulsification Stability Index (dimensionless)				
IA2020 Soybeans				
LFSPC, pH 7.5	62[d]	28[c]	25[f]	76[f]
LFSPC, pH 8.5	83[c]	26[c]	26[e,f]	97[e]
SPI	107[b]	38[a]	46[a]	175[b]
EWSPC	33[e]	25[c]	28[d,e,f]	41[g]
HS/LS Soybeans				
LFSPC, pH 7.5	71[d]	36[b]	31[c,d,e]	129[d]
LFSPC, pH 8.5	119[a]	34[b]	38[b]	157[c]
SPI	125[a]	45[a]	35[b,c]	191[a]
EWSPC	40[e]	49[a]	33[b,c,d]	48[g]
LSD	9	5	5	10

[a] n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose/low-stachyose soybeans; IA2020, a line of normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference.

acid-precipitated, neutralized and re-disolved. This additional handling of the protein may account for the different behaviors of these two ingredients.

The LFSPCs prepared from HS/LS soybeans had similar ECs over the pH range tested and superior ESIs than did LFSPCs prepared from IA2020 soybeans. This observation contrasts with thermal behavior, solubility, and surface hydrophobicity data. The LFSPCs prepared from HS/LS soybeans emulsified as much oil as did the control products but were more effective in stabilizing these emulsions. The improved emulsion stabilization of HS/LS LFSPCs was probably due to higher proportions of β-conglycinin present in the protein portion than the same products prepared from IA2020 soybeans. β-Conglycinin is reported to have superior emulsification properties than glycinin (8, 10).

Foaming properties. Proteins are polymers of amino acids that have hydrophilic and hydrophobic side chains. The amphipathic character that these side chains confer to proteins is responsible for their adsorption at interfaces. To efficiently form foam, the protein needs to rapidly adsorb at the air-liquid interface during the transient stage of foam formation. The adsorption of proteins at interfaces is controlled by three processes, the transport from bulk solution to the interface, penetration into the surface layer, and reorganization of the protein structure in the adsorbed layer (16). Foaming capacity, stability and rate of foaming results are shown in Table 5. Foaming capacity is expressed in mL of foam formed per mL of a 0.5% solids dispersion. Foam stability is expressed by k, which is the time for one-half of the liquid to drain from the foam. The smaller that k is, the more stable the foam. Rate of foaming is a measure of speed of foam formation.

The LFSPC prepared from HS/LS soybeans extracted at pH 8.5 had lower FC, formed more stable foams and was slower forming foams compared to the LFSPC extracted at pH 7.5, but these differences were significant only at $p<0.1$ and insignificant at $p<0.05$. The higher stability of the LFSPC extracted at pH 8.5 was attributed to its higher solubility and surface hydrophobicity, both properties are fundamental for stabilizing foam. The LFSPC prepared from IA2020 soybeans extracted at pH 7.5 had significantly higher FC, formed foams faster and had a similar foaming stability compared to the LFSPC extracted at pH 8.5.

The LFSPCs prepared from HS/LS soybeans had significantly higher FC, K and Vi than did the traditional soy protein ingredients. The EWSPC had the lowest values for all three foaming properties mainly because this protein concentrate contains insoluble aggregates lacking the molecular flexibility to efficiently form foams and stabilize them. The protein fractions prepared from HS/LS soybeans had similar or superior foaming properties, with the exception of the foaming rate for IA2020 SPI that was significantly higher than for the SPI prepared from HS/LS soybeans. Similar emulsion stability, the higher content of β-conglycinin probably accounts for the improved foaming properties of the LFSPCs prepared from HS/LS soybeans.

TABLE 5
Foaming Properties of Protein Ingredients Prepared from Normal and High-sucrose/Low-stachyose Soybeans[a].

Soybeans/Protein Fraction	Foaming Capacity (mL/mL)	Foaming Stability [K=1/(mL*min)]	Rate of Foaming (mL/min)
IA2020 Soybeans			
LFSPC, pH 7.5	1.425[a]	0.0104[d,e,f]	21.8[a]
LFSPC, pH 8.5	1.258[c]	0.0122[d,e]	16.1[b]
SPI	1.096[d]	0.0133[c,d]	11.1[c]
EWSPC	0.949[e]	0.0216[b]	3.8[e]
HS/LS Soybeans			
LFSPC, pH 7.5	1.360[a,b]	0.0092[e,f]	22.5[a]
LFSPC, pH 8.5	1.299[b,c]	0.0086[f]	20.7[a]
SPI	1.377[a,b]	0.0157[c]	6.5[d]
EWSPC	0.963[e]	0.0735[a]	5.1[d,e]
LSD	0.090	0.0031	2.3

[a]n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. HS/LS denotes high-sucrose/low-stachyose soybeans; IA2020, a line of normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference.

Dynamic viscosity. Dynamic viscosity results are shown in Table 6. The data for EWSPC were not included in the statistical analysis because the correlation coefficient for the data in the power-law regression analysis was < 0.8. These low correlation coefficients introduced sufficient variability to the data set that we could not compare the rest of the

products among themselves. Still, the power-law model was the best fit for the EWSPC data. The correlation coefficients for the rest of the protein fractions were >0.998. The LFSPC extracted at pH 7.5 had a similar consistency coefficient and flow behavior index as did the LFSPC extracted at pH 8.5. Both flours behaved the same.

TABLE 6
Dynamic Viscosity Properties of Protein Ingredients Prepared from Normal and High-sucrose/Low-stachyose Soybeans [a].

Soybeans/Protein Fraction	Flow Consistency Index (k, mPa*s)	Flow Behavior Index (n, dimensionless)
IA2020 Soybeans		
LFSPC, pH 7.5	0.004[c]	0.975[a]
LFSPC, pH 8.5	0.008[c]	0.923[a]
SPI	0.151[a]	0.728[c]
EWSPC	232.000[*]	0.561[*]
HS/LS Soybeans		
LFSPC, pH 7.5	0.004[c]	0.972[a]
LFSPC, pH 8.5	0.007[c]	0.947[a]
SPI	0.070[b]	0.811[b]
EWSPC	470.000[*]	0.660[*]
LSD	0.045	0.055

[a]n=3. Means within a column followed by different superscripts are significantly different at $p<0.05$. [*]Samples not included in statistical analysis. HS/LS denotes high-sucrose/low-stachyose soybeans; IA2020, a line of normal soybeans; LFSPC, low-fiber soy protein concentrate prepared by alkali extracting, neutralizing and drying; pH 7.5 and 8.5, extraction pH for LFSPC; SPI, soy protein isolate; EWSPC, ethanol-washed soy protein concentrate; and LSD, least significant difference.

The LFSPCs had lower viscosity and flow behavior more like a Newtonian fluid than did SPI. We attributed this to the lower protein content of the LFSPCs. The rheological behavior of soy protein dispersions are highly sensitive to protein concentration (17). In order to compare the LFSPCs to SPI at the same protein concentration, we dispersed the LFSPCs at the same protein concentration as in the original 10% SPI dispersion and the resulting slurries of the LFSPCs contained more solids. The k, and n values for the LFSPC extracted at pH 8.5 were 0.0322 and 0.838 and for the LFSPC extracted at pH 7.5 were 0.010, and 0.938, respectively. The LFSPCs, at the same protein concentration, had lower viscosities than did

traditional SPI prepared from IA2020 soybeans (LSD = 0.031 and 0.019 for k and n, respectively). We attributed these differences to less denaturation in the glycinin component and similar or higher degree of denaturation in the β-conglycinin component of the LFSPCs than in the traditional SPI. In addition, the LFSPCs had higher ash contents than did the SPIs (3). Higher salt concentrations reduce apparent viscosity of soy protein dispersions, probably due to increased proteins solubilities (17). The SPI prepared from HS/LS soybeans had a significantly lower consistency factor and higher flow behavior index than did the SPI prepared from IA2020 soybeans. We attributed this later observation to the fact that SPI made from IA2020 had more native β-conglycinin than did the SPI prepared from HS/LS. Rickert et al. (8) reported that native β-conglycinin dispersions are more viscous than native glycinin dispersions. This later observation was also reported by Bian et al (10).

Data interpretation for the EWSPCs was difficult due to the low correlation coefficient. The readings for these products were not consistent because slurry sampling was highly variable due to poor solubility. These samples had suspended particles that probably interfered with viscosity readings.

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CHAPTER 8. FRACTIONATION OF GLYCININ AND β-CONGLYCININ FROM HIGH-SUCROSE/LOW-STACHYOSE SOYBEANS

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ABSTRACT

There is increased interest in understanding the health benefits and functional properties of the two major storage proteins of soybeans, glycinin and β-conglycinin. The carbohydrate contents of some soybean lines have recently been genetically modified and improved by elevating sucrose and reducing oligosaccharide content, especially stachyose. Reducing stachyose is key to reducing or eliminating flatulence caused by many soybean products. The objectives of the present study were to evaluate the fractionation behavior of these soybean lines in two different fractionation procedures, the three-step procedure of Wu et al., which employs SO_2, NaCl and precipitations at pH 6.4 and 4.8, and a two-step procedure of Deak et al., which employs SO_2, $CaCl_2$ and precipitations at pH 6.4 and 4.8. The later procedure was evaluated with and without a chilling step. Both soybean variety and fractionation procedure significantly affected fraction yields, purities and functional properties. The Wu fractionation procedure gave glycinin and β-conglycinin-rich fractions with 100% purity and high yields of solids (15.4 and 10.5%) and protein (31.7 and 22.3%, respectively) from high-sucrose/low-stachyose (HS/LS) soy flour. These yields and purities were significantly higher than those achieved when using regular soybeans (83.7 and 83.8% purity, 11.6 and 11.5% solids, and 22.3 and 18.5% protein, for glycinin and β-conglycinin respectively). The other two procedures, in contrast with earlier reports for normal soybeans, were less efficient in fractionating these proteins from HS/LS soybeans producing protein fractions with purities ranging from only 71 to 80%. The two-step procedures yielded products with unique functional properties such as solubility, surface hydrophobicity, emulsification and foaming. These properties, in most cases, were similar or superior to the same fractions produced with the Wu procedure.

INTRODUCTION

Soybeans are a good source of high quality protein. Yet, <5% of the available soy protein is currently used for human consumption (*1*). Although the health benefits of consuming soy protein, especially the β-conglycinin fraction, are becoming recognized, poor functionality, undesirable taste/flavor, and the presence of flatus-causing indigestible oligosaccharides are significant limitations to consuming higher levels of soybean products and soy protein ingredients. Recent advances in genetic engineering have overcome the presence of indigestible sugars by developing soybean lines low in stachyose and high in sucrose (*2*). These soybean lines allow the production of new soy protein ingredients with unique chemical composition and functional properties (*2, 3*). Recently, we reported on the properties of soy protein ingredients prepared from defatted soy flour of high-sucrose/low-stachyose (HS/LS) soybeans (*4, 5*). In spite of having similar protein profiles in the flours prepared from a normal soybean line and HS/LS soybeans, we observed that some of the protein fractions had higher amounts of β-conglycinin when using HS/LS soy flour (*4*). This observation and others indicated that products made from HS/LS soy flour had unique functional behavior compared to similar products made from normal soybeans (*5*) and prompted us to examine the fractionation behavior of HS/LS soybeans and the compositions and functionalities of the fractions that result.

Producing protein fractions enriched in one of the two major storage proteins, glycinin and β-conglycinin, has been of interest for quite some time. Recent research has suggested that the β-conglycinin component of soy proteins has health benefits (*6, 7*), more so than the glycinin. Several fractionation methods have been reported but have achieved mixed success. One such method reported by Wu et al. (*8*) is a modification of methods of Nagano et al. (*9*), and this method has been improved and scaled-up to produce Kg quantities sufficient for human feeding trials (*10*). This three-step fractionation procedure (Wu procedure) is based on adjusting the ionic strength of an extract of soluble soy protein and isoelectric precipitation. Three fractions are obtained by this procedure, a glycinin-rich fraction, a β-conglycinin-rich fraction, and an intermediate fraction, which is a mixture of the two storage proteins. This procedure is complex and requires several centrifugation steps and a dilution step, which make the procedure very expensive to conduct on commercial scale.

We evaluated two simplified procedures and characterized the functional properties of the protein fractions produced (*11*). These new procedures are based on differential precipitation of the storage proteins by means of adjusting the pH of a soluble protein extract in which mM amounts of calcium ions and sulfites are used.

The functional properties of a protein ingredient will determine its performance in various food systems and, as a consequence, its value (*12*). The objectives of the present study were to apply three different fractionation procedures to HS/LS soy flour and evaluate their products yields, purities, and functional properties.

MATERIALS AND METHODS

Materials. Air-desolventized, hexane-defatted HS/LS white flakes were prepared from HS/LS soybeans (Low Stachyose, Lot-980B0001 OPTIMUM, 1999 crop years, Pioneer-DuPont, Johnston, IA) and from a line of normal soybeans (IA2020, 1999 crop year, Iowa State University) in the extraction pilot plant at the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). The white flakes were milled with a Krups grinder (Distrito Federal, Mexico) until 100% of the material obtained passed through a 50-mesh screen by using small quantities (~10 g) to preserve the native protein state. The HS/LS defatted soy flour contained 58.3% protein with 95.0 protein dispersibility index. The flours were stored in sealed containers at 4°C until used.

Wu soybean protein fractionation procedure. The soy protein fractionation procedure utilized as the control procedure has been reported by Wu et al. (*8*) and modified by Nagano et al. (*9*). A flowchart of this procedure is shown in **Figure 1**. About 100 g defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the slurry was stirred for 1 h. After centrifuging the slurry at 14,300 x g and 15°C for 30 min, the protein extract (first protein extract) was decanted, and the amount of insoluble fiber residue was determined and sampled for proximate composition. Sufficient $NaHSO_3$ was added to the protein extract to achieve 10 mM SO_2 and the pH was adjusted to 6.4 with 2N HCl. This slurry was stored at 4°C for 12-

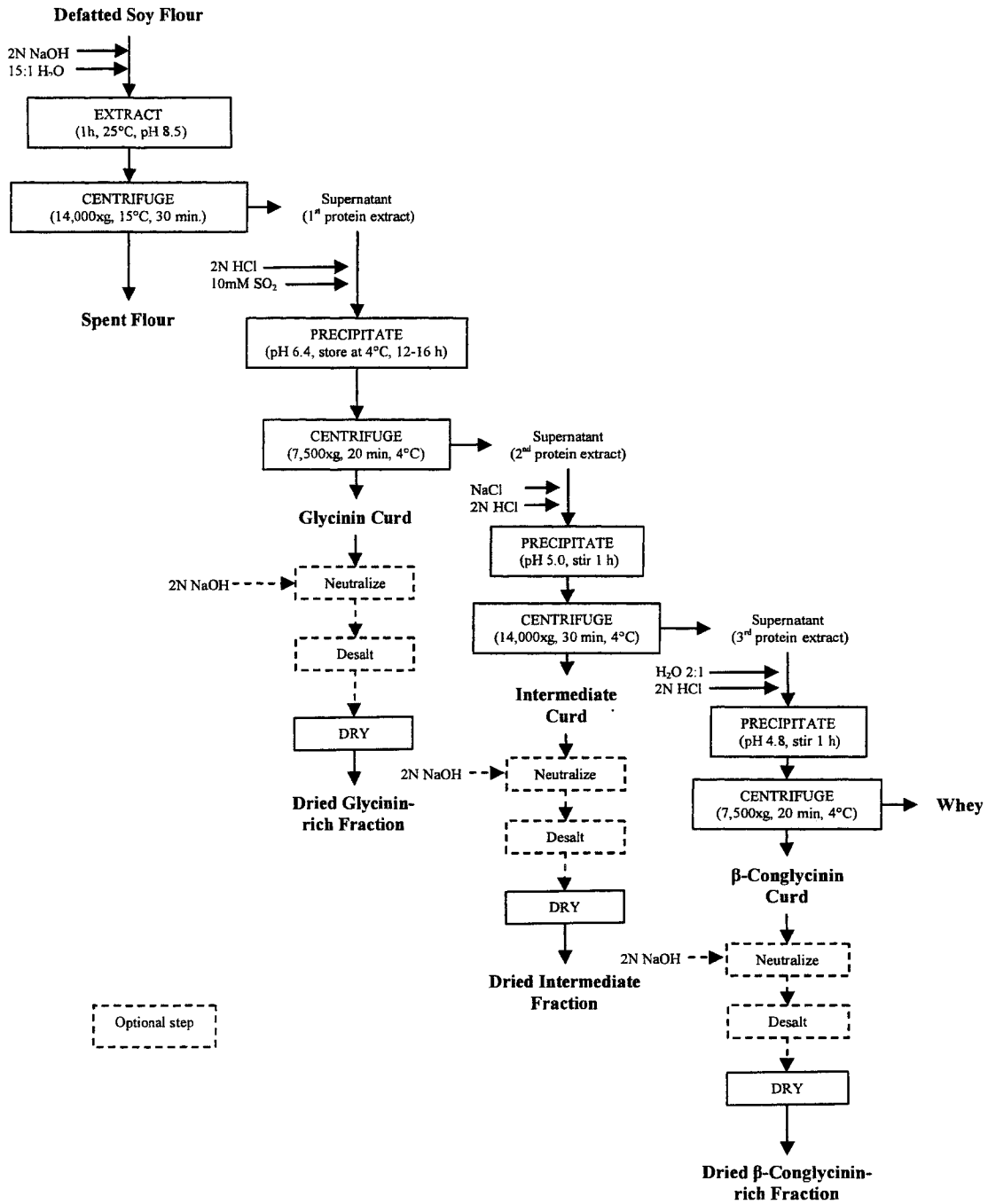

Figure 1. Three-step soy protein fractionation procedure of Wu et al (8)

16 h and then centrifuged at 7,500 x g and 4°C for 20 min. A glycinin-rich fraction was obtained as the precipitated curd, which was redisolved in de-ionized water and the pH was adjusted to 7 with 2N NaOH. The protein fraction was sampled and stored in sealed containers at -80°C until freeze-dried. Sufficient NaCl was added to the supernatant (second protein extract) to achieve 250 mM. The pH was adjusted to 5 with 2N HCl and the resulting slurry was stirred for 1 h. The slurry was centrifuged at 14,000 x g and 4°C for 30 min. An intermediate fraction, a mixture of glycinin and β-conglycinin, was obtained as the precipitated curd and treated as described above. The supernatant (third protein extract) was diluted with de-ionized water in a ratio of 2 times the volume of the third protein extract and adjusted to pH 4.8. This slurry was centrifuged at 7,500 x g and 4°C for 20 min and a β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as described above and the amount of supernatant (whey) was determined and sampled for proximate composition. The fractionation procedure was replicated twice and means reported.

New simplified fractionation procedure. A flow chart for this procedure is shown in **Figure 2**. About 50 g defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the slurry was stirred for 1 h. After centrifuging at 14,300 x g and 15°C for 30 min, the protein extract (first protein extract) was decanted and the amount of insoluble fiber residue was determined and sampled for proximate composition. To this extract, $NaHSO_3$ and $CaCl_2$ were added to obtain 5mM SO_2 and 5 mM Ca^{2+}. The pH was adjusted to 6.4 with 2N HCl. In one case, the slurry was stored at 4°C for 12-16 h (this treatment is identified as New 4C) and, in another case, the slurry was stirred for 1 h at ~25°C (this treatment is identified as New RT). In both cases, the fractionation procedure was continued by centrifuging the slurry at 14,000 x g and 4°C for 30 min. A glycinin-rich fraction was obtained as the precipitated curd, which was neutralized and treated as described above. The supernatant (second protein extract) was adjusted to pH 4.8 with HCl and the slurry was stirred for 1 h. The slurry was then centrifuged at 14,000 x g and 4°C for 30 min. A β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as described above, the amount of supernatant (whey) was determined,

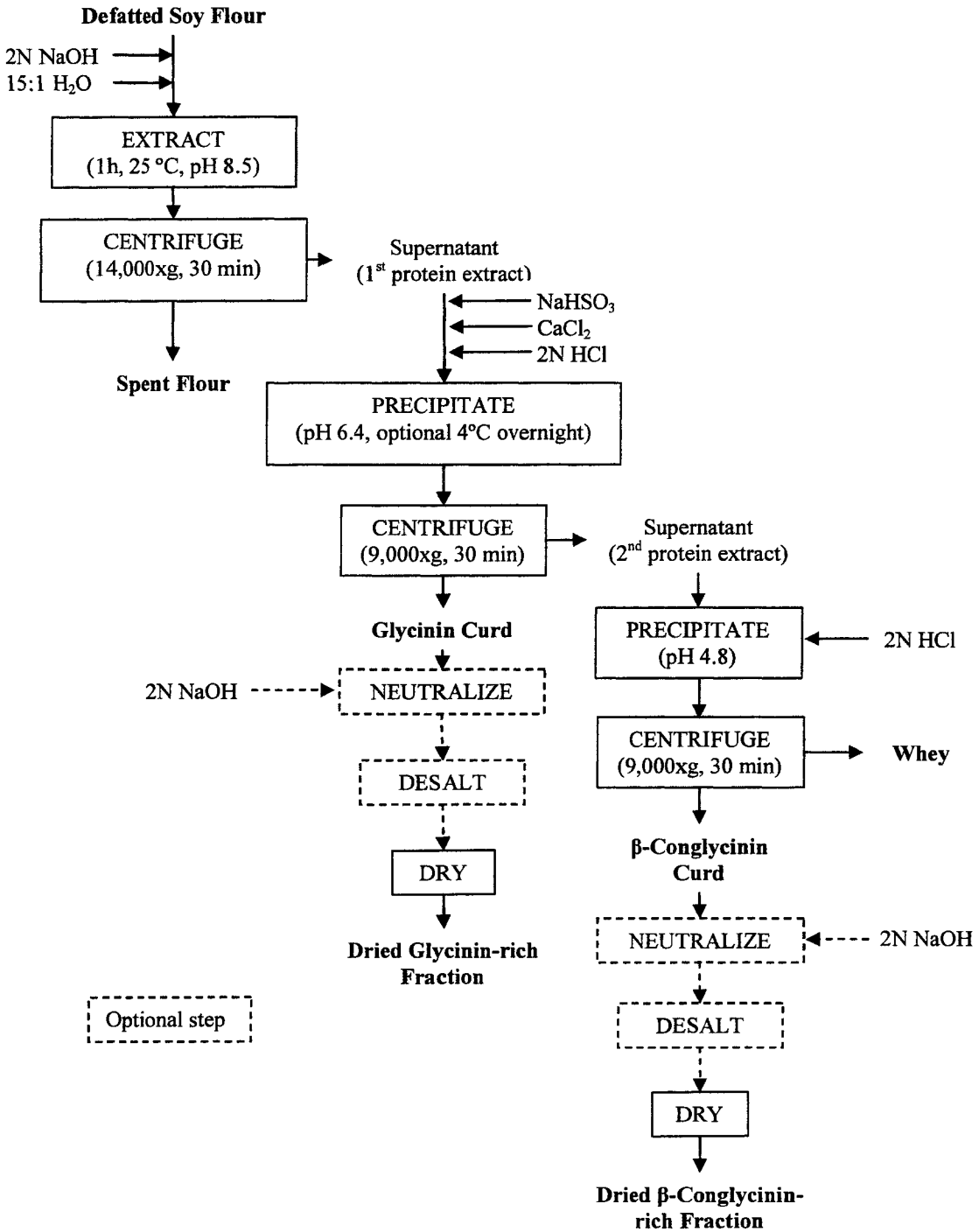

Figure 2. New soy protein fractionation procedure

and the whey was sampled for proximate composition. Both treatments (New 4C and New RT) were replicated twice and means reported.

Freeze-drying. All samples were kept at –80°C and placed into a Virtis Ultra 35 (Gardnier, NY) freeze-dryer with shelves cooled to -20°C. High vacuum was then applied and the temperature was held constant until the vacuum dropped to 100 mTorr. Secondary drying was achieved by heating the freeze-dryer shelves to 26°C at high vacuum. The complete freeze-drying cycle lasted for 120 h. Samples were placed in sealed containers until analyzed.

Proximate analyses and mass balance. The nitrogen contents of the soy flour and each protein fraction and byproduct stream were measured by using the combustion or Dumas method (*13*) with a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). These values were converted to Kjeldahl nitrogen by using the conversion equations of Jung et al. (*14*). The conversion factor used to convert percentage of nitrogen to protein content was 6.25. Moisture content was determined by oven-drying for 3 h at 130°C (*15*). Ash content was determined by using AACC methods (*16*). Mass balances of solids and protein were determined for each procedure. All measurements were replicated in triplicate and means reported.

Protein profile analysis. Urea-sodium-dodecylsulfate polyacrylamide gel electrophoresis (urea-SDS-PAGE) was performed by using the methods of Rickert et al. (*10*) to determine the protein composition profiles of all fractions. Electrophoretic bands were identified by using a pre-stained SDS-PAGE low-range molecular-weight standard (Bio-Rad Laboratories, Hercules, CA). Glycinin and β-conglycinin subunit bands were confirmed by using purified standards produced according to methods of O'Keefe et al. (*17*). Densitometry was carried out by using the Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on images scanned by a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). SDS-PAGE results were calculated as % composition: total storage protein in a given fraction = [(sum of storage protein subunit bands)/(sum of all bands)] x 100, fraction

purity/composition = [(sum of subunit bands)/(sum of storage protein bands)], and subunit composition of a specific protein = [(subunit band)/(sum of subunits for the specific protein)]. All measurements were replicated at least four times and means reported.

Thermal behavior. Thermal behaviors of the protein fractions were evaluated by differential scanning calorimetry (DSC). Samples (15-20 mg) of 10% (w/w, dry basis) dispersion were hermetically sealed in aluminum pans. A sealed, empty pan was used as reference. The samples were analyzed from 25 to 120°C at 10°C/min by using an SII Exstar 6000 (Seiko Instrument, Inc., Tokyo, Japan). All samples were analyzed at least three times and means reported.

Solubility. Protein solubility was determined according to the method of Rickert et al. (*10*) by preparing 1% (w/w dry basis) sample dispersions in de-ionized water. The pH was adjusted to 7.0 by using 2N HCl or NaOH and the dispersions were stirred for 1.0 h. Aliquots (25 mL) of the dispersions were transferred to 50-mL centrifuge tubes and centrifuged at 10000 x g and 20°C for 10 min. The protein contents of the supernatants were measured by using the Biuret method with bovine serum albumen (Sigma, St. Louis, MO) as the reference standard. Solubility was calculated as % Solubility = (protein in supernatant/initial protein content) x 100.

Surface hydrophobicity. Surface hydrophobicity was determined by using the method of Wu et al. (*8*) with 1-anilino-8-naphthalene sulfonic acid magnesium salt monohydrate (ANS, ICN Biomedicals, Inc., Aurora, OH). Protein dispersions prepared as in the solubility test were stirred, adjusted to pH 7.0, and centrifuged as described above. An aliquot of soluble protein (supernatant) was serially diluted with 0.1 M phosphate buffer (pH 7.0) to obtain 6.25 to 100 μg/mL protein. 40 μL of ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was dispersed in 3-mL aliquots of each dilution. Fluorescence intensity units (FIU) were measured with a Turner Quantech® spectrophotometer (Barnstead Thermolyne, Dubuque, IA) by using 440 nm (excitation) and 535 (emission) filters. FIU were standardized by using a solution of 40-μL ANS in 3-mL phosphate buffer as the zero point and 15-μL

ANS in 3-mL methanol assigned an arbitrary value of 80 FIU. FIUs were plotted versus percentage protein concentration. The slope of the regression line was reported as surface hydrophobicity. Samples were run in triplicate and means reported.

Emulsification properties. Emulsification capacity (was measured according to the method of Bian et al. (*18*) with modifications. Twenty-five mL of 2 % (w/w, dry basis) sample dispersions were adjusted to 7.0 with 2 N HCl or NaOH and transferred to a 400-mL plastic beaker. Soybean oil, dyed with approximately 4-ppm Sudan Red 7B (Sigma, St. Louis, MO), was continuously blended into the dispersion at 37 mL/min flow rate by using a Bamix wand mixer (ESGE AG Model 120, Mettlen, Switzerland) at low setting until phase inversion was observed. Emulsification capacity (g oil/g sample) was calculated as g of oil used to cause inversion multiplied by 2. Samples were run at least in triplicate and means reported.

Emulsification activity and emulsification stability index were measured according to the methods of Rickert et al. (*10*). Twenty-one mL of 2 % (w/w, dry basis) sample dispersions adjusted to 7.0 were blended with 7 mL of refined soybean oil (Bakers and Chefs Vegetable Oil, North Arkansas Wholesale Company Inc., Bentonville, AK) in a 250-mL glass beaker for 1.0 min by using the Bamix wand mixer at low speed. Immediately after mixing, the emulsion was diluted 1:1000 with 0.1% sodium dodecyl sulfate. Absorbance was measured at 500 nm and reported as emulsification activity. After 15 min, the absorbance was measured again. These two absorbance readings were used to calculate emulsion stability index (ESI) as ESI (min) = $(A_0/A_0\text{-}A_{15})$*t, where A_0 and A_{15} are absorbances at time 0 and 15 min, respectively, and t is the time interval. Samples were run in triplicate and the means reported.

Foaming properties. Foaming properties were measured according to methods of Sorgentini et al. (*19*) with modifications (4). Dispersions of protein fractions (0.5% w/w, dry basis) were prepared and pH adjusted to 7.0. Aliquots (95-mL) were loaded into a custom-designed glass column (58.5 cm x 2 cm) fitted with a coarse glass frit at the bottom, and N_2 was purged through the sample at 100 mL/min flow rate. Time for the foam to reach the 300-

mL mark, time for one-half of the liquid incorporated into the foam to drain back, and the volume of the liquid incorporated into the foam were measured. Three parameters were calculated:

Foaming capacity (FC) = $V_f/(f_r \times t_f)$

Specific rate constant of drainage (K) = $1/(V_{max} \times t_{1/2})$

Rate of liquid conversion to foam (V_i) = V_{max}/t_f

where V_f = a fixed volume of 300 mL, f_r = the flow rate of the gas, t_f = time to reach V_f, V_{max} is the volume of liquid incorporated into foam, and $t_{1/2}$ is the time to drain one-half of the liquid incorporated into the foam. Samples were run in triplicate and means reported.

Statistical analysis. The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM). Least significant differences (LSD) were calculated at $p<0.05$ to compare treatment means by using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Protein fraction yields and proximate compositions. The fractionation procedure of Wu and others (Wu) yielded slightly less total solids (34.7%) when all protein fractions were considered. The new fractionation procedure with chilling to 4°C (N4C) yielded 38.5% of the solids and the new fractionation procedure without chilling (NRT) yielded 36.5% of the solids (**Table 1**). These results differ slightly with our previous findings for normal soybeans (*11*) where the yields of solids for the Wu procedure were slightly higher than were the yields when using the new fractionation procedures. The total protein yields were higher when using the Wu procedure (69.9%) than were those for N4C (55.0%) and NRT (54.2%), but 23% of the recovered protein in the Wu procedure was recovered in the intermediate fraction, which is much less useful due to the mixture of predominantly denatured proteins. Similar results were obtained in our previous work using normal soybeans (*11*). Significantly more protein was recovered in the intermediate fraction when using the Wu procedure and the new fractionation procedures yielded slightly more protein when the defatted soy flour was from normal soybeans (*11*) compared to using defatted flour from HS/LS soybeans.

Table 1. Yields and Compositions (dry basis) of Soy Protein Fractions Prepared by Using the Wu and New Procedures [a].

Fraction/Treatment	Solids Yield (%)	Protein Yield (%)	Protein Content (%)	Ash (%)
Wu glycinin	15.4[b]	31.7[a]	96.4[a]	4.1[a]
N4C glycinin	18.0[a]	25.7[b]	97.3[a]	3.6[b]
NRT glycinin	14.3[b]	25.5[b]	94.7[a]	3.4[b]
LSD	1.8	3.5	4.4	0.3
Wu Intermediate	8.8±0.3	15.9±0.7	80.9±0.7	14.8±0.1
Wu β-conglycinin	10.5[c]	22.3[b]	95.6[a]	11.2[a]
N4C β-conglycinin	20.5[b]	29.3[a]	92.2[b]	6.1[b]
NRT β-conglycinin	22.2[a]	28.7[a]	92.0[b]	5.8[b]
LSD	1.1	3.3	1.4	0.5
LSD[b]	1.1	2.4	2.3	0.3

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference to compare all fractions within a column.

When comparing the glycinin-rich fractions produced by using the three fractionation procedures, the N4C procedure yielded slightly more solids than did the other two procedures; however, the Wu procedure yielded significantly more protein. The protein contents of the glycinin-rich fractions were well above 90% for all three procedures. The ash content for the glycinin-rich fraction of the Wu procedure was slightly higher than those contents of the glycinin-rich fractions produced by using the other two procedures. The glycinin-rich fraction of the Wu procedure yielded almost 10% more protein when using the HS/LS flour than was obtained when using normal flour (*11*). The ash contents of the glycinin-rich fractions obtained when using HS/LS soy flour were slightly higher than for the same fractions obtained when using flour from normal soybeans (*11*).

When comparing the β-conglycinin-rich fractions produced by using the three fractionation procedures, both new procedures yielded significantly more solids and protein.

The protein contents of the β-conglycinin-rich fractions for all treatments were >90%. The protein yield of the β-conglycinin-rich fraction obtained when using the Wu procedure was ~4.0% higher than that obtained for the β-conglycinin-rich fraction when using soy flour prepared from normal soybeans (*11*).

The Wu procedure also yielded an intermediate fraction, whose protein content was ~15% lower than those of the other two fractions obtained by using this procedure making this fraction much less desirable than the other fractions. The ash content of this fraction was also the highest. Considerable amounts of solids and proteins were recovered in the less desirable protein fraction. These losses of solids and protein were 9.4 and 10.9% lower, respectively, when using HS/LS soy flour compared to those of the same fractions prepared from soy flour of normal soybeans (*11*).

The Wu procedure yielded higher amounts of solids and protein in both the glycinin-rich and β-conglycinin-rich fractions than reported by Nagano et al. (*9*), Wu et al. (*8*), and Rickert et al. (*10*), which used similar procedures. Rickert et al. (*10*) reported on an optimized fractionation procedure that yielded more solids and protein in the β-conglycinin-rich fraction, but their purities were lower (~68%). The yields of solids and protein when using our procedures were similar to those reported by Wu et al. (*8*) and Rickert et al. (*10*).

Protein compositions. The total storage protein contents of the glycinin-rich fractions from each procedure were different (**Table 2**). The Wu procedure produced a glycinin-rich fraction with 10% more storage protein than did the N4C procedure and ~20% more than did the NRT procedure. The purity of the glycinin-rich fraction was also affected by the fractionation method used. The Wu procedure yielded a glycinin-rich fraction with 20% higher purity than did the N4C procedure and 26.7% higher purity than did the NRT procedure. The glycinin subunit composition was significantly different for each procedure. The Wu procedure yielded a glycinin-rich fraction with 10.1 and 6.3% more acidic subunits, respectively, than did the glycinin-rich fractions obtained by using the N4C and NRT procedures. All three procedures yielded more acidic than basic subunits in the glycinin precipitated in this fraction. The subunit compositions of the contaminant β-conglycinin using the new procedures were not different from that obtained when using the Wu procedure

Table 2. Protein Compositions and Subunit Profiles (%) of Soy Protein Fractions Prepared by Using the Wu and New Procedures [a].

Fraction/ Treatment	Storage Protein in Fraction (%)	β-Conglycinin				Glycinin		
		%	Subunit Composition (%)			%	Subunit Composition (%)	
			α'	α	β		A	B
Wu glycinin	100.0[a]	0.0[c]	0.0[b]	0.0[b]	0.0[b]	100.0[a]	63.6[a]	36.4[c]
N4C glycinin	88.9[b]	20.0[b]	29.7[a]	22.4[a]	48.0[a]	80.0[b]	53.5[c]	46.5[a]
NRT glycinin	81.5[c]	26.7[a]	23.3[a]	24.2[a]	52.5[a]	73.3[c]	57.2[b]	42.8[b]
LSD	0.7	3.1	8.3	3.0	6.3	3.1	2.1	2.1
Wu intermediate	69.6±2.3	51.0±3.6	28.6±1.0	28.0±0.9	43.4±0.2	49.0±3.6	45.1±3.3	54.9±3.3
Wu β-conglycinin	100.0[a]	100.0[a]	32.7[a]	38.5[a]	28.8[b]	0.0[b]	0.0[b]	0.0[b]
N4C β-conglycinin	78.2[c]	73.1[b]	29.5[a]	32.6[a,b]	37.9[a]	26.9[a]	49.5[a]	50.5[a]
NRT β-conglycinn	81.2[b]	71.9[b]	30.9[a]	28.9[b]	40.1[a]	28.1[a]	46.7[a]	53.3[a]
LSD	0.3	1.5	10.7	6.4	4.7	1.5	3.4	3.4
LSD[b]	2.1	3.6	6.7	3.5	3.8	3.6	3.6	3.6

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; A, acidic subunits of glycinin; B, basic subunits of glycinin; and LSD, least significant difference at $p<0.05$.

[b] Least significant difference to compare all fractions within a column.

(**Table 2**). Comparing these observations for soy flour from HS/LS soybeans with the results obtained by Deak et al. (*11*) using soy flour from normal soybeans, the glycinin-rich fraction obtained from HS/LS flour with the Wu process was more pure, whereas, the new procedures produced fractions with similar purities for normal and HS/LS soybeans.

The β-conglycinin-rich fraction recovered from HS/LS soybeans by using the Wu procedure had ~20% more storage protein than did the same fraction obtained by using the new procedures. The highest purity was achieved by using the Wu procedure, followed by the new procedures (**Table 2**). The subunit composition for β-conglycinin in the β-conglycinin-rich fraction obtained by using the Wu procedure was different from those of the β-conglycinin-rich fractions obtained by using the new procedures. The Wu procedure produced a β-conglycinin-rich fraction with no glycinin contamination. The amounts of the contaminant glycinin, in the β-conglycinin-rich fraction, using the new procedures were higher for HS/LS soybeans than the amounts reported earlier for normal soybeans (*11*).

One possible explanation for the different fractionation behavior of HS/LS soybeans compared to normals soybeans that is consistent with the fractionation mechanisms that we have proposed in earlier studies (*11, 20, 21*) is phytate affects soy protein fractionation. The myo-inositol metabolism is altered in HS/LS soybeans (*2*) in a way that also reduces synthesis of phytic acid. We attribute that this difference in behavior to the HS/LS soy flour having lower phytate content. The Wu procedure then would be more efficient in fractionating glycinin from β-conglycinin as per the model for the Wu fractionation procedure presented by Deak et al. (*20*). This also fits the model previously reported (*21*) for the new two-step fractionation procedure (22). If less phytate is present, more calcium remains free to bind to soy protein resulting in a higher contamination of β-conglycinin in the glycinin-rich fraction. These proteins have different calcium-binding affinities that depend upon calcium concentration.

The intermediate fraction produced from HS/LS soy flour when using the Wu procedure contained ~30% less storage protein than did the other two fractions. The intermediate fraction contained nearly equal amounts of glycinin and β-conglycinin, but with different subunit compositions. The β subunit was the main component of the β-conglycinin present. Comparing this fraction to the same fraction obtained from soy flour prepared from

normal soybeans (*11*), HS/LS flour gave an intermediate fraction with less storage protein and different β-conglycinin subunit composition. The Wu procedure was more effective in fractionating glycinin and β-conglycinin from HS/LS soy flour than it was when using soy flour from normal soybeans while the new procedures were more effective fractionating storage proteins from soy flour prepared from normal soybeans than from HS/LS soybeans.

The glycinin-rich and β-conglycinin-rich fractions recovered by using the Wu procedure were 100% electrophoretically pure, which are much more pure than those found in earlier reports for similar procedures (*8, 9, 10*). In addition, Rickert et al. (*10*) reported a significant amount of lipoxygenase contamination in their β-conglycinin-rich fraction (3-4%), we could not detect any lipoxygenase. Our intermediate fraction contained only about 70% storage proteins, lipoxygenase probably being the principal component of the remaining 30% (data not shown).

Thermal behavior. The thermal properties of the glycinin-rich fractions for all fractionation procedures are shown in **Table 3**. The peak denaturation temperature was slightly lower for the glycinin portion and slightly higher for the contaminant β-conglycinin portion when using the Wu procedure than for the same fractions obtained when using the new procedures. The contaminant β-conglycinin comprised 1.6, 2.7, and 6.7% of the total denaturation enthalpy in the glycinin-rich fractions when using the Wu, N4C, and NRT procedures, respectively. Although no protein contamination was detected by SDS-PAGE when using HS/LS soy flour in the Wu procedure, some denaturation enthalpy for β-conglycinin was detected. Gel electrophoresis is a less sensitive test than is DSC for detecting the presence of small quantities of contaminant proteins. This fraction had significantly higher denaturation enthalpy than all fractions in all treatments. This was probably due to calcium-mediated interactions with the protein present in this fraction that increase denaturation enthalpies (23). The glycinin-rich fractions of the new procedures had about 2.5 and 2.8 times more denaturation enthalpy than did the β-conglycinin-rich fractions when using the N4C and NRT procedures, respectively. The denaturation enthalpies and peak temperatures observed for the fractions obtained when using HS/LS soy flour were similar to those of soy flour produced from normal soybeans (*11*).

Table 3. Thermal Behaviors of Soy Protein Fractions Prepared by Using the Wu and New Procedures[a].

Fraction/Treatment	β-Conglycinin Td (°C)	Glycinin Td (°C)	β-Conglycinin Enthalpy (mJ/mg)	Glycinin Enthalpy (mJ/mg)
Wu glycinin	74.9[a]	89.5[b]	0.26[b]	15.96[b]
N4C glycinin	73.8[a,b]	91.5[a]	0.51[b]	18.65[a]
NRT glycinin	73.5[b]	91.3[a]	1.33[a]	18.62[a]
LSD	1.3	1.0	0.47	1.70
Wu intermediate	75.5±0.3	93.6±0.2	1.06±0.13	3.06±0.10
Wu β-conglycinin	75.3[a]	90.0[b]	10.33[a]	0.17[c]
N4C β-conglycinin	75.8[a]	89.8[b]	6.48[b]	1.03[b]
NRT β-conglycinin	75.3[a]	91.7[a]	5.35[b]	1.77[a]
LSD	1.4	1.4	1.25	0.37
LSD[b]	0.9	0.9	0.66	0.85

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; Td, peak denaturation temperature; and LSD, least significant difference at $p<0.05$. [b]Least significant difference to compare all fractions within a column.

The peak denaturation temperatures for the β-conglycinin-rich fractions were approximately the same for all treatments and components with the exception of the contaminant glycinin in the β-conglycinin-rich fraction produced by using the NRT procedure, which was significantly higher than for the other two procedures (**Table 3**). The contaminant glycinin comprised 1.6, 13.7, and 24.8% of the total denaturation enthalpy in this fraction for the Wu, N4C, and NRT procedures, respectively. These denaturation enthalpies were significantly higher than those of soy flour prepared from normal soybeans (*11*). This was probably due to the higher contamination in the fractions produced by the new procedures. The Wu procedure yielded a β-conglycinin-rich fraction with the highest denaturation enthalpy.

The intermediate fraction produced when using the Wu procedure had the lowest total denaturation enthalpy among all protein fractions indicating substantial denaturation. The β-conglycinin component of the intermediate fraction comprised 26% of the total denaturation enthalpy, while the glycinin component comprised 76%. This 3:1 ratio was not observed by SDS-PAGE (where the proportion was 1:1). This difference was probably due to partial denaturation of the β-conglycinin component recovered in this fraction.

Comparing our results for the Wu fractionation procedure with those reported by Rickert et al. (*10*) for a similar procedure, we found several differences in thermal behavior of our fractions. Our glycinin-rich fraction, in spite of being electrophoretically pure contained some native β-conglycinin. In contrast, Rickert et al. (*10*) found no native β-conglycinin contamination in their glycinin-rich fraction by DCS, but significant contamination (~10%) by urea-SDS-PAGE. Our denaturation enthalpy was lower that that found in their study (16 versus 22 mJ/mg of protein). On the other hand, our β-conglycinin-rich fraction had higher denaturation enthalpy (10.3 mJ/mg of protein) than was reported by Rickert el al. (*10*) (7.5 mJ/mg of protein). Comparing the intermediate fractions from both studies, the β-conglycinin component had the same denaturation enthalpy, while the glycinin component of our intermediate fraction had about two times the denaturation enthalpy reported by Rickert et al. (*10*). In spite of these differences, the intermediate fraction produced protein with the lowest denaturation enthalpies among all three fractions collected in boh studies.

Solubility. The fractionation procedure used significantly affected the solubilities of the fractions recovered (**Table 4**). There were no significant differences in solubilities among the glycinin-rich fractions. In our previous study with normal soybeans (11), the new procedures produced glycinin-rich fractions with lower solubility and the temperature used to precipitate this fraction significantly affected solubility.

The Wu procedure produced a β-conglycinin-rich fraction having significantly higher solubility. The new procedures yielded β-conglycinin-rich fractions with 17.0 and 22.8% less solubility than the glycinin-rich fractions produced by using the N4C and NRT procedures,

respectively. The solubilities of the β-conglycinin-rich fractions were similar for normal soybeans and HS/LS soybeans (*11*).

Table 4. Solubilities and Surface Hydrophobicities of Soy Protein Fractions Prepared by Using the Wu and New Procedures[a].

Fraction/Treatment	Solubility (%)	Surface Hydrophobicity (Dimensionless)
Wu glycinin	88.9[a]	152[a]
N4C glycinin	92.9[a]	148[a]
NRT glycinin	93.2[a]	154[a]
LSD	5.6	33
Wu intermediate	41.6 ± 0.8	179 ± 5
Wu β-conglycinin	92.8[a]	185[a]
N4C β-conglycinin	75.9[b]	180[a]
NRT β-conglycinin	70.4[b]	130[b]
LSD	6.2	23
LSD[b]	4.1	20

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference to compare all fractions within a column.

The intermediate fraction was the least soluble fraction because this fraction contained more denatured protein as indicated by having low denaturation enthalpies for both the glycinin and β-conglycinin components. Similar conclusions were drawn for the intermediate fraction prepared from soy flour of normal soybeans (*11*).

The solubilities (pH 7.0) of the glycinin-rich fractions prepared by using the Wu procedure had similar solubilities to those reported by Bian et al. (*18*) and were lower than those reported by Rickert et al. (*10*). The differences with this later study probably were attributed to our glycinin-rich fraction being more denatured as indicated by having lower denaturation enthalpy. The solubilities of the β-conglycinin-rich fractions produced by using the Wu procedure were slightly higher than those reported in earlier studies for the same

procedure (*10, 18*). We attributed this to our β-conglycinin-rich fraction being less denatured. When comparing the solubilities of the intermediate fractions with those of earlier reports, we found no differences in solubility behavior in spite of the intermediate fraction containing more native protein,

Surface hydrophobicity. There were no differences in surface hydrophobicities for the glycinin-rich fractions (**Table 4**). The β-conglycinin-rich fraction of the NRT procedure had the lowest surface hydrophobicity. The fractions obtained when using HS/LS soy flour had lower hydrophobicities than did the same fractions from soy flour prepared from normal soybeans (*11*) with the exception of the intermediate and β-conglycinin-rich fractions produced by using the Wu procedure.

When comparing our results for the fractions produced with the Wu procedure with those of earlier studies (*8, 10*), we found several differences. The β-conglycinin-rich and intermediate fractions had the highest surface hydrophobicities, while glycinin had the lowest. Wu et al. (*8*) indicated that their β-conglycinin-rich fraction had the highest surface hydrophobicity and no difference between their glycinin-rich and intermediate fractions. Rickert et al (*10*) reported no differences between their glycinin-rich and intermediate fractions, both having higher surface hydrophobisities than the β-conglycinin-rich fraction. In all studies the method used was the ANS probe, which only measures the surface hydrophobicity of soluble protein, making data interpretation for the intermediate fraction difficult. When we discarded the data for intermediate fraction, our findings agree with those of Wu et al. (*8*) and but not those of Rickert et al. (*10*).

Emulsification properties. Emulsification capacity, activity, and stability index results are shown in **Table 5**. The glycinin-rich fractions produced by using the new procedures had significantly higher emulsification capacities than did the same fraction produced by the Wu procedure. The higher emulsification capacities of the fractions produced by using the new procedures may have been due to the new procedures producing fractions with more native proteins as indicated by higher denaturation enthalpies. Alternatively, calcium ions may have introduced structural changes (*20*) allowing these

proteins to be better surfactants. The emulsification capacities for the glycinin-rich fractions produced from HS/LS soy flour were lower than those of the same fractions produced from soy flour of normal soybeans (*11*). Emulsification activities and stability indices were similar for the glycinin-rich fractions among all three procedures and were significantly lower than for the β-conglycinin-rich fractions. This phenomenon may have been partially due to the glycinin-rich fractions having lower surface hydrophobicities.

Table 5. Emulsification Properties of Soy Protein Fractions Prepared by Using the Wu and New Procedures[a].

Fraction/Treatment	Emulsification Capacity (g of oil emulsified/g of product)	Emulsification Activity (absorbance at 500 nm)	Emulsification Stability Index (dimensionless)
Wu glycinin	307[c]	0.155[a,b]	76[b]
N4C glycinin	618[a]	0.177[a]	103[a]
NRT glycinin	547[b]	0.151[b]	83[a,b]
LSD	62	0.026	22
Wu intermediate	219 ± 5	0.194 ± 0.012	69 ± 6
Wu β-conglycinin	612[a]	0.311[a]	216[a]
N4C β-conglycinin	564[b]	0.301[a]	216[a]
NRT β-conglycinin	633[a]	0.322[a]	240[a]
LSD	41	0.038	147
LSD[b]	36	0.025	73

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference to compare all fractions within a column.

The emulsification capacities of the β-conglycinin-rich fractions produced by using the Wu and NRT procedures were significantly higher than that of the β-conglycinin-rich fraction produced by using the N4C procedure (**Table 5**). There were no differences among treatments for emulsification activity and stability index. These finding are somewhat

different from our previous findings for fractions produced from soy flour of normal soybeans (*11*). The HS/LS soybeans produced β-conglycinin-rich fractions with the highest emulsification activities and stability indexes. This was probably because these fractions also had the highest surface hydrophobicities. The intermediate fraction was a poor emulsifier.

In all prior studies (*10, 11, 18*) as well as the present one, the β-conglycinin-rich fraction had the best emulsification properties, followed by the glycinin-rich fraction, and then the intermediate fraction. We did find, however, significant differences regarding to the amount of oil that these fractions were able to emulsify. While our and Rickert's β-conglycinin-rich fractions emulsified about 600 g of oil/g of product, the same fraction of Bian et al. (*18*) emulsified less than half that amount.

Foaming properties. Foaming capacity is a measure of foaming efficiency; foaming stability is related to the ability of foam to hold air; and rate of foaming gives a measure of the speed that foam is formed. In general, the fractions prepared by using the NRT procedure had the best foaming properties (**Table 6**). The foaming rates for the glycinin-rich fractions were similar for all processes although the fractions produced by using the NRT procedure foamed at twice the rate as did the same fractions produced by using the Wu procedure. Foaming capacity followed the same order, but the stabilities of the foam formed by the fractions made with the NRT procedure were significantly lower than were the same fractions produced by the Wu procedure and similar to the fractions produced by the N4C procedure.

The β-conglycinin-rich fraction produced by using the NRT procedure had the best foaming properties. In general, the foams of β-conglycinin-rich fractions were more stable than were those of the glycinin-rich fractions, probably due to their higher surface hydrophobicities. The intermediate fraction produced when using the Wu procedure had the highest foaming stability with high foaming rate and low foaming capacity.

When comparing the fractions produced from HS/LS soy flour with the same fractions produced from soy flour of normal soybeans (*11*), the glycinin-rich fractions prepared from HS/LS soy flour formed less stable foams with similar foaming capacities. Protein fractions produced by using the new procedures had significantly slower rates of

foaming. The β-conglycinin-rich fractions prepared from HS/LS soy flour had similar foaming properties to the β-conglycinin-rich fractions produced by using the Wu and NRT procedures and significantly poorer than when using the N4C procedure. The intermediate fractions for both flours had similar foaming properties.

Table 6. Foaming Properties of Soy Protein Fractions Prepared by Using the Wu and New Procedures[a].

Fraction/Treatment	Foaming Capacity (mL/mL)	Foaming Stability (k=1/(mL*min))	Rate of Foaming (Vi = mL/min)
Wu glycinin	1.090[c]	0.092[a]	2.3[a]
N4C glycinin	1.300[b]	0.173[b]	4.4[a]
NRT glycinin	1.514[a]	0.164[b]	5.0[a]
LSD	0.083	0.035	2.9
Wu intermediate	1.141±0.062	0.005 ± 0.001	21.9 ± 0.7
Wu β-conglycinin	1.184[c]	0.018[a]	13.7[b]
N4C β-conglycinin	1.396[b]	0.035[b]	14.2[b]
NRT β-conglycinin	1.671[a]	0.012[a]	30.4[a]
LSD	0.186	0.007	4.5
LSD[b]	0.113	0.017	2.6

[a] n=2. Means within a column for a specific fraction followed by different superscripts are significantly different at $p<0.05$. Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step; glycinin, glycinin-rich fraction; intermediate, intermediate fraction; β-conglycinin, β-conglycinin-rich fraction; and LSD, least significant difference at $p<0.05$.
[b] Least significant difference to compare all fractions within a column.

Foaming properties of the fractions prorduced by using the Wu procedure were different from those reported by Bian et al. (*18*) and Rickert et al. (*10*). In our study, the intermediate fraction had the best combination of foaming properties followed by the β-conglycinin-rich fraction, and then the glycinin-rich fraction. Rickert et al (*10*) found no differences between their intermediate and glycinin-rich fractions, but both were better than their β-conglycinin-rich fraction. Bian et al. (*18*) reported their β-conglycinin-rich fraction to be the best foaming agent followed by their intermediate fraction and then their glycinin fraction. One possible explanation for these discrepancies is that Rickert's β-conglycinin had

the lowest surface hydrophobicity among all studies considered, which is an important property for foaming of proteins.

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CHAPTER 9. FATE OF PHYTIC ACID DURING PREPARATION OF SOY PROTEIN INGREDIENTS

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ABSTRACT

Phytic acid is present in amounts of 1-2% in soybeans and soy protein products, and is responsible for poor absorption of essential electrolytes and minerals. Phytate binds to proteins and co-precipitates when producing soy protein ingredients. The objectives of the present study were to develop an understanding of how phytic acid partitions when producing different soy protein ingredients and to relate phytic acid partitioning to functional properties of proteins when using two different soybean varieties. Processing method and soybean variety significantly affected phytic acid content and recovery. High-sucrose/low-stachyose soybeans contained significantly ($p<0.05$) less phytate than did normal soybeans. Ethanol-washed soy protein concentrate had the highest phytate contents and yields when using both soybean varieties. A fractionated glycinin-rich fraction had the lowest phytic acid content. Protein content of the protein ingredients was negatively correlated with phytic acid content. Significant correlation was found between isoflavone and phytic acid contents. When ethanol-washed soy protein concentrate was excluded from the correlation study, phytic acid content positively correlated with solubility, surface hydrophobicity, emulsification and foaming properties.

INTRODUCTION

Phytic acid (myo-inositol 1,2,3,4,5,6, hexakis dihydrogen phosphate) is present in soybeans and soybean products in concentrations between 1.0 and 1.5% (*1*). At pH values normally encountered in food systems, phytic acid is highly negatively charged, having potential to complex with or bind to positively charged molecules such as cations and proteins (*2*). Phytate binds nutritionally important minerals, such as iron, zinc, and calcium, impeding their absorption (*3*) and may also bind to protein.

The phytic acid solubility profile over a pH range is quite different in the presence of proteins and different mechanisms for interaction have been suggested at three different pH regions (*2*). At low pH (<4.5), soy proteins possess net positive charge while phytic acid is negatively charged and, consequently, protein-phytate interaction at low pH is a result of strong electrostatic interaction. At intermediate pH (5-7), both proteins and phytic acid have net negative charge; however, some protein-phytate complexes still form (*2*). At high pH (>7), multivalent cations, such as calcium, are essential for establishing protein-phytate complexes (*4*). Saio et al. (*5*) found that a single protein molecule may bind many molecules of calcium and phytic acid. Phytate behavior at alkaline pH appears to be strongly affected by salt linkages or alkaline-earth ion bridges (*2*). This mechanism also explains why phytic acid is soluble in the presence of protein above pH 6, even though phytate salts by themselves are insoluble at alkaline pH (*5*).

Considerable research has focused on removing phytic acid from soy protein products because protein-bound phytate may dissociate from protein in the digestive system to bind with free minerals impeding their absorption because these complexes are not soluble. Addition of NaCl has been used to disrupt the alkaline-earth ion bridges to produce phytate-reduced soy protein products (*6*). Ford et al. (*7*) used low pH in combination with $CaCl_2$ to remove 90% of the phytate from protein concentrates. Omosaiye et al. (*8*) developed an ultrafiltration method to eliminate phytate from soy protein isolates and full-fat protein concentrates. Kumagai et al. (*9*) removed phytate by using ion-exchange resins. Saito et al. (*10*) reported on a method for separating soy glycinin and β-conglycinin using phytase and suggested that phytate may affect protein solubility and functional properties. Honing et al. (*11*) studied the effectiveness of dialysis for removing phytate from several soy protein isolates and fractions, and suggested that processing conditions were involved in forming phytate-protein complexes. In spite of this research, very little has been published about the fate of phytic acid during soy protein ingredient processing.

During the course of our protein fractionation research (*12, 13*), we have also observed that two different soybean varieties, IA2020, a normal soybean variety, and a genetically modified variety high in sucrose and low in stachyose (HS/LS) fractionated differently when subjected to the same fractionation procedure. We hypothesized that

differences in phytic acid may have caused these differences, since the myo-inositol metabolism was genetically modified to improve the sugar contents in the HS/LS soybean line (*14*). The objectives the present study was to evaluate the fate of phytic acid during processing soy protein ingredients and to understand how phytate partitioning differs between these two soybean varieties.

MATERIALS AND METHODS

Materials. Soy protein ingredients were prepared from air-desolventized, hexane-defatted white flakes prepared from normal soybeans (IA2020 variety, 1999 harvest) and HS/LS soybeans (2 HS Soybeans, Low Stachyose, Lot-980B0001 OPTIMUM, 1999 harvest, Pioneer-DuPont, Johnston, IA). Defatted white flakes were prepared in the pilot-plant extraction facility at the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). The flakes were milled with a Krups grinder (Distrito Federat, Mexico) until 100% of the material obtained passed through a 50-mesh screen by using small quantities of about 10 g to preserve the native protein state. The flours were stored in sealed containers at 4°C until used. The IA2020 soy flour contained 57.3% protein (db) with 93.8 protein dispersibility index (PDI) and the HS/LS soy flour contained 58.3% protein (db) with 95.0 PDI.

Preparation of ethanol-washed soy protein concentrate (EWSPC). About 100 g defatted soy flour was extracted with 60% ethanol/40% de-ionized water at 10:1 solvent-to-flour ratio and 40°C by stirring the slurry for 30 min in sealed containers to avoid ethanol evaporation. After centrifuging at 14,300 x g for 30 min, EWSPC was obtained as the solids fraction and the extract (supernatant, primarily soluble sugars) was discarded. The resulting EWSPC was air-desolventized at 25°C for 24 h. The samples were then freeze-dried and stored in sealed containers until used. These procedures were replicated three times with each flour. The EWSPC prepared from IA2020 soybeans contained 70.0% protein (db) and the EWSPC from HS/LS soybeans contained 69.4% protein (db).

Preparation of soy protein isolate (SPI). About 150 g of defatted soy flour was extracted with de-ionized water at 10:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 30 min at 60°C. After centrifuging at 14,300 x g for 30 min, a protein extract was obtained and the insoluble fiber residue was discarded. The protein extract was adjusted to pH 4.5 with 2N HCl and centrifuged as described above. A protein isolate curd was obtained as a precipitate and the supernatant (whey) was discarded. The curd was re-dissolved in de-ionized water and 2N NaOH was added to achieve pH 7 with approximately 10% solids content. The resulting slurry was freeze-dried and stored in sealed containers until used. These procedures were replicated three times with each flour. The SPI prepared from IA2020 soybeans contained 91.3% protein (db), and the SPI prepared from HS/LS soybeans contained 92.1% protein (db).

Preparation of low-fiber soy protein concentrates. Low-fiber soy protein concentrates (LFSPC) were prepared from each flour according to methods of Deak et al. (*15*), which simulated details of the Crank and Kerr patent (*14*) in which protein is extracted at 7.5 and of the Johnson patent (*16*) in which protein is extracted at 8.5, and the extracts were neutralized and dried. About 100 g defatted soy flour was extracted with de-ionized water at a 10:1 water-to-flour ratio, the pH was adjusted to 7.5 or 8.5 with 2N NaOH, and the resulting slurry was stirred for 30 min at 60°C. After centrifuging at 14,300 x g for 30 min, a protein extract was obtained and the insoluble fiber residue was re-extracted with additional de-ionized water at 5:1 water-to-insoluble fiber ratio. The pH was adjusted as before and the resulting slurry was stirred for 30 min. After centrifuging at 14,300 x g for 30 min, the resulting second protein extract was combined with the first extract, and the insoluble fiber was discarded. The combined extracts were adjusted to pH 7.0 with 2N HCl and freeze-dried. The dry products were stored in sealed containers until used. These procedures were replicated three times for each flour and means reported. The protein contents were 62.3, 62.7, 66.6, and 66.3% for LFSPCs extracted at pH 7.5 and 8.5 from IA2020 soybeans and for LFSPCs extracted at pH 7.5 and pH 8.5, respectively.

Preparation of fractionated soy proteins. Soy protein was fractionated into glycinin-rich and β-conglycinin-rich fractions by using the procedure of Nagano et al. (*17*) and modified by Wu et al. (*18*). About 100 g defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the slurry was stirred for 1 h. After centrifuging at 14,300 x g and 15°C for 30 min, the protein extract (first extract) was decanted. The amount of insoluble fiber residue was determined and sampled for proximate composition. Sufficient $NaHSO_3$ was added to the resulting protein extract to achieve 10 mM SO_2 and the pH was adjusted to 6.4 with 2N HCl. This slurry was stored at 4°C for 12-16 h and then centrifuged at 7,500 x g and 4°C for 20 min. The glycinin-rich fraction was obtained as the precipitated curd. This fraction was redisolved in de-ionized water, the pH was adjusted to 7 with 2N NaOH, and the fraction was sampled and stored in sealed containers at -80°C until freeze-dried. To the supernatant, second protein extract, sufficient NaCl was added to achieve 250 mM concentration, the pH was adjusted to 5 with 2N HCl, and the resulting slurry stirred for 1 h. The slurry was then centrifuged at 14,000 x g and 4°C for 30 min. An intermediate fraction (mixture of glycinin and β-conglycinin) was obtained as the precipitated curd, whcih was treated as described for the previous fraction. The supernatant, third protein extract, was combined with de-ionized water in a ratio of 2 times the volume of the third protein extract and the pH was adjusted to 4.8. The resulting slurry was centrifuged at 7,500 x g and 4°C for 20 min. The β-conglycinin-rich fraction was obtained as the precipitated curd and this fraction was treated as described for the previous fractions. The amount of supernatant (whey) was determined and sampled for proximate composition. This procedure was replicated two times for each type of flour and means reported. The protein contents were 96.7, 80.3, 92.2, 96.4, 80.9, and 95.6% for the IA 2020 glycinin-rich, intermediate, and β-conglycinin-rich fractions prepared from IA2020 soybeans and for the glycinin-rich, intermediate, and β-conglycinin-rich fractions prepared from HS/LS soybeans, respectively.

Preparation of soy protein fractions by using a new simplified procedure. About 100 g defatted soy flour was extracted with de-ionized water at 15:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 1 h. After

centrifuging at 14,300 x g and 15°C for 30 min, the protein extract (first extract) was decanted, and the amount of insoluble fiber residue was determined and sampled for proximate composition. To this extract, we added sufficient $NaHSO_3$ and $CaCl_2$ to obtain 5mM SO_2 and 5 mM Ca^{2+} and the pH was adjusted to 6.4 with 2N HCl. In one case, the resulting slurry was stored at 4°C for 12-16 h (this treatment is identified as New 4C) and, in another case, the slurry was stirred at ~25°C for 1 h (this treatment is identified as New RT). In both cases, the fractionation procedure was continued by centrifuging the slurry at 14,000 x g and 4°C for 30 min. The glycinin-rich fraction was obtained as the precipitated curd, which was neutralized and treated as described for the other fractions until analyzed. The supernatant, second protein extract, was adjusted to pH 4.8 with HCl and the slurry was stirred for 1 h. The slurry was then centrifuged at 14,000 x g and 4°C for 30 min. The β-conglycinin-rich fraction was obtained as the precipitated curd. This fraction was treated as described above for the other fractions, and the amount of supernatant (whey) was determined and sampled for proximate composition. Both treatments (New 4C and New RT) were duplicated for each type of flour and means reported. The protein contents of the glycinin-rich fractions prepared from IA2020 soybeans by using the New 4C and New RT procedures were 98.9 and 96.6%, respectively; the protein contents of the β-conglycinin-rich fractions prepared from IA2020 soybeans by using the New 4C and New RT procedures were 90.0 and 91.2%, respectively; the protein contents of the glycinin-rich fractions prepared from HS/LS soybeans by using the New 4C and New RT procedures were 97.3 and 94.7%, respectively; and the protein contents of the β-conglycinin-rich fractions prepared from HS/LS soybeans by the New 4C and New RT procedures were 92.2 and 92.0%, respectively.

Composition analyses and mass balances. Moisture contents were determined by oven-drying for 3 h at 130°C (*19*). Nitrogen contents were determined by using the combustion or Dumas method (*20*) with a Rapid NIII Analyzer (Elementar Americas Inc., Mt. Laurel, NJ). These values were converted to Kjeldahl nitrogen using the conversion formula of Jung et al. (*21*). The factor used to convert percentage nitrogen to protein content was 6.25.

Phytate contents were determined by HPLC according to methods of Kwanyuen et al. (*22*). Phytate data were converted to dry basis and yields were calculated based on the initial phytate content of the starting soy flours. All measurements were replicated in triplicate and means reported.

Statistical analysis. The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM). Least significant differences (LSD) were calculated at $p<0.05$ to compare treatment means. Pearson correlation coefficients were calculated with the (CORR) function to test relationship among variables and phytic acid content by using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Soy flours. The soy flour prepared from IA2020 soybeans contained 25.6 ± 0.5 mg/g phytic acid, whereas the soy flour prepared from HS/LS soybeans contained 22.1 ± 0.5 mg/g phytic acid on dry-weight basis. These phytic acid contents were statistically different (LSD = 1.8%, p = 0.0054). We attributed this difference in phytic acid content (13.7%) to the genetic modification in the myo-inositol metabolism of HS/LS soybean (*14*). Unusually high amounts (more than four times) of galactinol (galactopyranosyl-myo-inositol), a precursor in the biosynthesis of raffinose and stachyose, were detected in flour prepared from HS/LS soybeans (0.71%, db) than detected in soy flour prepared from normal soybeans (0.16% db) (*15*). Myo-inositol is a common substrate for both galactinol and phytic acid synthesis (**Figure 1**) (*23*). We hypothesized that the high galactinol accumulation probably allowed less myo-inositol to be used for synthesizing phytate.

Phytic acid in EWSPC. EWSPC had the highest phytate content among all protein products produced (**Table 1**). There were no differences in the phytate contents of the EWSPCs prepared from IA2020 and HS/LS flours; however, significantly higher phytate yields were observed in EWSPC prepared from HS/LS flour partially because the later had lower phytate content and because almost none was solubilized by the ethanol-water solvent. The differences in phytate partitioning suggested that phytic acid was complexed differently

in IA2020 soy flour than in HS/LS soy flour. In the EWSPC procedures, we were able to account for almost all the phytate that entered the system, partially because phytase activity was inhibited by the ethanol and because the pH used did not hydrolyze phytic acid.

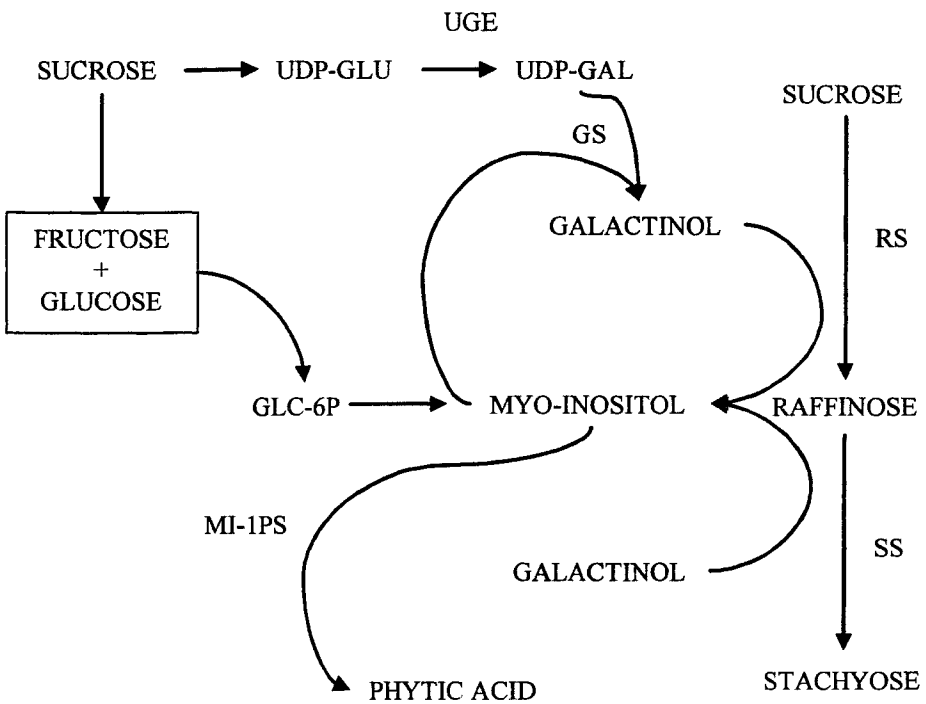

Figure 1. Biosynthetic pathway of oligosaccharide synthesis in soybeans. Adapted from Wilson (*23*). UGE denotes UDP-glucose-4'-epimerase; GS, galactinol synthase; RS, raffinose synthase; SS, stachyose synthase; and MI-1PS, myo-inositol phosphate synthase.

Phytic acid in SPI. There were no significant differences among the phytate contents of the spent flours for the two soybean varieties (**Table 1**). In spite of this, some differences were observed in phytate partitioning. HS/LS soy flour yielded significantly more phytate in the spent flour and whey fractions. Significant differences were observed in total phytate recovery in preparing SPI. There was 11.7% higher recovery of phytate in the SPI procedure when using HS/LS soy flour, suggesting that IA2020 flour might have higher phytase activity, since all procedure variables (pH adjustments, temperatures, extraction and processing times) were the same for both flours. There were lower total phytate recoveries in

Table 1. Phytic acid contents and mass balances solids and phytic acid of soy protein concentrates and isolates prepared from IA2020 and HS/LS soy flours [a].

Process/Product	IA 2020			HS/LS			LSD	
	Phytate (mg/g)	Solids Yield (%)	Phytate Yield (%)	Phytate (mg/g)	Solids Yield (%)	Phytate Yield (%)	Phytate (mg/g)	Phytate (%)
EWSPC								
Concentrate	27.7 ± 0.4	76.1 ± 0.7	82.1 ± 2.1	27.4 ± 0.5	78.4 ± 0.2	97.4 ± 2.1	1.1	4.8*
Extract	15.8 ± 0.3	24.8 ± 0.4	15.3 ± 0.4	3.1 ± 0.2	22.2 ± 0.7	2.1 ± 0.3	1.3*	0.8*
Total		100.8 ± 1.0	97.4 ± 1.8		100.7 ± 0.6	99.5 ± 1.9		3.9
SPI								
Spent flour	27.4 ± 1.2	36.5 ± 0.5	39.1 ± 1.2	28.6 ± 1.0	34.8 ± 0.8	45.1 ± 2.5	2.6	4.4*
Isolate	12.6 ± 0.7	40.7 ± 0.7	20.0 ± 1.0	12.2 ± 1.4	42.4 ± 0.4	23.4 ± 2.9	2.5	4.9
Whey	9.8 ± 0.4	24.4 ± 0.4	9.3 ± 0.5	10.8 ± 0.3	23.4 ± 0.4	11.5 ± 0.3	0.7*	0.8*
Total		101.6 ± 1.0	68.3 ± 0.7		100.6 ± 1.6	80.0 ± 4.9		7.9*
LFSPC, pH 7.5								
Spent flour	22.6 ± 1.0	29.7 ± 0.5	26.2 ± 1.6	28.6 ± 1.0	32.8 ± 0.3	42.5 ± 1.8	2.2*	3.8*
Concentrate	18.0 ± 0.4	70.4 ± 0.3	49.5 ± 1.4	14.6 ± 1.2	67.4 ± 0.3	44.8 ± 3.7	2.0*	6.3
Total		100.1 ± 0.2	75.8 ± 1.3		100.2 ± 0.2	87.3 ± 3.8		6.5*
LFSPC, pH 8.5								
Spent flour	30.4 ± 1.7	28.9 ± 0.7	34.3 ± 2.8	28.7 ± 1.1	30.1 ± 0.7	39.2 ± 2.3	3.3	5.8
Concentrate	15.9 ± 0.3	71.5 ± 1.5	44.3 ± 1.6	14.1 ± 0.4	69.1 ± 1.0	44.1 ± 1.3	0.8*	3.3
Total		100.4 ± 0.9	78.6 ± 1.8		99.2 ± 1.0	83.4 ± 3.6		6.4

[a]n=3. *denotes significant difference at $p<0.05$. HS/LS denotes high-sucrose/low-stachyose soybeans; IA2020, a line of normal soybeans; EWSPC, ethanol-washed soy protein concentrate; SPI, soy protein isolate; LFSPC, low-fiber soy protein concentrate prepared by alkali extraction, neutralizing and drying; pH 7.5 and 8.5, extraction pH for LFSPC; and LSD, least significant difference.

the SPI procedure than in the EWSPC and LFSPC procedures, which also suggested some hydrolysis of phytic acid. Comparing the SPI process to the LFSPC at pH 8.5, two major processing differences must be noted, one is that two extraction steps were used in the LFSPC procedure, but more extraction did not result in significantly more phytate being extracted. Another difference was the acid precipitation step during SPI production that would cause the reduced phytate recovery in this process. Phytic acid is soluble at the pH of SPI precipitation (*2*), yet significant amounts of phytate were found in SPI. We attributed this phenomenon to protein-phytate complex formation at pH<5.0 and, consequently, leading to co-precipitation.

Phytic acid in LFSPC. Phytate contents and partitioning for these procedures for both flours are shown in **Table 1**. There were significant differences between the two soybean varieties for the NSPC extracted at pH 7.5. IA2020 flour produced significantly less phytate in the spent flakes and significantly more phytate in the LFSPC than did the HS/LS flour. We attributed this difference (16.3%) to more protein-phytate complex formation at pH 7.5 for the IA2020 flour, since all other processing variables for both flours remained the same. The phytate yields in the LFSPCs from both flours were about the same, but the IA2020 produced LFSPC with significantly higher phytate content, probably due to the higher phytic acid content in the IA2020 flour.

Total phytate recoveries throughout this procedure were significantly different for both flours and were less than 100%. For both flours, more phytate entered the procedure than was accounted for in products. Alkali hydrolysis and/or phytase activity can cause this. There is conflicting evidence about endogenous phytase activity in soybeans. Although early research failed to show phytase activity in soybeans (*2*), Selle et al. (*24*) reported phytase activities ranging from 10 to 95 FTU/Kg for 22 different soybean meal samples.

There were no significant differences between soybean varieties in phytate partitioning when preparing LFSPC extracted at pH 8.5. The LFSPC obtained from IA2020 soy flour, however, contained slightly more phytate (11.3%) than was contained in LFSPC obtained from HS/LS soy flour, and this difference was consistent with the differences in phytate contents of the starting soy flours. When using HS/LS soy flour, there were no

differences in phytate contents and partitioning when preparing LFSPC extracted at pH 8.5 and LFSPC extracted at pH 7.5. Significant differences occurred, however, when IA2020 flour was used. Significantly more (LSD = 3.1 mg/g) phytate remained in the spent flour when the extraction pH was 8.5 and, as a consequence, this procedure yielded LFSPC with significantly (LSD = 1.0 mg/g) lower phytate content. Phytate partitioning followed the same pattern as for spent flour phytate yield (LSD = 6.3%). These differences might be due to differences in strength of the phytate-protein complex at these pHs, the protein of LFSPC extracted at pH 7.5 seemed to have higher affinity for phytate than did the LFSPC extracted at pH 8.5. Additionally, phytate is more insoluble at higher pHs (*2*), which could explain the difference in phytate extractability for the IA2020 flour. This phenomenon was not observed for any procedures in which HS/LS soybean were used.

Phytic acid in fractionated soy protein. Phytate contents and yields were determined for three different soy protein fractionation procedures and both flours (**Table 2**). In general, there were significant differences among procedures and among soybean varieties for both phytic acid content and partitioning. All three procedures involved the same extraction procedure using 15:1 water-to-flour ratio, pH 8.5, and room temperature for 1 h. This extraction procedure was significantly more efficient in extracting phytate from IA2020 soy flour than it was when using HS/LS soy flour. The IA2020 soy flour not only had higher phytate content but also more readily released phytate to the extraction media, producing a first extract with significantly higher phytic acid content. There were no significant differences among the phytate contents of the spent flours from all fractionation procedures using the same variety. The HS/LS spent flour, however, had similar phytate contents to those of the LFSPC and SPI procedures, and the spent IA2020 flours contained less phytate than did the spent flours for LFSPC at pH 8.5 and SPI. For this soybean variety, the extraction temperature may have influenced the efficiency of extracting phytate, since the fractionation procedures were extracted at room temperature while the other two processes started at 60°C.

Table 2. Phytic acid contents and mass balances for solids and phytic acid in soy protein fractionation procedures using IA2020 and HS/LS soy flours[a].

Process/Product	IA 2020			HS/LS			LSD	
	Phytate (mg/g)	Solids Yield (%)	Phytate Yield (%)	Phytate (mg/g)	Solids Yield (%)	Phytate Yield (%)	Phytate (mg/g)	Phytate (%)
Wu								
Spent flour	17.1 ± 0.1	27.5 ± 0.7	18.4 ± 0.4	31.1 ± 2.6	25.0 ± 0.2	35.1 ± 2.7	7.8*	8.2*
Glycinin	2.9 ± 0.1	11.1 ± 1.2	1.3 ± 0.2	1.2 ± 0.1	15.4 ± 0.6	0.8 ± 0.1	0.5*	0.5
Intermediate	3.2 ± 0.0	17.3 ± 0.4	2.2 ± 0.0	1.7 ± 0.2	8.8 ± 0.3	0.7 ± 0.1	0.7*	0.4*
β-Conglycinin	10.3 ± 0.2	10.7 ± 0.3	4.3 ± 0.1	7.7 ± 0.1	10.5 ± 0.2	3.7 ± 0.0	0.5*	0.2*
Whey	18.4 ± 0.3	36.4 ± 0.7	26.1 ± 0.1	11.2 ± 0.3	40.6 ± 0.9	20.6 ± 1.0	1.4*	3.1*
Total		103.0 ± 0.6	52.2 ± 0.2		100.3 ± 0.8	60.9 ± 3.5		7.4*
N4C								
Spent flour	21.9 ± 0.9	30.2 ± 0.3	25.9 ± 0.8	30.1 ± 1.3	35.5 ± 0.3	48.5 ± 1.6	4.7*	5.4*
Glycinin	10.9 ± 0.4	15.5 ± 0.7	6.6 ± 0.5	7.7 ± 0.1	18.0 ± 0.0	6.3 ± 0.1	1.1*	1.5
β-Conglycinin	14.5 ± 0.1	23.1 ± 0.5	13.0 ± 0.4	10.1 ± 0.3	20.5 ± 0.2	9.3 ± 0.2	1.0*	1.3*
Whey	24.4 ± 3.4	31.2 ± 0.4	29.7 ± 3.7	10.7 ± 1.4	26.0 ± 0.5	12.5 ± 1.4	11.2*	12.2*
Total		100.0 ± 0.0	75.2 ± 3.1		100.0 ± 1.0	76.6 ± 2.9		9.3
NRT								
Spent flour	22.3 ± 1.6	30.8 ± 0.4	26.8 ± 2.3	31.1 ± 2.6	37.8 ± 0.5	53.2 ± 5.1	5.8*	17.0*
Glycinin	18.9 ± 0.3	15.7 ± 1.6	11.6 ± 1.0	9.6 ± 0.1	14.3 ± 0.8	6.2 ± 0.3	1.1*	3.1*
β-Conglycinin	12.0 ± 0.1	23.3 ± 0.8	10.9 ± 0.5	8.1 ± 0.6	22.2 ± 0.6	8.1 ± 0.8	1.8*	2.8*
Whey	10.4 ± 0.5	30.1 ± 0.4	12.2 ± 0.8	9.1 ± 2.0	25.7 ± 0.7	10.6 ± 2.6	6.2	8.2
Total		99.9 ± 0.9	61.6 ± 2.6		100.1 ± 1.1	78.3 ± 2.0		9.9*

[a]n=2. *denotes significant difference at $p<0.05$. HS/LS denotes high-sucrose low-stachyose soybeans; IA2020, a line of normal soybeans; Wu denotes fractions produced by using the Wu procedure; N4C, fractions produced by using the new fractionation procedure with a chilling step; NRT, fractions produced by using the new fractionation procedure without a chilling step;

Phytic acid in the Wu soy protein fractionation procedure. During the Wu protein fractionation procedure, almost 50 and 40% of the phytate present in the IA2020 and HS/LS flours, respectively, were lost. This difference in loss between soybean varieties may be due to different phytase activities of the soy flours. The Wu procedure also yielded the lowest amount of total phytate in the protein fractions among all protein fractionation procedures for both varieties. The phytic acid contents and yields were unique. The protein fractions obtained by using the Wu fractionation procedure had the lowest phytate contents and yields among all procedures evaluated. This is an interesting finding since this procedure started with the highest phytate content in the system. The glycinin-rich fraction had the lowest phytic acid content of the three fractions, followed by the intermediate fraction, and with considerably higher phytate content in the β-conglycinin-rich fraction. This trend was observed for both soybean varieties.

The low phytate content of the glycinin-rich fraction was probably due to the pH at which this fraction was precipitated (6.4), which is supported by work of Okubo et al. (*4*) where no specific binding was found to occur between phytic acid and glycinin at pH 6.0 to 10.0. This fraction was precipitated in the intermediate pH range of phytate-protein complex formation where data interpretation is difficult (*2*). In addition, the Wu fractionation procedure uses sulfites as reducing agents, which may have altered protein structure (*25*) and phytate-binding specificity. The glycinin-rich fraction prepared from IA2020 flour had 2.4 times more phytate than did the same fraction prepared from HS/LS flour. This difference may be due to the higher phytic acid content in the IA2020 flour. Both varieties yielded the same amount of phytate in this fraction ($p<0.05$), but IA2020 yielded more phytate at $p<0.1$ (LSD=0.2%).

The phytate contents of the intermediate fraction were difficult to interpret. During the precipitation of the intermediate fraction, 0.25 mM NaCl was added to the system. This fraction was precipitated at pH 5.0, which is between the low and intermediate pH for data interpretation of phytate binding to protein (*2*). Phytic acid is not as tightly bound to the protein as at lower pHs. deRham et al. (*6*) reported that adding NaCl to a protein extract could disrupt alkaline-earth ion bridges yielding proteins low in phytic acid content. During

the Wu fractionation procedure, this latter mechanism was likely for the intermediate fraction. Both phytic acid yield and content were higher for the intermediate fraction prepared from IA2020 soy flour, probably due to the higher initial phytic acid content.

Higher phytate contents were found in the β-conglycinin-rich fraction than in the other two fractions (**Table 2**). This observation was consistent for both soybean varieties and statistically significant (LSD = 0.9 mg/g and 1.1 mg/g for IA2020 and HS/LS soybeans, respectively). The β-conglycinin-rich fraction produced from IA2020 soybeans contained 25% more phytate than did the same fraction prepared from HS/LS soybeans, however, the difference in yields was lower (13.9%). In both cases, these differences were statistically significant. The higher phytate contents for these β-conglycinin-rich fractions were probably due to precipitating this fraction at pH 4.8 after two-fold addition of de-ionized water that drove the NaCl concentration of the system to 1/3 of that when the intermediate fraction is precipitated. This pH falls in the region where phytic acid is thought to be tightly bound to the protein, since phytate is negatively charged at this pH and the protein is positively charged allowing strong protein-phytate complex formation (*2*).

Phytic acid in the new soy protein fractionation procedure. In previous studies, we developed a new soy protein fractionation procedure (*26*) and reported on compositional and functional characteristics of the fractions obtained from IA2020 soy flour (*12*) and HS/LS soy flour (*13*). Our procedure was based on differences in calcium binding to glycinin and β-conglycinin in the presence of a reducing agent. The two soybean varieties fractionated differently when using this new procedure, producing enriched soy protein fractions with higher purities when the initial flour was IA2020 soy flour than when using HS/LS soy flour (*12, 13*). Preferential calcium-binding to glycinin and the consequential precipitation of this fraction have been widely reported (*27, 28, 29*) and calcium-binding to phytic acid and protein has already been discussed. Graf (*30*) reported that calcium-binding to phytic acid is temperature, pH, and ionic strength dependent. He found that calcium affinity for phytate increased at higher temperatures, while 2 mM free Ca^{2+} ions was critical for phytate precipitation. Cheryan (*2*) reported that an excess of calcium ions displaces the phytate-protein complex. The difference in fractionation behavior between these two soybean

varieties in the presence of calcium ions may be due to differences in the phytate contents of the soy flours that were used. The IA2020 soy flour had higher phytate content and its phytate was more readily extracted than that of the HS/LS soy flour. Consequently, greater amounts of phytate were present in extracts prepared from IA2020 soy flour than those of the extracts from HS/LS soy flour. In our procedures, we added 5 mM of Ca^{2+} ions, this amount worked better for IA2020 soy extracts than for HS/LS extracts. We also found that mM amounts of reducing agent increased the purities of the fractions (*26*).

The total phytate recovery for the New 4C procedure was similar to those of the previous procedures (LFSPC and SPI) and significantly higher than the total recovery when using the Wu procedure. This was probably due to chilling the extract to 4°C after the initial extraction and enzyme activity should have been negligible. This observation explains why, in this case, there were no significant differences in total phytate recovery between soybean varieties.

The phytate content of the glycinin-rich fraction produced by using the New 4C procedure was significantly higher than for the same fraction produced by using the Wu procedure. The increased phytate content and yield were probably due to insolubilization of phytate by calcium and co-precipitation of the phytate salt with the glycinin-rich fraction. The glycinin-rich fraction prepared from IA2020 flour contained more phytate than did the same fraction prepared from HS/LS flour. This difference was not observed in phytate yield, indicating that phytate partitioned similarly with both flours. The lower phytic acid content of the glycinin-rich fraction obtained from HS/LS soybeans indicated that the amount of free calcium ions available to specifically bind to soy proteins was higher than for the same fraction prepared from IA2020 soy flour. This finding probably explains why HS/LS soy flour produced fractions with lower purities (*13*). If there had been more free calcium ions available to bind to protein, at constant pH, there would have been more co-precipitation of glycinin and β-conglycinin.

The β-conglycinin-rich fraction contained more phytic acid than did the glycinin-rich fraction. These differences, however, were less significant when using the Wu procedure, where the β-conglycinin-rich fraction yielded between three and four times as much phytate as did the glycinin-rich fraction. The β-conglycinin-rich fraction produced by using the New

4C procedure had higher phytate content than did the same fraction produced by using the Wu procedure for both flours. Because the β-conglycinin-rich fraction was precipitated at pH 4.8, phytic acid was tightly bound to the protein and the amounts of calcium remaining might account for the increased phytate contents and yields. The β-conglycinin-rich fraction produced from IA2020 soy flour had significantly higher phytate content and yield than did the same fraction prepared from HS/LS flour, probably due to the higher phytate content in the first extract prepared from IA2020 flour.

For the New RT fractionation procedure, the only variable different from the New 4C procedure was precipitation temperature. Phytase would be more active at 25 than at 4°C, which would explain the lower total phytate recovery from the IA2020 flour. In all procedures in which more enzyme activity was possible, significantly lower amounts of phytate were observed with IA2020 flour than in the same fractions prepared from HS/LS flour, suggesting different phytase activities. Calcium affinity for phytate increases as temperature increases, which explains why more phytate was precipitated in the glycinin-rich fraction than in the same fraction when using the New 4C procedure. The phytate contents of the β-conglycinin-rich fractions were approximately the same for both proceduress (New 4C and New RT), probably because calcium-binding to this protein was not influenced by temperature or calcium concentration, since similar amounts of phytate were also present in this fraction when using the Wu procedure.

Effect of phytic acid on compositions of soy protein ingredients. To study the relationships between phytic acid content and specific compositional variables, we used Pearson's correlation test (**Table 3**). Six different variables were tested against phytate content including protein, storage protein, glycinin, β-conglycinin, and isoflavone contents, and the ratio of glycinin-to-β-conglycinin. The rationale for the selected variables was based on reports of phytate binding to soy proteins (*6, 7, 8, 11, 24*), phytate binding to storage proteins (*2, 5, 9*), phytate binding to glycinin (*4, 9,10*), and phytate binding to β-conglycinin (*10, 27*). Additionally, we wanted to test isoflavone content and phytic acid relationships based on our observation that the low-phytate soybean flour had higher isoflavone content (*15*).

This correlation study was first performed by pooling all data collected throughout our previous studies (*12, 13, 15*). Protein content was negatively correlated with phytate content, the products with higher protein contents had lower phytic acid contents. This was probably due to contributions from the purified fractions and the EWSPC. When data for EWSPC were excluded, the correlation coefficient was reduced by 10% because these products had the highest phytate contents and low protein contents. Phytic acid content was correlated with any specific protein components or their ratios.

Isoflavone content was also negatively correlated to phytate content. When EWSPC data were excluded, the isoflavone correlation coefficient changed sign because of the contribution of these concentrates. EWSPC had the highest phytate content and the lowest isoflavone content (*15*).

Table 3. Pearson's correlation coefficients between phytic acid content and different sample variables[a].

Variables	Pooled Samples			Pooled Samples (-EWSPC)		
	N	r	p-value	N	r	p-value
Component						
Protein content	52	- 0.57	0.000	46	- 0.47	0.001
Storage protein content	52	0.00	0.978	46	0.04	0.804
β-Conglycinin content	52	- 0.03	0.836	46	0.10	0.521
Glycinin content	52	0.03	0.836	46	- 0.10	0.521
Glycinin/β-conglycinin ratio	52	- 0.15	0.299	46	- 0.13	0.402
Isoflavone content	38	- 0.45	0.005	32	0.47	0.006
Functional Property						
7S protein enthalpy	52	- 0.17	0.226	46	- 0.02	0.901
11 S protein enthalpy	52	- 0.05	0.734	46	0.02	0.911
Total enthalpy	52	- 0.17	0.240	46	0.01	0.931
Solubility	52	- 0.43	0.001	46	0.50	0.004
Surface hydrophobicity	52	- 0.11	0.452	46	0.55	0.000
Emulsification capacity	52	0.04	0.799	46	0.56	0.000
Emulsification activity	52	- 0.36	0.009	46	0.26	0.078
Emulsification stability	52	- 0.28	0.046	46	0.11	0.464
Foaming capacity	52	0.14	0.306	46	0.46	0.001
Foaming stability	52	- 0.14	0.317	46	- 0.29	0.049
Rate of foaming	52	- 0.09	0.522	46	0.32	0.032

[a]N denotes sample size for linear regression; r, correlation coefficient; pooled samples, all samples tested for a particular variable and phytate from both soybean varieties; and pooled samples (-EWSPC), all samples tested for a particular variable excluding ethanol-washed soy protein concentrate.

Effect of phytic acid on functionality of soy protein ingredients. Phytic acid content has been reported to affect functional properties of protein (*2, 10*). To confirm if phytic acid content was related to functional properties in our samples, we pooled all samples and determined correlation coefficients (**Table 3**). When all samples were pooled, solubility was highly correlated with phytic acid content, while emulsification activity and stability were negatively correlated. All other functional properties were not significantly correlated with phytic acid content. Because EWSPC had very high phytate content and, in general, very poor functional properties, data for EWSPC were excluded and correlation coefficients re-calculated (**Table 3**). In this case, the number of significant correlation coefficients increased. The thermal behavior of theprotein was not significantly correlated with phytate content, suggesting that the native state of the protein does not affect phytate content. Significant correlation coefficients were found for solubility, surface hydrophobicity, emulsification capacity, and foaming properties. All correlations were positive, indicating that those soy protein fractions having good functional properties also have high phytate contents. None of the correlation coefficients were above 0.6 indicating that these functional properties are probably also affected by other factors in addition to phytate content.

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CHAPTER 10. EFFECTS OF EXTRACTION TEMPERATURE AND PRESERVATION METHOD ON FUNCTIONALITY OF SOY PROTEIN ISOLATES

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Abstract

The effects of extraction temperature and preservation method on the functional properties of soy protein isolate (SPI) were determined. Four extraction temperatures (25, 40, 60, and 80°C) were used to produce SPI and yields of solids and protein contents were determined. Four preservation methods were tested, spray-drying, freeze-drying, fresh (undried), and freezing-thawing, for each extraction temperature. No differences in yields of solids and protein were observed among SPIs extracted at 25, 40, and 60°C; however, SPI extracted at 80°C yielded significantly less solids and protein. Extraction temperature significantly affected SPI functionality. As extraction temperature increased, solubility and emulsification capacity decreased; surface hydrophobicities, emulsification activities and stabilities, and dynamic viscosities increased; and foaming properties improved. Preservation method also significantly affected SPI functionality. The drying method did not affect the denaturation enthalpies of SPIs. Spray-dried SPIs had higher solubilities, surface hydrophobicities, and emulsification stabilities, and lower viscosities, emulsification activities and rates of foaming than did freeze dried SPI. Emulsification and foaming capacities and foaming stabilities were similar for both methods of drying. There was significant interaction between extraction temperature and preservation method factors for all functional properties but emulsification capacity. Each preservation method gave unique characteristics to the SPI and these characteristics were not related to one another.

Introduction

A substantial body of research has prompted the Food and Drug Administration to recently approve a cholesterol-lowering health claim for soy protein indicating that daily consumption of 25 g soy protein (6.5 g of soy protein per serving) may lower LDL

cholesterol in individuals who have high cholesterol and who also adhere to a low-fat diet (US FDA 1999). Soy protein isolates (SPIs) are the most highly refined soy protein products commercially available for use as food ingredients and the use of SPI has been steadily increasing for the last two decades (Golbitz 2003). SPI is prepared from defatted soy meal and contains more than 90% (dry basis) protein. SPI is used as ingredients in high-protein foods, especially in dairy products, nutritional supplements, meats, infant formulas, nutritional beverages, soups, sauces, and snacks. SPI utilization is based on the wide range of highly desirable functional properties such as solubility, hydrophobicity, emulsification, foaming, fat and water absorption, gelling, and viscosity control. These important functional properties affect their suitability in various applications and, as a result, their value in the marketplace. Stabilization for storage and distribution of SPI is critical for food safety, but to also preserve good functionality. Generally, long-term protein preservation is accomplished by drying to less than 8% moisture.

Heat denaturation is a major factor influencing protein functionality (Wu and Inglett 1974). Usually, SPI is produced by extracting defatted soy flakes/flour with alkali at temperatures between 20 and 80°C (60°C being usual). Freeze-drying uses mild temperatures for extended periods (Fagain 1997), while spray-drying utilizes high temperatures for short periods of time (Lusas and Rhee 1995). Drying affects protein functionality because it usually involves the use of high temperatures, and proteins are thermally unstable and denature (Fagain 1997). For this reason, we hypothesized that both factors, extraction temperature and drying method, significantly affect SPI functionality. Two commonly utilized methods to obtain acceptable long-term storage stability of SPI are freeze-drying and spray-drying.

Freeze-drying is widely used in scientific research. The process involves removing water from frozen protein dispersions by sublimation under vacuum followed by controlled heating to moderate temperatures for removing the remaining water. Residual moisture levels are often less than 1%. Freeze-drying is believed to be the best method to stabilize protein functionality (Fagain 1997).

On the other hand, spray-drying is the primary method used by the food industry for commercial production, especially for producing milk powder, dairy products, and food

protein ingredients such as SPI. Spray-drying rapidly dries solutions or slurries to particulate forms by atomizing the liquid in a heated chamber. Spray-drying typically consists of pre-concentrating the liquid (for more economic operation, since evaporation is expensive), atomizing (creation of droplets), drying in a stream of hot, dry gas (usually air), separating the powder from the moist gas, cooling, and packaging.

Surprisingly, little has been published about the effects of extraction temperature and preservation method on the functional properties of SPI. Boatright and Hettiarachchy (1995) found that spray-dried SPI had higher solubilities than did freeze-dried SPI. The objectives of the present study were to evaluate the effects of extraction temperature and preservation method on the yield, composition and functional properties of SPI.

Materials and Methods

Soy flour

SPIs were produced from air-desolventized, hexane-defatted white flakes (IA 2020 variety, 1999 harvest) extracted in the pilot plant of the Center for Crops Utilization Research by using a French Oil Mill Machinery extractor-simulator (Piqua, OH). The defatted flakes were milled with a Krups grinder (Distrito Federal, Mexico) to achieve 100% of the material passing through a 50-mesh screen by using small quantities (10 g) to preserve the native protein state. The protein content of the flour was 57.3% on dry-weight basis with 93.8 protein dispersibility index (PDI) as determined by Silliker Laboratories (Minnetonka, MN). The flour was stored in sealed containers at 4°C until used.

SPI preparation

SPI was prepared as shown in Figure 1. About 200 g defatted soy flour was extracted with de-ionized water at 10:1 water-to-flour ratio, the pH was adjusted to 8.5 with 2N NaOH, and the resulting slurry was stirred for 30 min. Four different extraction temperatures (25, 40, 60, and 80°C) were evaluated in triplicate. After centrifuging at 14,300 x g for 30 min, the protein extract was decanted and the amount of insoluble fiber residue was determined and sampled for proximate composition. The protein extract was adjusted to pH 4.5 with 2N HCl

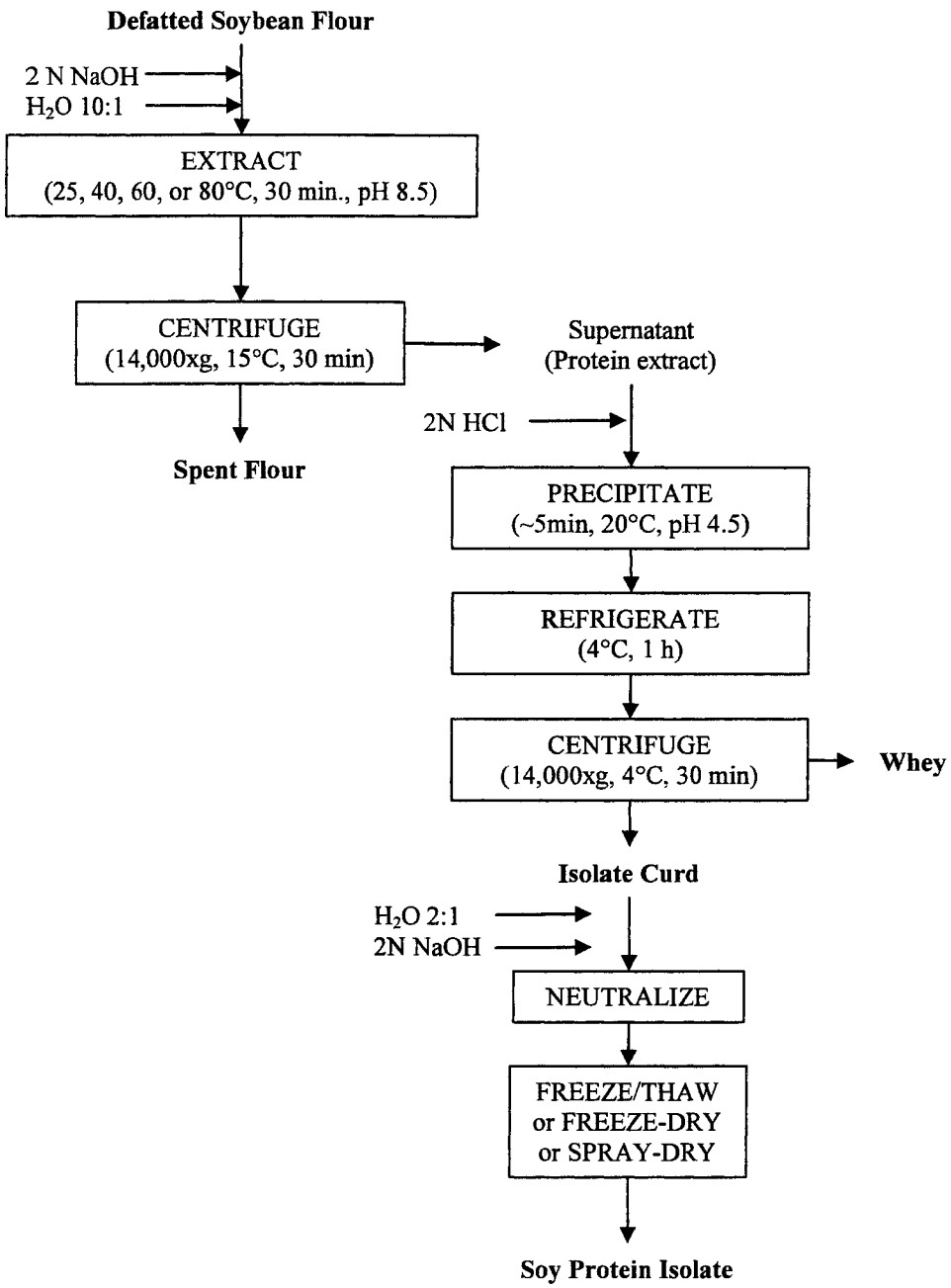

Figure 1-Soy Protein Isolation Procedure

and centrifuged as described above. Protein curd was obtained as a precipitate and the amount of supernatant (whey) was determined and sampled for proximate composition. The curd was re-dissolved in de-ionized water and sufficient 2N NaOH was added to achieve pH 7 with approximately 10% solids.

Preservation

Four samples were taken from each slurry. One was analyzed fresh (within 24 h of preparation), another was analyzed after freezing for at least 2 days and then thawing, a third was analyzed after freeze-drying, and the last sample was analyzed after spray-drying. For freezing and freeze-drying, the samples were frozen at -80°C for at least 48 h. One frozen sample was then placed in a Vartis Ultra 35 (Gardnier, NY) freeze-dryer with shelves cooled at -20°C. High vacuum was then applied while the temperature was held constant (-20°C) until the vacuum dropped to 100 mTorr. Secondary drying was achieved by heating the freeze-dryer shelves to 26°C at high vacuum. The complete freeze-drying cycle lasted for 120 h.

For spray-drying, the samples were fed at about 7 mL/min and 25°C to a Yamato Pulvis spray-dryer (Model GB-21, Yamato Scientific Co. LTD, Yamanashi, Japan). The air-inlet temperature was held at 160°C, the air-outlet temperature was 80°C, and the pulverizer air flow was set at 2.5 Kgf/cm^2. All preservation treatments were replicated three times for each extraction temperature.

Proximate analyses and mass balances

Nitrogen contents of the soy flour, isolated products and byproduct streams were measured by using the combustion or Dumas method (AOAC 1995a) with a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). The nitrogen values were converted to Kjeldahl nitrogen by using the conversion formula of Jung and others (2003). All measurements were determined at least three times and means reported. The factor used to convert percentage nitrogen to protein content was 6.25. Moisture was determined by oven drying for 3 h at 130°C (AOAC 1995b). Mass balances of solids and protein were determined for all SPI treatments. Analyses were replicated in triplicate and means reported.

Thermal behavior

Thermal behavior of the isolated proteins was assessed by using differential scanning calorimetry (DSC). Sample dispersions (15-20 mg) of 10% (w/w, dry basis) protein were hermetically sealed in aluminum pans. A sealed, empty pan was used as reference. The samples were heated from 25 to 120°C at 10°C/min using an SII Exstar 6000 (Seiko Instrument, Inc., Tokyo, Japan). All samples were analyzed at least three times and means reported.

Solubility

Solubility was evaluated according to methods of Rickert and others (2004). The samples were tested at pH 7.0. Solubility was calculated as: % Solubility = (amount of protein in supernatant/amount of initial protein in the sample) x 100. All samples were analyzed at least three times and means reported.

Surface hydrophobicity

Surface hydrophobicity was measured by using methods of Wu and others (1999) with modifications. Protein dispersions were prepared as in the solubility test and aliquots of the soluble protein (supernatant) were serially diluted to obtain 6.25 to 100 μg/mL protein with 0.1 M phosphate buffer (pH 7.0) as diluent. To 3-mL aliquots of each dilution, 40 μL of 1-anilino-8-naphthalene sulfonic acid magnesium salt monohydrate (ANS, ICN Biomedicals, Inc., Aurora, OH) (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was dispersed. Fluorescence intensity units (FIU) were measured with a Turner Quantech® spectrophotometer (Barnstead Thermolyne, Dubuque, IA) by using 440 nm (excitation) and 535 (emission) filters. FIU were standardized using a solution of 40 μL of ANS in 3 mL of phosphate buffer as the zero point and 15 μL of ANS in 3 mL of methanol assigned an arbitrary value of 80 FIU. FIUs were plotted against percentages of protein concentration. The slope of the regression line was reported as surface hydrophobicity. Samples were run in triplicate and means reported.

Emulsification properties

Emulsification capacity was measured according to methods of Bian and others (2003) with modifications. Twenty-five mL of a 2% (w/w, dry basis) sample dispersion adjusted to pH 7.0 with 2 N HCl or NaOH was transferred to a 400-mL plastic beaker. Soybean oil, dyed with approximately 4 ppm Sudan Red 7B (Sigma, St. Louis, MO), was continuously blended into the dispersion at 37 mL/min flow rate by using a Bamix wand mixer (ESGE AG Model 120, Mettlen, Switzerland) at the low setting until phase inversion was observed. Emulsification capacity (g oil/g sample) was calculated as g of oil used to cause inversion multiplied by 2. Emulsification activity and emulsification stability index were measured according to methods of Rickert and others (2004). All analyses were replicated at least three times and means reported.

Foaming properties

Foaming properties were measured according to methods of Sorgentini and others (1995) with modifications developed by Rickert and others (2004). A 0.5% (w/w, dry basis) sample dispersion was prepared and the pH adjusted to 7.0. A 95-mL aliquot was loaded into a custom-designed glass column (58.5 cm x 2 cm) fitted a coarse fritted glass at the bottom, and N_2 was purged through the sample at 100 mL/min flow rate. Time for the foam to reach 300-mL volume, time for one-half of the liquid incorporated into the foam to drain back, and volume of the liquid incorporated into the foam were measured. Three parameters were calculated:

Foaming capacity (FC) = $V_f/(f_r \times t_f)$

K (specific rate constant of drainage) = $1/(V_{max} \times t_{1/2})$

V_i (rate of liquid conversion to foam) = V_{max}/t_f

where V_f = a fixed volume of 300 mL, f_r = the flow rate of the gas, t_f = time to reach V_f, V_{max} = volume of liquid incorporated into foam, and $t_{1/2}$ = time to drain one-half of the liquid incorporated into the foam. Samples were run in triplicate and means reported.

Dynamic viscosity

A 10% (w/w, dry basis) sample dispersion was prepared at pH 7.0 (Rickert and others 2004). The sample was applied to the plate of a RS-150 Rheo Stress (Haake, Karlsruhe, Germany) and shear applied with a 60-mm 2° titanium cone (C60/2 Ti) over the range of 10-500/s shear rate at constant temperature (23°C). Shear rate (γ) and shear stress (τ) over the course of the analysis and application the power law formula were used to determine the consistency coefficient (k) and flow behavior index (n), where $\tau = k\gamma^n$. Using k, n, and γ, apparent viscosity (η) was estimated by $\eta = k\gamma^{n-1}$. Samples were run in triplicate and means reported.

Statistical analysis

The data was analyzed according to the split-plot experimental design by Analysis of Variance (ANOVA) and the Mixed Model by SAS system (version 8.2, SAS Institute Inc., Cary, NC). Least Significant Differences (LSD) were calculated at the 5% level to compare whole-plot and split-plot treatment means for each response variable.

Results and Discussion

Yields and protein contents

SPIs extracted at 25, 40, and 60°C gave similar yields of solids (~42%) and protein (~72%) and had similar protein contents (~92%) (Table 1). SPIs extracted at 80°C had lower yields of solids (~39%) and protein (~63%) and protein contents (~88%) due to protein denaturation during extraction and, as a consequence, loss of protein solubility.

Thermal properties

There were no statistically significant differences among denaturation onset temperatures or denaturation peak temperatures for any of the SPIs prepared at any of the extraction temperatures and by any of the preservation methods (denaturation onset temperatures and peak temperatures were 67.1±1.0 and 73.9±0.6 for β-conglycinin, and 83.0±0.9 and 91.1±0.5 °C for glycinin, respectively). Extraction temperature, however, significantly affected denaturation enthalpies. SPIs extracted at 60°C had reduced

denaturation enthalpies (greatly reduced β-conglycinin and slightly reduced glycinin enthalpies). The SPI extracted at 80°C had no thermally active native structure remaining. These reduced enthalpies were probably caused by the combination of temperature and alkaline environment (pH 8.5 during extraction step), since onset denaturation temperatures at pH 7.0 were above 60 and 80°C, for β-conglycinin and glycinin, respectively, the extraction temperatures used for samples where significant enthalpy reductions were observed.

Table 1-Solids Yields, Protein Yields and Protein Contents of Soy Protein Isolate before Preservation[a]

Extraction Temperature (°C)	Solids Yield (%)	Protein Yield (%)	Protein Content (%, N = 6.25)
25	42.62 a	71.88 a	91.07 a
40	41.64 a	71.59 a	92.83 a
60	42.70 a	71.95 a	91.23 a
80	39.11 b	63.27 b	88.21 b
LSD	2.50	1.99	1.86

[a] Means in the same column followed by different letters are significantly different ($p < 0.05$, N=3). LSD denotes least significant difference.

Reduced denaturation enthalpies for both β-conglycinin and glycinin were observed after both methods of drying at all extraction temperatures (Table 2). There were no significant differences between the denaturation enthalpies of the freeze-dried and the spray-dried samples extracted at the same temperature. These findings indicate that both drying methods denature soy proteins to the same extent despite major differences in time/temperature exposure. Denaturation enthalpy was significantly reduced by freezing-thawing; however, this reduction could not account for the total loss of enthalpy observed in freeze-dried samples. Significant denaturation must have occurred during the sublimation phase of freeze-drying.

Significant interaction was observed between extraction temperature and method of preservation. For denaturation enthalpy of β-conglycinin, there was weak interaction evidence with an F-value of 2.75 and a p-value of 0.044. For the glycinin component, there was stronger interaction evidence with an F-value of 21.06 and a p-value of <0.0001. The error degrees of freedom for all cases were 18. The interaction between extraction

temperature and method of preservation was probably due to the denaturation caused by preservation method depending on the previous thermal history of the protein. The lower significance level for the β-conglycinin component was probably due to this protein being more sensitive to denaturation and, as a consequence, denatured to similar extents regardless of methods of extraction and preservation. On the other hand, the glycinin component was less sensitive to denaturation and was partially denatured to different extents depending on extraction temperature and preservation method. This significant interaction means that the preservation method and extraction temperature are non-additive factors affecting denaturation and each preservation method has to be compared individually to each of the extraction temperatures.

Table 2-Effects of Extraction Temperature and Preservation Method on Denaturation Enthalpies (ΔH, mJ/mg) of Soy Protein Isolate[a]

Treatment	Extraction Temperature (°C)		
	25	40	60
Denaturation enthalpy of β-conglycinin[b]			
Fresh	2.58[a] a	2.63[a] a	0.70[a] b
Frozen/thawed	2.23[b] a	2.29[b] a	0.65[a,b] b
Freeze-dried	2.07[b] a	2.25[b] a	0.52[b,c] b
Spray-dried	2.16[b] a	2.20[b] a	0.44[c] b
Denaturation enthalpy of glycinin[c]			
Fresh	7.79[a] a	7.04[a] b	6.72[a] c
Frozen/thawed	7.51[b] a	6.83[b] b	6.49[b] c
Freeze-dried	6.51[c] a	6.53[c] a	6.21[c] b
Spray-dried	6.50[c] a	6.53[c] a	6.26[c] b

[a]LSD denotes least significant difference, $p<0.05$, N = 3.

[b]LSD for means within the same row is 0.14, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 0.15, means followed by different lower case superscript letters within a column are significantly different.

[c]LSD for means within the same row is 0.18, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 0.16, means followed by different lower case superscript letters within a column are significantly different.

Solubility

Solubility is the fundamental functional property of protein ingredients, since solubility affects most other functional properties (Kinsella 1979). The highest solubilities were for SPIs extracted at 25, 40, and 60°C (no significant difference among them) followed by the isolates extracted at 80°C (Table 3). The solubility of the SPI extracted at 25°C was more affected by preservation method than were SPIs extracted at 40 and 60°C, probably because the former wet products had significantly higher amounts of native protein.

Table 3-Effects of Extraction Temperatures and Preservation Method on Protein Solubility (%) of Soy Protein Isolate at pH 7.0[a]

Treatment	Extraction Temperature (°C)			
	25	40	60	80
Fresh	94.9^{a} a	94.9^{a} a	93.4^{a} a	83.5^{a} b
Frozen/thawed	89.4^{c} b	92.0^{b} a	$92.0^{a,b}$ a	66.7^{c} c
Freeze-dried	85.1^{d} b	92.1^{b} a	90.6^{b} a	58.8^{d} c
Spray-dried	91.7^{b} b	95.3^{a} a	93.7^{a} a	77.8^{b} c

[a]LSD denotes least significant difference, $p < 0.05$, $N = 3$. LSD for means within the same row is 2.1, means followed by different full case letters within a row are significantly different. LSD for means within columns is 2.2, means followed by different lower case superscripts within a column are significantly different.

Freeze-dried SPIs were significantly less soluble than spray-dried SPIs, confirming previous observations of Boatright and Hettiatachchy (1995). Freeze-dried SPIs were easier to disperse in water than were spray-dried SPIs, probably due to the electrostatic charges on the particle surfaces of spray-dried SPI.

There was significant interaction between extraction temperature and preservation method on the solubility of SPI. The F-value for interaction was 38.05 with a corresponding p-value of <0.0001, the error degrees of freedom were 24. Thermal treatment at 80°C caused a large loss in solubility, probably by a high degree of aggregation.

The formation of different sizes of aggregates would partially explain the preservation method effects on solubility. In general, there was an important drop in solubility for the SPIs that were freeze-thawed. This behavior could be caused by increased size of aggregates were formed during alkali extraction and acid precipitation. This model would also explain why the freeze-dried SPIs at similar degrees of denaturation had lower

solubilities than spray-dried SPIs. Evidently, the degree of aggregation was dependant on the original amount of denatured protein present in the SPI, which in turn was highly dependant on extraction temperature. The degree of aggregation depends on thermal treatment and the size of aggregates formed in SPI dispersions affects solubility (Petrucelli and Anon 1994, 1995,1996). The formation of soluble and insoluble aggregates of SPI on heating has been widely reported as has been studied the nature of these aggregates and the interaction of different soy protein components (Damodaran and Kinsella 1982, German and others 1982, Utsumi and others 1984, Petrucelli and Anon 1995, Sorgentinin and others 1995). We propose that both freezing and freeze-drying induce the formation of insoluble aggregates regardless of extractions temperature. Freezing is not instantaneous. Initially, only water is frozen increasing the protein concentration in the unfrozen water (Franks 1991). High protein concentrations induce protein-protein interactions and, as a consequence, larger aggregates (Sorgentini and others 1995). In addition, during sublimation of freeze-drying the sublimation front moves down into the product and the "liberated" water molecules have to pass through a layer of dried product. Some of these molecules are adsorbed and allow molecular mobility for further aggregation (Franks 1991). On the other hand, spray-drying is a much faster process that does not allow for such interactions to occur. Spray-drying causes some denaturation, as evidenced by thermal behavior and surface hydrophobicity data, but speed and shear of in this process prevents the proteins from forming large aggregates. Similar shear-temperature treatment models have been previously proposed for hydrothermal processing (Wang and Johnson 2001). The degree of denaturation and extent of aggregation depend on both extraction temperature and preservation method.

Surface hydrophobicity

Many of the molecular and functional properties of food proteins are related to the relative proportions of hydrophobic and hydrophilic amino acids, and their distribution in the primary structure (Damodaran 1989). The amount of hydrophobic regions exposed by a given protein significantly affects intermolecular interactions, such as binding of small ligands or the association with other macromolecules (including protein-protein or protein-

lipid interactions), which in turn affect surface active functional properties (Nakai and others 1996).

The SPIs extracted at higher temperatures (80 and 60°C) had significantly higher surface hydrophobicity than did the SPIs extracted at lower temperatures (25 and 40°C) (Table 4). There were also significant differences in hydrophobicity between freeze-dried and spray-dried SPIs. Higher hydrophobicities were achieved with spray-drying than with freeze-dried SPI. Fresh SPIs had significantly different hydrophobicity compared to dried SPIs.

Table 4-Effects of Extraction Temperatures and Preservation Method on Surface Hydrophobicity (dimensionless) of Soy Protein Isolate at pH 7.0[a]

Treatment	Extraction Temperature (°C)			
	25	40	60	80
Fresh	278[a,b] c	275[b,c] c	388[b] b	425[b] a
Frozen/thawed	262[b] c	294[b] b	389[b] a	413[b] a
Freeze-dried	205[c] c	255[c] b	339[c] a	346[c] a
Spray-dried	290[a] c	323[a] b	458[a] a	470[a] a

[a]LSD denotes least significant difference, $p < 0.05$, $N = 3$. LSD for means within the same row is 29.8, means followed by different full case letters within a row indicate means are significantly different. LSD for means within the same column is 23.7, means followed by different lower case superscript letters within a column are significantly different.

These results were consistent with the DSC results indicating SPIs extracted at 60 and 80°C were more extensively denatured. Higher proportions of hydrophobic regions are exposed in these products probably due to unfolding. This mechanism can also explain the higher hydrophobicities of spray-dried SPIs, but fails to explain why the freeze-dried SPIs had significantly lower hydrophobicities. Electrostatic charges produced during spray-drying may partially account for the increased hydrophobicity as measured by ANS (Nakai and others1996).

There was interaction between extraction temperature and drying method for surface hydrophobicity, having an F-value of 3.55, p-value of 0.0063, and 24 degrees of freedom for error. The interaction in hydrophobicity also fits in our proposed model of higher extents of insoluble aggregates being formed by freezing and freeze-drying. Surface hydrophobicity not only depends on the extent of denaturation, which increases surface hydrophobicity, but also on the extent of aggregation, which tends to decrease surface hydrophobicity (Petrucelli and

Anon 1996). The decrease in surface hydrophobicity due to freezing and freeze-drying can be explained by the formation of larger aggregates, not allowing the ANS probe to reach the hydrophobic regions exposed by denaturation. In contrast, the spray-dried samples having approximately the same degree of denaturation and higher solubility due to smaller aggregates will have higher surface hydrophobicity.

Emulsification properties

Emulsification capacity. Proteins are often used to aid emulsion formation and increase emulsion stability of foods. Proteins are much larger and more complex than simple emulsifier molecules and the formation of protein-stabilized emulsions requires that the protein molecule migrate to the water/lipid interface and unfold such that its hydrophobic regions can contact the lipid phase (Mangino 1989). In order to achieve this, protein molecules must have both hydrophilic and hydrophobic regions and retain flexibility in order to unfold.

SPIs extracted at 25 and 40°C had the highest emulsification capacities, followed by the SPI extracted at 60 and 80°C (Table 5). This indicates that emulsification capacity was influenced by the amount of native β-conglycinin present in the SPI and the protein solubility. Higher contents of native β-conglycinin and higher protein solubility favor higher emulsification capacities.

The emulsification capacities for freeze-dried and spray-dried SPIs were not significantly different from each other for any extraction temperature. On the other hand, there were significant differences between the emulsification capacities for fresh and frozen/thawed SPIs extracted at 25°C.

There was no significant interaction between the extraction temperature and drying method for emulsification capacity having an F-value of 0.33, p-value of 0.9551, and 24 degrees of freedom. This indicated that the emulsification capacities for these SPIs were not affected by preservation method. When assessing emulsification capacity, any preservation method can be used. This was the only functional property that showed no significant interaction, indicating that both extraction temperature and preservation method had additive effects and were independent from each other.

Table 5-Effects of Extraction Temperatures and Preservation Method on Emulsification Properties of Soy Protein Isolate at pH 7.0[a]

Treatment	Extraction Temperature (°C)			
	25	40	60	80
Emulsification capacity (g of oil emulsified by 1 g of SPI)[b]				
Fresh	546[b] a	578[a] a	481[a,b] b	455[a] b
Frozen/thawed	587[a] a	611[a] a	517[a] b	483[a] b
Freeze-dried	563[a,b] a	585[a] a	478[b] b	459[a] b
Spray-dried	590[a] a	592[a] a	498[a,b] b	475[a] b
Emulsification activity (Absorbance at 500 nm)[c]				
Fresh	0.248[a] d	0.257[a] c	0.285[a] b	0.334[a] a
Frozen/thawed	0.236[b] d	0.252[a] c	0.271[b] b	0.322[b] a
Freeze-dried	0.234[b] d	0.252[a] c	0.270[b] b	0.311[c] a
Spray-dried	0.233[b] d	0.242[b] c	0.264[b] b	0.294[d] a
Emulsification stability index (dimensionless)[d]				
Fresh	103[c] b	117[c] b	320[a] a	335[a] a
Frozen/thawed	155[b] c	199[a] b	170[c] c	233[c] a
Freeze-dried	112[c] d	159[b] b	142[d] c	188[d] a
Spray-dried	169[a] d	190[a] c	229[b] b	253[b] a

[a]LSD denotes least significant difference, $p < 0.05$, $N = 3$.
[b]LSD for means within the same row is 35.7, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 36.1, means followed by different superscrips within a column are significantly different.
[c]LSD for means within the same row is 0.009, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 0.008, means followed by different superscripts within a column are significantly different.
[d]LSD for means within the same row is 16.3, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 15.7, means followed by different superscripts within a column are significantly different.

Emulsification activity and emulsification stability index. Emulsions are thermodynamically unstable. Once formed, an emulsion can undergo a number of changes. It is of interest to know not only how efficient a protein dispersion is in emulsifying but also the stability of the resulting emulsion. The factors involved in emulsification stability are many and complex (Mangino 1989).

The emulsification activities of the SPI extracted at 80°C were significantly higher than for SPI extracted at 60°C, followed by SPI extracted at 40°C and by SPI extracted at 25°C (Table 5). The freeze-dried SPIs had higher emulsification activities than did spray-

dried SPIa, but this phenomenon was only significant for SPIs extracted at 40 and 80°C. Fresh samples had the highest emulsification activity. There was significant interaction between the extraction temperature and preservation method, having an F-value of 3.77, p-value of 0.0044, and 24 degrees of freedom for error.

Emulsification stability indices were significantly affected by both extraction temperature and drying method. The SPIs extracted at higher temperatures had significantly better stabilities than those dried at lower temperatures (Table 5). Freeze-dried SPIs had consistently lower emulsion stability indices compared to spray-dried SPIs, but only SPIs extracted at 40 and 60°C were significantly different. Preservation method affected emulsion stability index to different extents. This behavior corresponded to both solubility and surface hydrophobicity. In order to produce stable emulsions, the molecule must be soluble in the continuous phase and have sufficient hydrophobic patches exposed to the dispersed oil phase. There was significant interaction between extraction temperature and preservation method having an F-value of 99.58, a p-value of <0.0001, and 24 degrees of freedom for error.

Foaming properties

Extraction temperature and preservation method significantly influenced foaming capacity. For fresh and frozen samples, the SPIs extracted at higher temperatures had higher foaming capacities (Table 6). For freeze-dried and spray-dried samples, SPI extracted at 40°C had the highest foaming capacity. Fresh or frozen products do not predict dry SPI behavior. There was significant interaction between the extraction temperature and the preservation method factors having an F-value of 33.25, a p-value of <0.0001, and 24 degrees of freedom for error.

High K values indicate less stable foam is formed. Foaming stability was significantly affected by both extraction temperature and preservation method (Table 6). The foams prepared with SPI extracted at 80°C were highly stable. Tthis may be due to the higher surface hydrophobicity observed in these samples that allow proteins to move more efficiently to the water/air interface and form more stable foams. Freeze-dried products and spray-dried products were not significantly different from each other. There was significant

interaction between extraction temperature and preservation method factors having an F-value of 132.27, a p-value of <0.0001, and 24 degrees of freedom for error.

Table 6-Effects of Extraction Temperature and Preservation Method on Foaming Properties of Soy Protein Isolate at pH 7.0[a]

Treatment	Extraction Temperature (°C)			
	25	40	60	80
Foaming capacity (mL of foam formed by ml of a 0.5 % SPI dispersion)[b]				
Fresh	1.218[a] b	1.295[a] b	1.437[a] a	1.449[a] a
Frozen/thawed	0.888[b] b	1.114[b] b	1.319[a,b] a	1.387[a,b] a
Freeze-dried	1.250[a] b	1.377[a] a	1.163[c] b	1.192[c] b
Spray-dried	1.250[a] a,b	1.375[a] a	1.234[b,c] b	1.266[b,c] a,b
Foaming stability [K=1/(mL*min)][c]				
Fresh	0.013[a] a	0.013[a] a	0.013[a] a	0.008[a] b
Frozen/thawed	0.008[b] b,c	0.009[b] a,b	0.011[a,b] a	0.006[a,b] c
Freeze-dried	0.005[c] b	0.006[c] b	0.009[b] a	0.004[a,b] b
Spray-dried	0.007[b,c] b	0.007[b,c] b	0.011[a,b] a	0.007[a] b
Rate of foaming (mL/min)[d]				
Fresh	15.34[b] b	16.63[b] b	16.80[a] b	21.97[b] a
Frozen/thawed	12.86[c] b	13.26[c] b	12.87[b] b	19.43[c] a
Freeze-dried	21.54[a] b	19.15[a] c	18.36[a] c	28.41[a] a
Spray-dried	16.31[b] b	12.47[c] c	13.36[b] c	23.34[b] a

[a]LSD denotes least significant difference, $p < 0.05$, $N = 3$.
[b]LSD for means within the same row is 0.127, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 0.136, means followed by different superscripts within a column are significantly different.
[c]LSD for means within the same row is 0.0024, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 0.0020, means followed by different superscripts within a column are significantly different.
[d]LSD for means with the same row is 2.35, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 2.06, means followed by different superscripts within a column are significantly different.

Both extraction temperature and preservation method significantly affected rate of foaming. The SPIs extracted at 80°C were the fastest in forming foams (Table 6). The freeze-dried samples had significantly higher foaming rates than those of spray-dried SPIs. Drying increased the rate of foaming in all cases. There was significant interaction between

extraction temperature and preservation method having an F-value of 4.05, a p-value of 0.0029, and 24 degrees of freedom for error.

Dynamic viscosity

The SPIs extracted at 60°C had the lowest consistency factor (k) and the closest flow behavior index (n) to Newtonian fluid behavior of all SPIs tested. This may be due to the fact that viscosity was controlled by the native β-conglycinin component, and when this protein was denatured, viscosity dropped. Another viscosity change was observed with thermal denaturation of the glycinin that would account for the high viscosity obtained at 80°C. Apparent viscosities for these products were similar to results reported by Rickert and others (2004) and are consistent with their findings for β-conglycinin, glycinin, and soy protein isolates. Upon β-conglycinin denaturation in alkaline conditions, β-conglycinin trimers dissociate into individual subunits (Petrucelli and Anon 1995), which would account for the drop in viscosity. On the other hand, when glycinin is denatured in alkaline conditions it also dissociates in acidic and basic polypeptide components, which in presence of β-conglycinin will first form soluble aggregates of heterogeneous nature (Utsumi and others 1984). Upon cooling, they form highly organized complexes. Depending upon protein concentration, they gel (Utsumi and Kinsella 1985), which accounts for the high viscosity obtained with the 80°C extraction treatment.

Dynamic viscosity was affected by both extraction temperature and preservation method to different extents (Table 7). Drying method significantly affected k for those SPIs that had higher viscosities (40 and 80°C). For these samples, spray-drying produced the lowest consistency factors, which was consistent with solubility. In general, those samples with higher solubilities for the same extraction temperature were less viscous. For the SPIs prepared at 25 and 60°C, there were no differences among preservation methods for consistency factor. Flow behavior index (n) gives an idea of how close to a Newtonian fluid the dispersions are; the closer to 1, the closer to a true Newtonian fluid behavior. In general, those SPI dispersions with high consistency factor had a low flow behavior index.

There was significant interaction between extraction temperature and preservation method for dynamic viscosity having an F-value of 2.32, and a p-value of 0.0479 for

consistency factor (k), and an F-value of 175.16, and a p-value of <0.0001 for flow behavior index (n) with 24 degrees of freedom for error in both cases. Our proposed model also fit viscosity data, and explains why there was significant interaction between extraction temperature and preservation method. The previous thermal history and the size and distribution of the soluble-insoluble aggregates, in addition to the above described complex association-dissociation behaviors of glycinin and β-conglycinin, account for this interaction.

Table 7-Effects of Extraction Temperature and Preservation Method on Dynamic Viscosity Soy Protein Isolate at pH 7.0[a]

Treatment	Extraction Temperature (°C)			
	25	40	60	80
Flow consistency Index (k, mPa*s)[b]				
Fresh	0.27[a] c	1.25[b,c] b	0.05[a] c	9.11[a] a
Frozen/thawed	0.38[a] c	2.05[a,b] b	0.04[a] c	7.56[b] a
Freeze-dried	0.45[a] c	2.32[a] b	0.08[a] c	6.27[c] a
Spray-dried	0.65[a] b	0.84[c] b	0.03[a] b	2.24[d] a
Flow behavior index (n, dimensionless)[c]				
Fresh	0.675[a] b	0.450[b] c	0.925[a] a	0.172[d] d
Frozen/thawed	0.585[b] b	0.389[c] c	0.871[c] a	0.369[c] d
Freeze-dried	0.562[c] b	0.380[c] c	0.877[c] a	0.392[b] c
Spray-dried	0.513[d] b	0.489[a] c	0.897[b] a	0.464[a] d

[a]LSD denotes least significant difference, $p < 0.05$, $N = 3$.
[b]LSD for means within the same row is 0.90, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 0.81, means followed by different superscripts within a column are significantly different.
[c]LSD for means within the same row is 0.018, means followed by different full case letters within a row are significantly different. LSD for means within the same column is 0.019, means followed by different superscripts within a column are significantly different.

Conclusions

Functionality of SPI was significantly affected by both the temperature at which the soybean flour was extracted and the method used for preservation. As extraction temperature increased, solubility and emulsification capacity decreased; surface hydrophobicities, emulsification activities and stabilities, and dynamic viscosities increased; and foaming properties improved. Denaturation enthalpies of the SPIs were not affected to different

extents by drying method. Spray-dried SPIs had higher solubilities, surface hydrophobicities, and emulsification stabilities and lower viscosities, emulsification activities and rates of foaming than did freeze-dried SPIs. Emulsification and foaming capacities and foaming stabilities were the same for both methods of drying. There was significant interaction between extraction temperature and preservation method for all functional properties tested but emulsification capacity. We proposed that the size and extent of aggregation account for the interaction between preservation method and extraction temperature.

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CHAPTER 11. GENERAL CONCLUSIONS

General Discussion

This body of work sought improve soy protein ingredients by focusing on how using genetically modified (high-sucrose/low-oligosaccharide) soybeans can be used to produce new low-fiber soy protein concentrates (LFNSPC), how different soy flours and processing conditions affect soy protein fractionation and functionality, how calcium can be used as a fractionating agent, the fate of phytic acid during these procedures, and how extraction temperature and preservation method affects soy protein functionality.

The concentration of reducing agent significantly affected fraction yields, purities, subunit compositions and functional properties of fractionated soy protein. The glycinin-rich fraction was the most affected, with purity increasing but functional properties declining as reducing agent concentration increased. The best combination of yields, purities and functional properties were achieved at 5 mM SO_2. The proposed mechanism for action of reducing agents during soy protein fractionation was based on the preferential reduction of the disulfide bond between the basic and acidic polypeptides of glycinin that are preferentially exposed through calcium-phytate linkages to the β-conglycinin component. This calcium-mediated linkage was most likely to occur between acidic polypeptides of glycinin and α' or α subunits of β-conglycinin.

Salting-in and salting-out were also optimized for fractionating soy protein. The optimum NaCl concentration was 250 mM, at which good protein yield (18.5%) and purity (84.5%) were achieved for the β-conglycinin-rich fraction. Increasing NaCl concentration beyond 250 mM caused significant protein losses to the whey fraction. There were no significant differences in protein yields or purities when using one-fold or two-fold dilution. The different trimers of β-conglycinin had different salting-in and salting-out behaviors. Those β-conglycinin trimers rich in β subunits were the first to salt-in and the last to salt-out. Those β-conglycinin trimers rich in α subunits salted-in and salted-out second, and those β-conglycinin trimers rich in α' subunits were the last to salt-in and the first to salt-out. The glycinin basic polypeptide was generally associated with the β subunit of β-conglycinin.

The feasibility of using calcium ions as a fractionating agent was demonstrated, when used in combination with sulfites. For the three-step Wu fractionation scheme, calcium was an effective precipitating agent for glycinin in the intermediate fraction at 5 and 10 mM concentrations and pH 6.4. The use of calcium allowed the development of a simplified two-step soy storage protein fractionation procedure. The use of 5 mM SO_2 in combination with 5 mM $CaCl_2$ in a new two-step fractionation procedure produced the highest simultaneous purities for the glycinin-rich (85.2%) and β-conglycinin-rich (80.9%) fractions. The proposed mechanism for action of reducing agents in combination with calcium during the new two-step soy protein fractionation was based on preferentially reducing the disulfide bonds between the basic and acidic polypeptides of glycinin that are preferentially exposed through calcium-phytate linkages to the β-conglycinin component. The consequential addition of calcium ions first displaces calcium-mediated protein-phytate interaction and the excess calcium preferentially precipitates the glycinin component through calcium-mediated aggregates formation. The new two-step fractionation procedure yielded fractions with improved solids, protein, and isoflavone yields and similar purities to the three-step fractionation procedure. The new two-step procedure yielded twice as much isoflavones in the glycinin-rich fraction and more than ten times that amount in the β-conglycinin-rich fraction than did the three-step Wu fractionation procedure. The fractions produced with the two-step fractionation procedure had superior emulsification and foaming properties and similar dynamic viscosities as did the fractions produced by using the three-step fractionation procedure.

The LFSPCs had slightly lower solids and protein yields (~70 and ~80%, respectively) than did conventional ethanol-washed soy protein concentrate (EWSPC) (~77 and ~93%, respectively) and much higher than conventional SPI (~42 and 70%, respectively). The LFSPCs were higher in soluble sugars and lower in fiber than were the EWSPC and SPI. The sums of raffinose and stachyose of the LFSPC were ~1%. Total isoflavone contents of NSPC (~12 μmol/g) were significantly higher than those of EWSPC or SPI (~1.5 and ~10 μmol/g, respectively). The LFSPCs had significantly better functional properties (solubility, surface hydrophobicity, and emulsification and foaming properties) than did EWSPC.

High-sucrose/low-stachyose (HS/LS) soybean lines fractionated quite differently from normal soybeans. The three-step fractionation procedure gave glycinin and β-conglycinin-rich fractions with 100% purity and high yields of solids (15.4%) and protein (31.7%) when using HS/LS defatted soy flour. These yields and purities were significantly higher than those of normal soybeans. The new two-step procedures were less efficient in fractionating these proteins from HS/LS soybeans with purities ranging from only 71 to 80%. As in the case for normal soybeans, the new two-step procedures yielded protein fractions with similar or superior functional properties as fractions produced with the three-step Wu procedure.

Processing method and soybean variety significantly affected phytic acid (PA) contents and recoveries. HS/LS soybeans had significantly less phytate than did IA2020 normal soybeans. EWSPC had the highest PA contents and yields for both soybean varieties. A fractionated glycinin-rich fraction had the lowest PA content. Data on PA confirmed the proposed mechanisms for soy protein fractionation and also explained the significant role that PA played in the different fractionation behaviors of the two soybean lines. When all samples were pooled, protein content was the only component that consistently had a significant negative correlation with PA content. Isoflavone content had a negative correlation coefficient when all samples were pooled, but and a positive correlation coefficient when EWSPCs were excluded from the data set. The same trend was observed for solubility. When all samples were pooled, emulsification activity and stability correlated negatively with PA content. When EWSPCs were excluded from the pool, solubility, surface hydrophobicity, emulsification capacity, and all three foaming properties correlated positively with PA content.

In general, protein content was positively correlated to functional properties (Appendix B). Surface-active functional properties, such as foaming and emulsification, were correlated to protein composition and denaturation enthalpies. Total denaturation enthalpies and surface hydrophobicities were significantly correlated to functional behavior of soy protein ingredients. In general, there was a positive correlation between solubility and functional properties, with the exception of foaming stability and rate of foaming. Correlation coefficients were significantly influenced by both processing and soybean variety.

Functionality of soy protein isolates (SPI) was significantly affected by both the temperature at which the soybean flour was extracted and the method used for preservation. As extraction temperature increased, solubility and emulsification capacity decreased; surface hydrophobicities, emulsification activities and stabilities, dynamic viscosities increased, and foaming properties improved. Interestingly, the drying method did not affect the denaturation enthalpies of the SPIs. Spray-dried SPIs had higher solubilities, surface hydrophobicities, and emulsification stabilities; and lower viscosities, emulsification activities and rates of foaming, than did freeze-dried SPIs. Emulsification and foaming capacities and foaming stabilities were the similar for both methods of drying. There was significant interaction between extraction temperature and preservation method for all functional properties tested but emulsification capacity. The proposed mechanism to explain this significant interaction is that freezing and freeze-drying promoted the formation of large insoluble protein aggregates and, in contrast, spray-drying prevents these aggregates from happening. The extent of formation and size of these aggregates depend on the previous thermal history of SPIs.

Recommendations for Future Research

Several conclusions were drawn from this body of research, yet much must be done in order to achieve the goal of providing ideal soy protein ingredients. The following recommendations would enable researchers to further advance production of soy protein ingredients and use in food, and generate useful data to answer critical questions regarding these ingredients.

The use of HS/LS has been discussed and new products have been characterized. One area of research interest would be to use soy molasses from these soybeans as a high isoflavone-content food additive, with or without chemical or enzymatic modification. These molasses are much lower in indigestible oligosaccharides than those of regular soybeans making their usage more practical.

The use of sulfites as an extraction aide during soy protein ingredient production is another area for potential improvement. A hypothesis that needs testing is that the use of

sulfites increase extractable protein yield by solubilizing proteins from their matrix during extraction steps.

During the Wu protein fractionation procedure, a third protein extract is produced with ~93% of the protein present in this extract being β-conglycinin. It would be interesting to evaluate the use a membrane filtration step at this point to achieve a high purity non-isoelectrically precipitated fraction that would have unique functional properties. If the starting material was HS/LS soy flour, it would also be interesting to evaluate the third extract directly as is. Probably its protein content would be between 50 and 60%, but isoflavone recovery would be dramatically increased because it would contain the isoflavones normally lost to the whey fraction.

To conclusively identify β-conglycinin or some of its subunits as having health benefits, there is a critical need to produce large amounts of these materials for clinical feeding studies. A different approach to achieve this would be to develop an extraction buffer based on this protein's differential solubility in the presence of sulfites, NaCl, and calcium ions and apply it directly to soy flour, two products will be recovered, spent flour with a protein profile enriched in glycinin suitable for feeding those species that need high sulfur-rich amino acid profiles and a very valuable soy protein ingredient for human use (β-conglycinin). In addition, other divalent cations and their combinations should be evaluated to determine process efficiency.

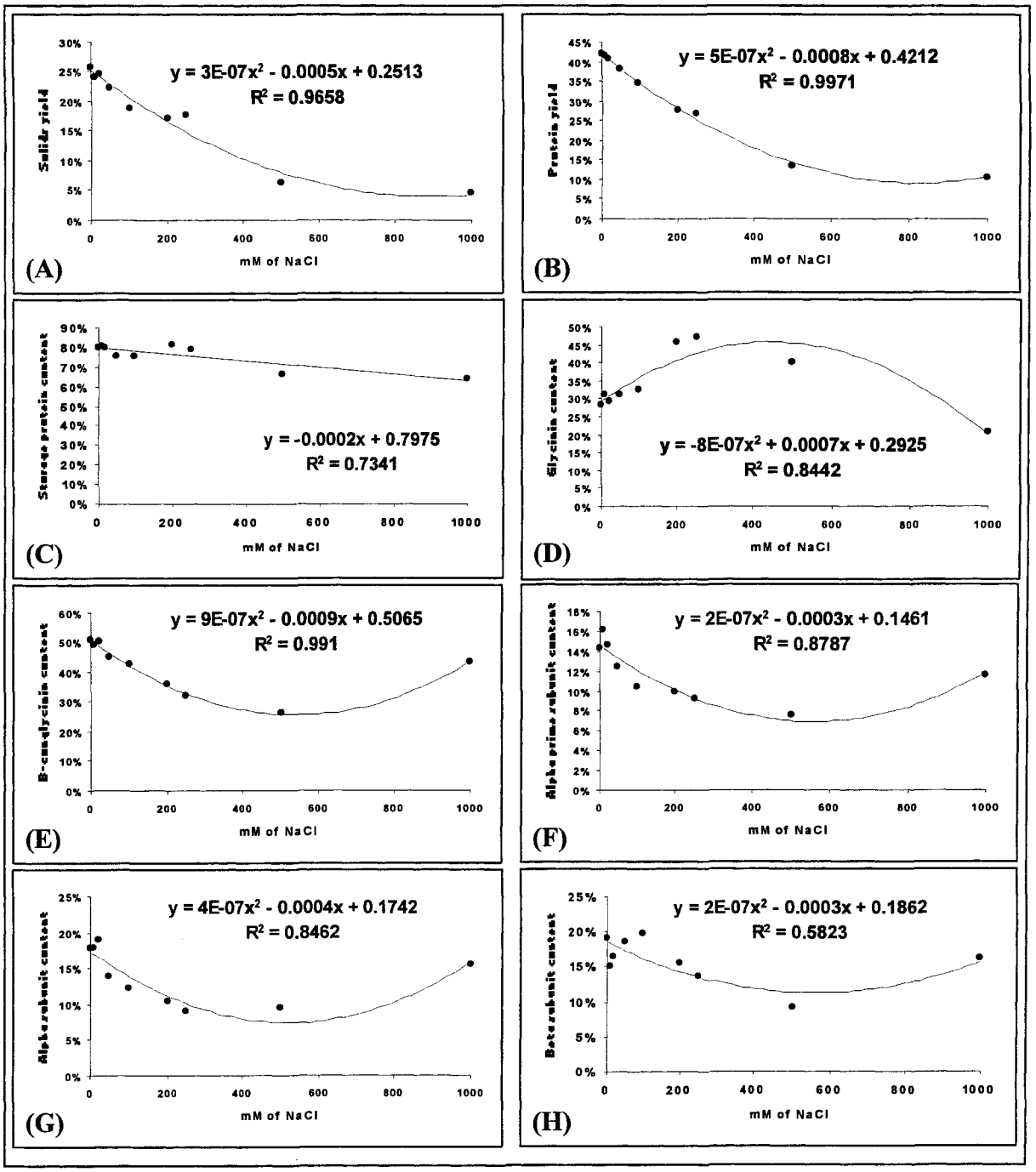
The new procedures developed in this body of work need further evaluation and scaling-up to pilot-plant and industrial scales. Some interesting questions to be answered are: 1. do these procedures work with denatured protein slurries, such as those obtained from low-PDI flakes; 2. could soymilk or full-fat soy flour be used as starting material; 3. could the procedure be improved by fine tuning precipitation pHs; and 4. could the procedure be improved by combining different divalent cations aiming at proportions such that the final product will not only be enriched in the individual soy storage protein but in essential minerals? The chilling step is essential to achieving high-purity fractions. Normally, laboratory procedures use overnight chilling for practical reasons. An interesting research line would be to evaluate the length of chilling time required to obtain acceptable purities.

Because phytate seems to significantly affect soy protein fractionation, it would be interesting to evaluate fractionation behavior of soybean lines that are low in phytate.

Sensory data is scarce for ingredients rich in either glycinin or β-conglycinin and consumer acceptance should not be overlooked. It would be a good idea to evaluate sensory properties because humans will only consume these products if they taste good. Appropriate delivery systems must be developed and evaluated for both sensory and health benefits.

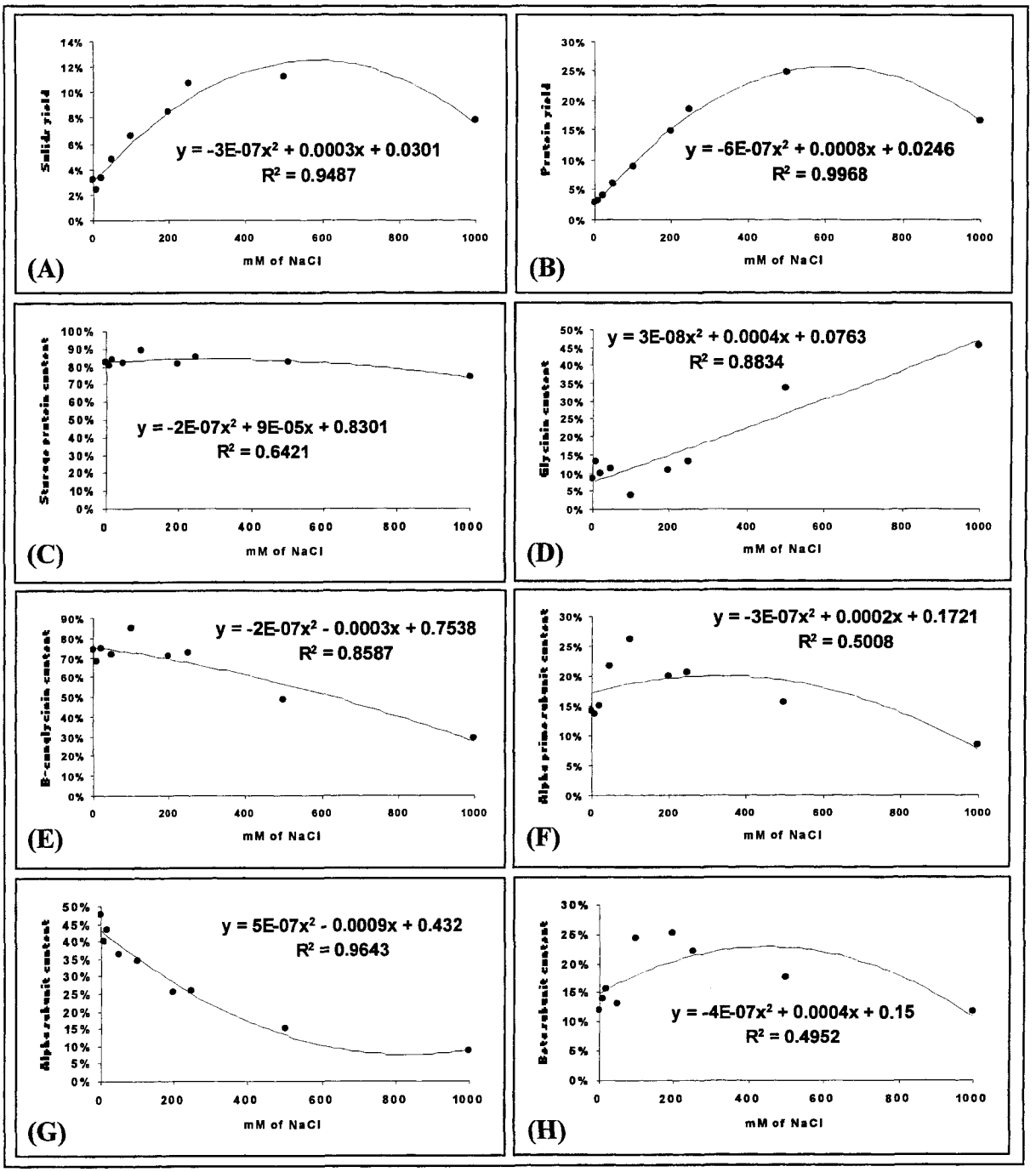
APPENDIX A. REGRESSION EQUATIONS AND PLOTS TO PREDICT THE EFFECTS OF NACL CONCENTRATION ON PROTEIN YIELDS AND COMPOSITIONS DURING SOY PROTEIN FRACTIONATION

Appendix A-1. Salting-in behavior of the intermediate fraction



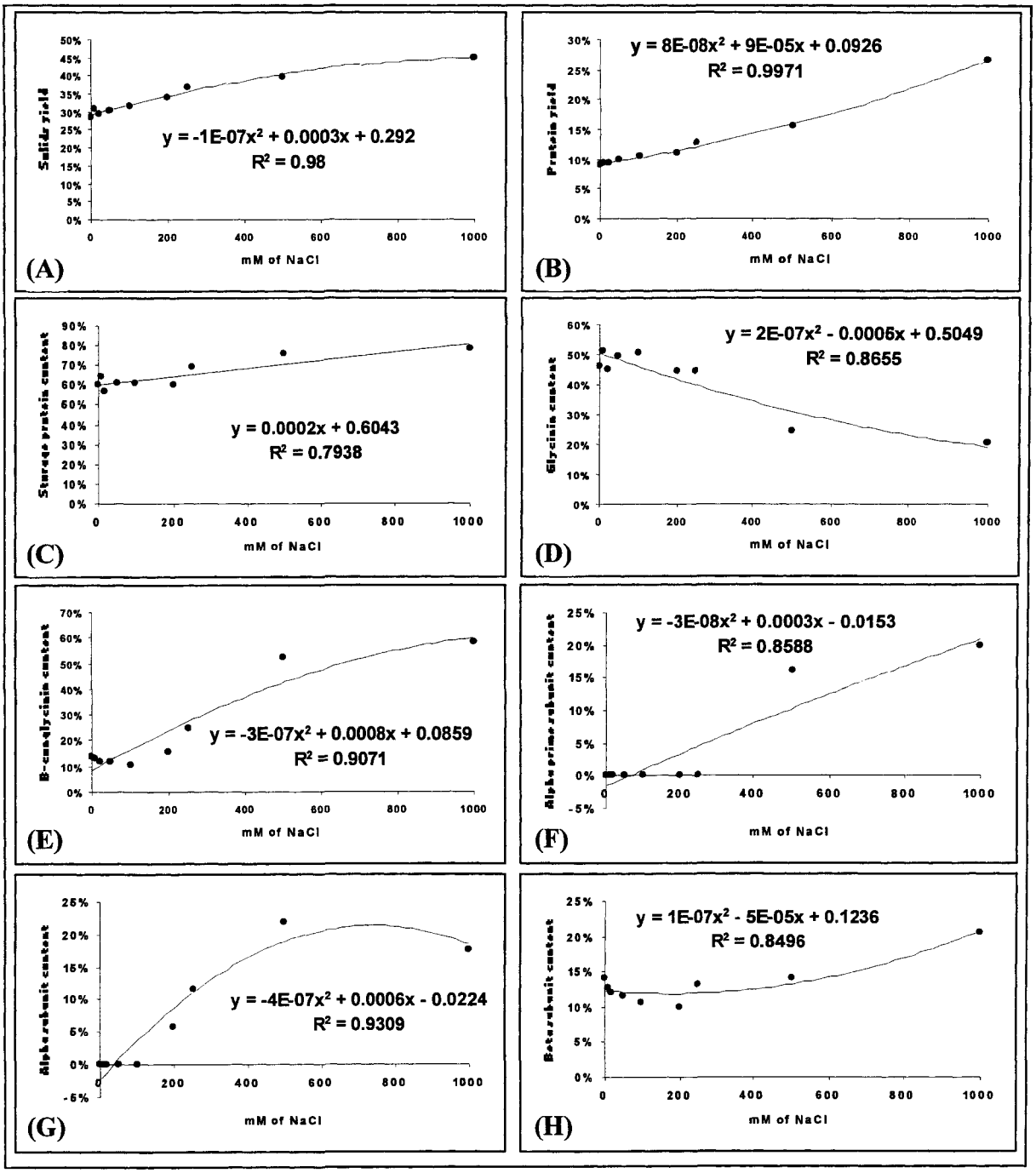
Data from Chapter 6; (A) solids yield of the fraction, (B) protein yield of the fraction, (C) storage protein content in the fraction, (D) glycinin content in the fraction (glycinin relative proportion*storage protein content), (E) β-conglycinin content in the fraction (β-conglycinin relative proportion*storage protein content), (F), (G), and (H), α', α, and β subunit content, respectively in the fraction (β-conglycinin content in the fraction*subunit proportion).

Appendix A-2. Salting-in behavior of the β-conglycinin-rich fraction



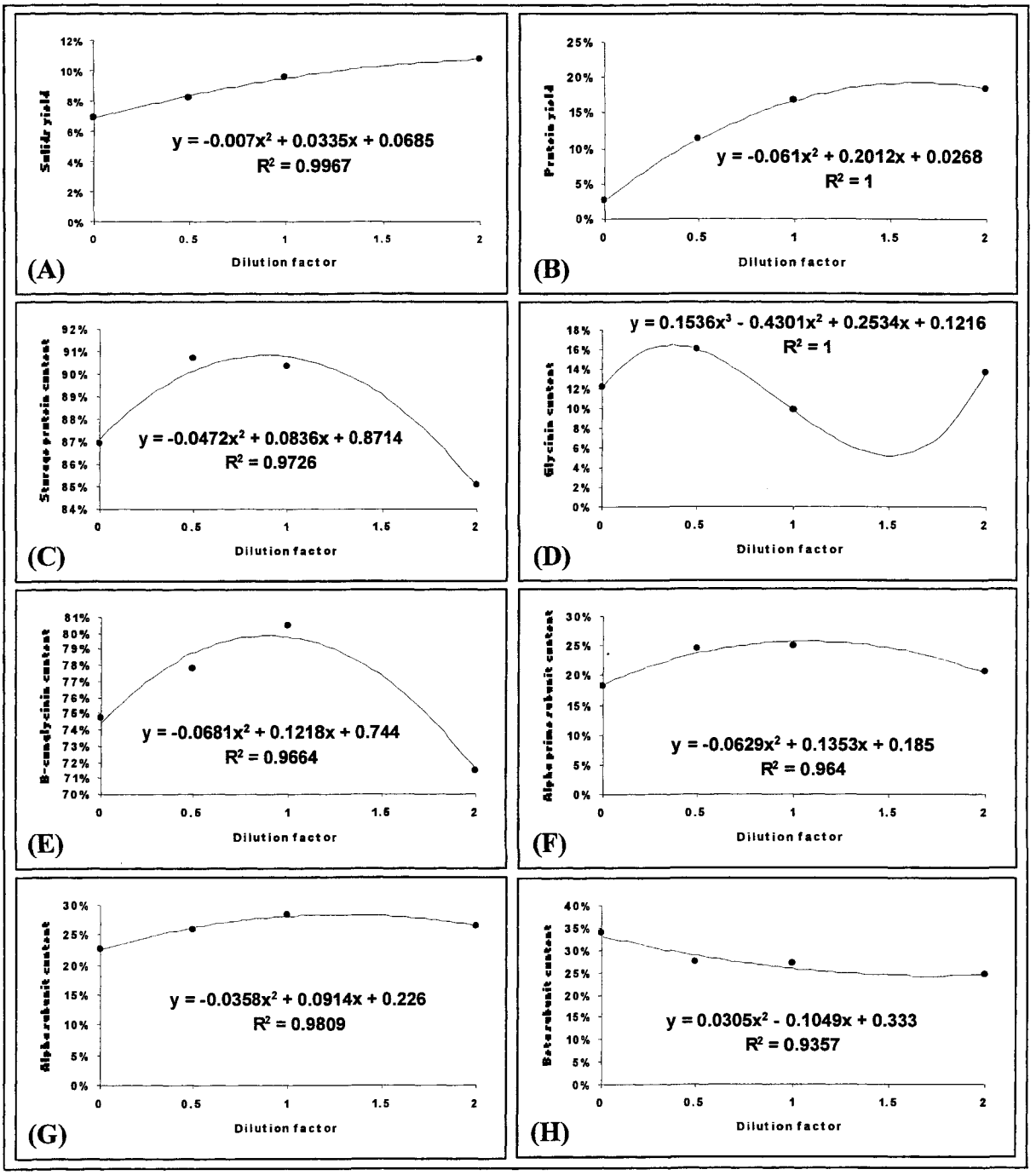
Data from Chapter 6; (A) solids yield of the fraction, (B) protein yield of the fraction, (C) storage protein content in the fraction, (D) glycinin content in the fraction (glycinin relative proportion*storage protein content), (E) β-conglycinin content in the fraction (β-conglycinin relative proportion*storage protein content), (F), (G), and (H), α', α, and β subunit content, respectively in the fraction (β-conglycinin content in the fraction*subunit proportion).

Appendix A-3. Salting-in behavior of the whey fraction



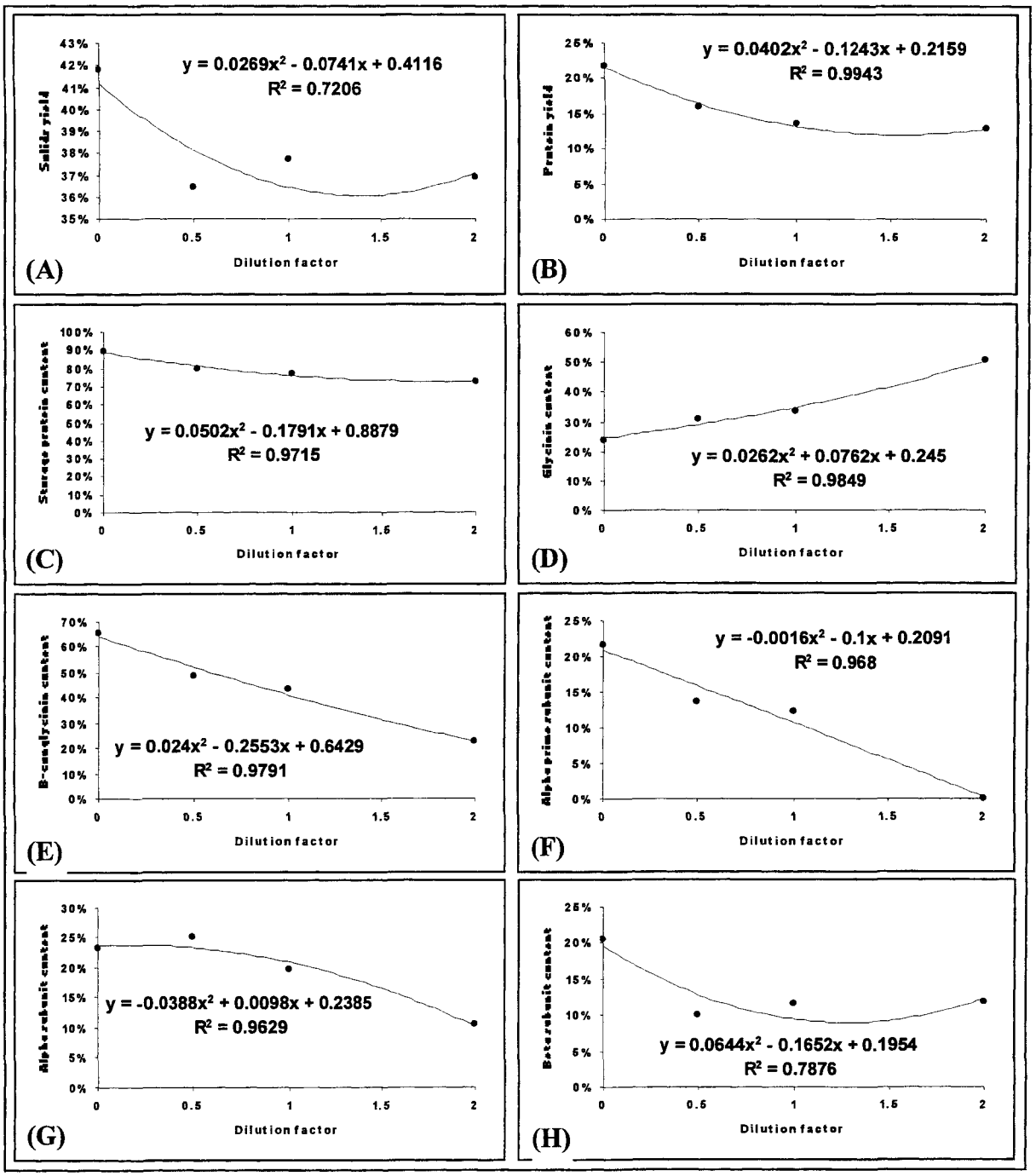
Data from Chapter 6; (A) solids yield of the fraction, (B) protein yield of the fraction, (C) storage protein content in the fraction, (D) glycinin content in the fraction (glycinin relative proportion*storage protein content), (E) β-conglycinin content in the fraction (β-conglycinin relative proportion*storage protein content), (F), (G), and (H), α', α, and β subunit content, respectively in the fraction (β-conglycinin content in the fraction*subunit proportion).

Appendix A-4. Salting-out behavior of the β-conglycinin-rich fraction



Data from Chapter 6; (A) solids yield of the fraction, (B) protein yield of the fraction, (C) storage protein content in the fraction, (D) glycinin content in the fraction (glycinin relative proportion*storage protein content), (E) β-conglycinin content in the fraction (β-conglycinin relative proportion*storage protein content), (F), (G), and (H), α', α, and β subunit content, respectively in the fraction (β-conglycinin content in the fraction*subunit proportion).

Appendix A-5. Salting-out behavior of the whey fraction



Data from Chapter 6; (A) solids yield of the fraction, (B) protein yield of the fraction, (C) storage protein content in the fraction, (D) glycinin content in the fraction (glycinin relative proportion*storage protein content), (E) β-conglycinin content in the fraction (β-conglycinin relative proportion*storage protein content), (F), (G), and (H), α', α, and β subunit content, respectively in the fraction (β-conglycinin content in the fraction*subunit proportion).

APPENDIX B. CORRELATION COEFFICIENTS FOR COMPOSITIONAL AND FUNCTIONAL VARIABLES OF SOY PROTEIN INGREDIENTS

Appendix B-1. Pearson's Correlation Coefficients for Comparisons of 20 Variables-Pooling All Samples[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	V k	V n	PA	Iso
Prot content	0.41**	-0.06	0.27**	0.15	0.24**	-0.05	0.33**	-0.32**	-0.36**	0.08	-0.57**	-0.04
Storage PC	0.48**	0.03	0.45**	0.10	0.31**	0.11	0.29**	-0.37**	0.11	-0.04	0.00	-0.08
BC	0.00	0.02	0.36**	0.74**	0.65**	0.11	-0.57**	0.48**	-0.40	0.41*	-0.03	0.05
GLY	0.00	-0.02	-0.36**	-0.74**	-0.65**	-0.11	0.57**	-0.48**	0.40	-0.41*	0.03	-0.05
B/G ratio	0.12	-0.04	0.17	0.28**	0.30**	-0.01	-0.13	0.06	-0.38	0.40	-0.15	-0.02
7S Enthalpy	0.16	-0.20*	0.35**	0.60**	0.44**	0.04	-0.25**	0.17	-0.28*	-0.03	-0.17	-0.12
11S Enthalpy	0.27**	-0.09	-0.02	-0.61**	-0.42**	0.02	0.75**	-0.59**	-0.19	0.34**	-0.05	0.04
T. Enthalpy	0.48**	-0.26**	0.24**	-0.33**	-0.21*	0.06	0.78**	-0.64**	-0.30*	0.19	-0.17	-0.02
Sol		0.43**	0.61**	0.39**	0.40**	0.18*	0.14	-0.15	-0.80**	0.87**	-0.43**	0.73**
So			0.31**	0.59**	0.65**	0.20*	-0.43**	0.36**	-0.64**	0.65**	-0.11	0.78**
EC				0.45**	0.49**	0.40**	-0.05	0.09	-0.60**	0.54**	0.04	0.46**
EA					0.80**	0.18*	-0.56**	0.48**	-0.73**	0.60**	-0.36**	0.67**
ESI						0.29**	-0.35**	0.32**	-0.41**	0.25*	-0.28*	0.47**
FC							0.04	0.47**	0.01	0.10	0.14	0.33*
K								-0.66**	0.83**	-0.61**	-0.14	-0.49**
Vi									-0.49**	0.40**	-0.09	0.63**
V k										-0.76**	0.79**	-0.82**
V n											-0.91**	0.97**
PA												-0.45**

[a] Prot. content, denotes protein content; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; V k, flow consistency coefficient; V n, flow behavior index; PA, phytic acid content; and Iso, isoflavone content. N = 145, for Prot content, Sol, So, EC, EA, ESI, FC, K, and Vi; N = 133, for 7S Enthalpy, 11S Enthalpy, and T. Enthalpy; N = 97, for Storage PC, BC, GLY, and B/G ratio; N = 72, for V k, and V n; N = 52 for PA; and N = 38, for Iso. * denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-2. Pearson's Correlation Coefficients for Comparisons of 20 Variables-Pooling All Samples, Excluding Ethanol-Washed Soy Protein Concentrates[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	V k	V n	PA	Iso
Prot. content	0.23**	-0.24**	0.22*	-0.06	0.15	-0.08	0.42**	-0.48**	0.14	-0.54**	-0.47**	-0.49**
Storage PC	0.63**	0.04	0.46**	0.13	0.33**	0.10	0.29**	-0.38**	0.26	-0.26	0.04	-0.21
BC	-0.07	-0.03	0.35**	0.80**	0.65**	0.11	-0.58**	0.47**	-0.24	0.22	0.10	-0.12
GLY	0.07	0.03	-0.35**	-0.80**	-0.65**	-0.11	0.58**	-0.47**	0.24	-0.22	-0.10	0.12
B/G ratio	0.10	-0.07	0.16	0.29**	0.29**	-0.01	-0.12	0.04	-0.25	0.24	-0.13	-0.26
7S Enthalpy	0.10	-0.27**	0.34**	0.62**	0.42**	0.04	-0.24**	0.14	0.60**	-0.73**	-0.02	-0.48**
11S Enthalpy	0.33**	-0.11	-0.02	-0.72**	-0.44**	0.02	0.77**	-0.62**	-0.32*	0.39**	0.02	-0.05
T. Enthalpy	0.51**	-0.34**	0.23*	-0.46**	-0.26**	0.06	0.82**	-0.71**	0.20	-0.27	0.01	-0.37*
Sol		0.29**	0.63**	0.15	0.31**	0.19*	0.27**	-0.39**	-0.53**	0.49**	0.50**	0.14
So			0.26**	0.51**	0.61**	0.19*	-0.42**	0.29**	0.32**	0.06	0.55**	0.56**
EC				0.42**	0.47**	0.41**	-0.03	0.05	-0.42**	-0.11	0.56**	0.12
EA					0.80**	0.18*	-0.58**	0.42**	0.70**	-0.41**	0.26	0.20
ESI						0.30**	-0.34**	0.27**	0.58**	-0.36**	0.11	0.06
FC							0.02	0.48**	0.31*	-0.04	0.46**	0.32
K								-0.66**	-0.33**	0.50**	-0.29*	-0.45**
Vi									0.39**	-0.21	0.32*	0.46**
V k										-0.68**	-0.68**	0.02
V n											0.79**	0.15
PA												0.47**

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; V k, flow consistency coefficient; V n, flow behavior index; PA, phytic acid content; and Iso, isoflavone content. N = 139, for Prot content, Sol, So, EC, EA, ESI, FC, K, and Vi; N = 127, for 7S Enthalpy, 11S Enthalpy, and T. Enthalpy; N = 91, for Storage PC, BC, GLY, and B/G ratio; N = 66, for V k, and V n; N = 46 for PA; and N = 32, for Iso. * denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-3. Pearson's Correlation Coefficients for Comparisons of 20 Variables-Pooling All Samples, Excluding Glycinin-rich and β-Conglycinin-rich Fractions[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	V k	V n	PA	Iso
Prot. content	0.30*	0.33**	0.48**	0.50**	0.53**	-0.17	-0.35**	-0.01	-0.36**	0.08	-0.38	0.08
Storage PC	-0.03	0.16	0.13	0.09	0.20	-0.10	0.19	-0.20	0.11	-0.04	-0.12	-0.02
BC	0.36	0.33	0.26	0.29	0.41*	0.22	-0.40	0.56**	-0.40	0.41*	-0.41*	0.39
GLY	-0.36	-0.33	-0.26	-0.29	-0.41*	-0.22	0.40	-0.56**	0.40	-0.41*	0.41*	-0.39
B/G ratio	0.35	0.32	0.25	0.27	0.40	0.23	-0.39	0.56**	-0.38	0.40	-0.40*	0.38
7S Enthalpy	0.32*	-0.21	0.52**	0.12	-0.17	0.06	-0.33**	0.33**	-0.28*	-0.03	-0.19	0.51*
11S Enthalpy	0.20	0.04	0.11	0.09	-0.23	-0.06	-0.16	0.26*	-0.19	0.34**	-0.60**	0.71**
T. Enthalpy	0.35**	-0.11	0.43**	0.14	-0.25	0.01	-0.33*	0.38**	-0.30*	0.19	-0.48*	0.70**
Sol		0.61**	0.76**	0.65**	0.33**	0.14	-0.70**	0.36**	-0.80**	0.87**	-0.92**	0.98**
So			0.31**	0.87**	0.76**	0.21	-0.57**	0.43**	-0.64**	0.65**	-0.97**	0.92**
EC				0.43**	0.16	-0.16	-0.49**	0.07	-0.60**	0.54**	-0.96**	0.85**
EA					0.74**	0.19	-0.71**	0.59**	-0.73**	0.60**	-0.96**	0.93**
ESI						0.25*	-0.41**	0.32**	-0.41**	0.25*	-0.90**	0.67**
FC							0.05	0.47**	0.01	0.10	0.02	0.23
K								-0.56**	0.83**	-0.61**	0.70**	-0.76**
Vi									-0.49**	0.40**	-0.52**	0.75**
V k										-0.76**	0.79**	-0.82**
V n											-0.91**	0.97**
PA												-0.91**

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; V k, flow consistency coefficient; V n, flow behavior index; PA, phytic acid content; and Iso, isoflavone content. N = 72, for Prot content, Sol, So, EC, EA, ESI, FC, K, Vi, V k, and V n; N = 60, for 7S Enthalpy, 11S Enthalpy, and T. Enthalpy; and N = 24, for Storage PC, BC, GLY, B/G ratio, PA, Iso. * denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-4. Pearson's Correlation Coefficients for Comparisons of 20 Variables-Pooling All Samples, Excluding Ethanol-washed Protein Concentrates and Glycinin-rich and β-Conglycinin-rich Fractions[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	V k	V n	PA	Iso
Prot. content	-0.21	0.02	0.29*	0.31*	0.40**	-0.29*	-0.07	-0.35**	0.14	-0.54**	-0.79**	-0.40
Storage PC	-0.11	0.55*	0.29	0.35	0.30	-0.38	0.22	-0.33	0.26	-0.26	-0.36	-0.13
BC	-0.26	-0.15	-0.21	-0.32	0.20	0.29	-0.60**	0.46	-0.24	0.22	-0.16	0.08
GLY	0.26	0.15	0.21	0.32	-0.20	-0.29	0.60**	-0.46	0.24	-0.22	0.16	-0.08
B/G ratio	-0.25	-0.16	-0.22	-0.34	0.19	0.30	-0.61**	0.47*	-0.25	0.24	-0.15	0.07
7S Enthalpy	0.06	-0.81**	0.47**	-0.52**	-0.44**	0.04	-0.32*	0.18	0.60**	-0.73**	0.80**	0.55*
11S Enthalpy	0.04	-0.19	-0.07	-0.28*	-0.41**	-0.05	0.29*	0.19	-0.32*	0.39**	0.39	-0.02
T. Enthalpy	0.08	-0.68**	0.29*	-0.55**	-0.56**	0.01	-0.04	0.23	0.20	-0.27	0.68**	0.29
Sol		-0.26*	0.42**	-0.63**	-0.34**	0.02	0.53**	-0.52**	-0.53**	0.49**	0.58*	0.32
So			-0.49**	0.65**	0.66**	0.14	0.17	-0.04	0.32**	0.06	-0.63**	-0.42
EC				-0.50**	-0.33**	-0.47**	0.22	-0.62**	-0.42**	-0.11	-0.80**	-0.51*
EA					0.70**	0.14	-0.06	0.20	0.70**	-0.41**	-0.54*	-0.17
ESI						0.21	-0.06	0.03	0.58**	-0.36**	-0.92**	-0.54*
FC							-0.18	0.49**	0.31*	-0.04	0.80**	0.52*
K								-0.60**	-0.34**	0.50**	-0.46	-0.38
Vi									0.39**	-0.21	0.70**	0.45
V k										-0.68**	-0.68**	0.02
V n											0.79**	0.15
PA												0.48*

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; V k, flow consistency coefficient; V n, flow behavior index; PA, phytic acid content; and Iso, isoflavone content. N = 66, for Prot content, Sol, So, EC, EA, ESI, FC, K, Vi, V k, and V n; N = 54, for 7S Enthalpy, 11S Enthalpy, and T. Enthalpy; and N = 18, for Storage PC, BC, GLY, B/G ratio, PA, Iso. * denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-5. Pearson's Correlation Coefficients for Comparisons of 20 Variables-Pooling All Soy Protein Isolates[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	V k	V n	PA	Iso
Prot. content	0.73**	-0.51**	0.69**	-0.68**	-0.43**	-0.19	0.32*	-0.59**	-0.57**	0.30*	0.24	-0.75
Storage PC	0.56	-0.32	-0.55	0.68	-0.55	0.83*	-0.42	0.62	0.57	-0.40	0.04	0.39
BC	0.33	-0.67	-0.50	-0.39	-0.56	0.23	-0.32	0.51	0.23	-0.24	0.41	0.67
GLY	-0.33	0.67	0.50	0.39	0.56	-0.23	0.32	-0.51	-0.23	0.24	-0.41	-0.67
B/G ratio	0.33	-0.68	-0.50	-0.39	-0.56	0.23	-0.33	0.51	0.23	-0.24	0.41	0.67
7S Enthalpy	0.16	-0.82**	0.57**	-0.73**	-0.53**	0.03	-0.36*	0.29	0.64**	-0.89**	0.44	0.90*
11S Enthalpy	-0.06	-0.15	0.43**	0.07	-0.21	-0.57**	0.51**	-0.49**	-0.13	-0.04	0.04	-0.84*
T. Enthalpy	0.11	-0.69**	0.65**	-0.54**	-0.51**	-0.24	-0.04	-0.04	0.41**	-0.70**	0.63	-0.13
Sol		-0.28*	0.52**	-0.65**	-0.23	-0.06	0.53**	-0.67**	-0.51**	0.43**	0.29	0.78*
So			-0.59**	0.70**	0.72**	0.23	0.18	0.04	0.32*	0.17	-0.37	-0.93**
EC				-0.70**	-0.51**	-0.44**	0.26	-0.62**	-0.49**	0.01	-0.15	-0.84*
EA					0.67**	0.31*	-0.04	0.41**	0.72**	-0.24	-0.68	0.29
ESI						0.42**	0.01	0.18	0.57**	-0.13	-0.23	-0.91*
FC							-0.21	0.43**	0.41**	-0.27	0.33	0.78*
K								-0.66**	-0.33*	0.56**	0.08	-0.86*
Vi									0.51**	-0.50**	0.39	0.90*
V k										-0.69**	-0.36	0.81*
V n											0.51	-0.77
PA												0.13

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; V k, flow consistency coefficient; V n, flow behavior index; PA, phytic acid content; and Iso, isoflavone content. N = 54, for Prot content, Sol, So, EC, EA, ESI, FC, K, Vi, V k, and V n; N = 42, for 7S Enthalpy, 11S Enthalpy, and T. Enthalpy; and N = 6, for Storage PC, BC, GLY, B/G ratio, PA, Iso. * Denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-6. Pearson's Correlation Coefficients for Comparisons of 18 Variables-Pooling All Glycinin-rich Fractions[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	PA	Iso
Prot. content	-0.40*	-0.50**	-0.08	-0.33	-0.16	-0.02	-0.08	-0.04	0.07	0.78*
Storage PC	-0.49**	-0.32	0.07	-0.46*	0.22	0.18	0.12	0.14	-0.13	0.68
BC	0.11	0.60**	0.61**	0.42*	0.16	0.30	-0.04	0.52**	0.81**	0.06
GLY	-0.11	-0.60**	-0.61**	-0.42*	-0.16	-0.30	0.04	-0.52**	-0.81**	-0.06
B/G ratio	0.13	0.65**	0.59**	0.49**	0.11	0.24	-0.12	0.50**	0.84**	0.03
7S Enthalpy	-0.03	0.13	0.58**	0.05	0.17	0.62**	0.30	0.62**	0.54	0.37
11S Enthalpy	-0.27	-0.15	0.73**	0.08	0.10	0.72**	0.18	0.78**	0.81**	0.74
T. Enthalpy	-0.23	-0.10	0.74**	0.08	0.12	0.74**	0.21	0.79**	0.81**	0.71
Sol		0.25	-0.14	0.34	0.37	-0.25	0.47*	-0.46*	-0.59*	-0.15
So			0.37	0.35	0.13	-0.12	-0.26	0.24	0.10	0.52
EC				0.15	0.34	0.63**	0.09	0.85**	0.74**	0.95**
EA					0.15	-0.23	-0.03	-0.04	-0.17	-0.43
ESI						0.13	0.69**	0.02	-0.24	-0.16
FC							0.35	0.79**	0.92**	0.53
K								-0.09	-0.19	-0.50
Vi									0.94**	0.63
PA										0.36

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; PA, phytic acid content; and Iso, isoflavone content. N = 27, for Prot. content, Storage PC, BC, GLY, B/G ratio, PA,7S Enthalpy, 11S Enthalpy, T. Enthalpy Sol, So, EC, EA, ESI, FC, K, and Vi; N = 12, for PA; and N = 6, for Iso. * Denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-7. Pearson's Correlation Coefficients for Comparisons of 18 Variables-Pooling All β-Conglycinin-rich Fractions[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	PA	Iso
Prot. content	0.41*	-0.18	0.00	0.33	0.21	-0.36	0.28	-0.40*	-0.79**	-0.83*
Storage PC	0.47*	0.14	0.00	0.34	0.07	-0.56**	0.02	-0.59**	-0.44	-0.71
BC	0.04	-0.01	-0.39*	-0.27	-0.34	-0.55**	-0.32	-0.33	-0.09	-0.18
GLY	-0.04	0.01	0.39*	0.27	0.34	0.55**	0.32	0.33	0.09	0.18
B/G ratio	0.15	0.01	-0.01	0.06	0.05	-0.16	0.02	-0.13	-0.50	-0.09
7S Enthalpy	0.68**	0.14	-0.08	0.35	0.11	-0.71**	0.30	-0.74**	-0.33	-0.93**
11S Enthalpy	-0.49**	-0.31	0.21	-0.01	0.15	0.71**	-0.10	0.62**	-0.12	0.74
T. Enthalpy	0.71**	0.10	-0.03	0.43*	0.19	-0.66**	0.35	-0.73**	-0.45	-0.95**
Sol		0.46*	0.54**	0.67**	0.49**	-0.34	0.50**	-0.49**	-0.33	-0.92**
So			0.58**	0.25	0.26	0.16	0.24	0.04	0.67*	0.41
EC				0.63**	0.75**	0.44*	0.27	0.27	0.54	0.92*
EA					0.82**	-0.13	0.64**	-0.41*	-0.74**	-0.74
ESI						0.15	0.53**	-0.10	-0.51	-0.15
FC							-0.18	0.88**	0.35	0.97**
K								-0.53**	-0.41	-0.98**
Vi									0.51	0.96**
PA										0.86*

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; PA, phytic acid content; and Iso, isoflavone content. N = 27, for Prot content, Storage PC, BC, GLY, B/G ratio, PA,7S Enthalpy, 11S Enthalpy, T. Enthalpy Sol, So, EC, EA, ESI, FC, K, and Vi; N = 12, for PA; and N = 6, for Iso. * Denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-8. Pearson's Correlation Coefficients for Comparisons of 20 Variables-Pooling All Samples Prepared from IA 2020 Soyflour[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	V k	V n	PA	Iso
Prot. content	0.43**	-0.04	0.29**	0.07	0.23*	0.03	0.41**	-0.33**	-0.39**	0.04	-0.51**	-0.10
Storage PC	0.55**	0.04	0.54**	0.16	0.41**	0.22	0.35**	-0.36**	-0.24	0.16	0.01	-0.06
BC	0.01	0.05	0.39**	0.78**	0.68**	0.10	-0.63**	0.45**	-0.12	0.05	-0.05	0.00
GLY	-0.01	-0.05	-0.39**	-0.78**	-0.68**	-0.10	0.63**	-0.45**	0.12	-0.05	0.05	0.00
B/G ratio	0.07	-0.11	0.24*	0.51**	0.43**	0.03	-0.36**	0.22	-0.13	0.06	-0.12	-0.04
7S Enthalpy	0.16	-0.20*	0.35**	0.60**	0.40**	0.02	-0.26**	0.13	-0.23	-0.18	-0.16	-0.19
11S Enthalpy	0.27**	-0.10	-0.03	-0.63**	-0.40**	0.02	0.78**	-0.60**	0.00	0.11	0.03	0.00
T. Enthalpy	0.48**	-0.29**	0.25*	-0.35**	-0.20*	0.04	0.82**	-0.68**	-0.16	-0.07	-0.05	-0.12
Sol		0.38**	0.63**	0.33**	0.39**	0.29**	0.24**	-0.22*	-0.80**	0.81**	-0.36	0.58**
So			0.31**	0.61**	0.70**	0.37**	-0.40**	0.38**	-0.60**	0.52**	-0.15	0.75**
EC				0.42**	0.46**	0.44**	-0.12	0.10	-0.62**	0.38**	0.02	0.39
EA					0.78**	0.26**	-0.57**	0.46**	-0.71**	0.43**	-0.29	0.54**
ESI						0.35**	-0.35**	0.30**	-0.35**	0.10	-0.28	0.31
FC							-0.10	0.51**	-0.41**	0.31*	0.10	0.53**
K								-0.70**	0.64**	-0.24	-0.23	-0.26
Vi									-0.50**	0.24	-0.09	0.60**
V k										-0.76**	0.88**	-0.93**
V n											-0.87**	0.97**
PA												-0.24

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; V k, flow consistency coefficient; V n, flow behavior index; PA, phytic acid content; and Iso, isoflavone content. N = 119, for Prot content, Sol, So, EC, EA, ESI, FC, K, and Vi; N = 107, for 7S Enthalpy, 11S Enthalpy, and T. Enthalpy; N = 71, for Storage PC, BC, GLY, and B/G ratio; N = 60, for V k, and V n; and N = 26 for PA, and Iso. * denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

Appendix B-9. Pearson's Correlation Coefficients for Comparisons of 20 Variables-Pooling All Samples Prepared from High-sucrose/Low-stachyose Soy Flour[a]

Variable	Sol	So	EC	EA	ESI	FC	K	Vi	V k	V n	PA	Iso
Prot. content	0.35	-0.23	0.32	0.28	0.29	-0.15	0.43*	-0.39	-0.20	0.14	-0.64**	0.08
Storage PC	0.34	-0.03	0.17	-0.03	0.05	-0.28	0.19	-0.43*	0.22	-0.21	-0.01	-0.22
BC	-0.02	0.00	0.36	0.67**	0.67**	0.23	-0.56**	0.56**	-0.59*	0.64*	-0.02	0.67*
GLY	0.02	0.00	-0.36	-0.67**	-0.67**	-0.23	0.56**	-0.56**	0.59*	-0.64*	0.02	-0.67*
B/G ratio	0.18	-0.08	0.25	0.36	0.37	-0.15	-0.18	0.03	-0.57*	0.63*	-0.15	0.66*
7S Enthalpy	0.15	-0.19	0.40*	0.63**	0.66**	0.22	-0.28	0.30	-0.38	0.48	-0.20	0.50
11S Enthalpy	0.33	-0.04	-0.03	-0.54**	-0.53**	-0.04	0.81**	-0.58**	-0.74**	0.89**	-0.12	0.90**
T. Enthalpy	0.51**	-0.17	0.22	-0.29	-0.26	0.08	0.85**	-0.55**	-0.67*	0.82**	-0.28	0.84**
Sol		0.59**	0.62**	0.55**	0.47*	-0.16	0.06	0.06	-0.88**	0.99**	-0.53**	0.98**
So			0.37	0.51**	0.39*	-0.47*	-0.51**	0.24	-0.85**	0.94**	-0.08	0.92**
EC				0.64**	0.73**	0.19	0.02	0.11	-0.79**	0.84**	0.02	0.81**
EA					0.93**	-0.04	-0.58**	0.55**	-0.84**	0.93**	-0.40*	0.91**
ESI						0.07	-0.49*	0.43*	-0.80**	0.86**	-0.21	0.84**
FC							0.19	0.41*	0.43	-0.35	0.24	-0.29
K								-0.69**	0.85**	-0.99**	-0.04	-1.00**
Vi									-0.54	0.68*	-0.13	0.72**
V k										-0.91**	0.86**	-0.87**
V n											-0.96**	0.99**
PA												-0.95**

[a] Prot. content, denotes protein content ; Storage PC, storage protein content ; BC, β-conglycinin content; GLY, glycinin content; B/G ratio, β-conglycinin to glycinin ratio; 7S Enthalpy, denaturation enthalpy of the 7S proteins; 11S Enthalpy, denaturation enthalpy of the 11S proteins; T. Enthalpy, total denaturation enthalpy of a given fraction; Sol, solubility; So, surface hydrophobicity; EC, emulsification capacity; EA, emulsification activity; ESI, emulsification stability index; FC, foaming capacity; K, foaming stability; Vi, rate of foaming; V k, flow consistency coefficient; V n, flow behavior index; PA, phytic acid content; and Iso, isoflavone content. N = 26, for Prot content, Storage PC, BC, GLY, B/G ratio, 7S Enthalpy, 11S Enthalpy, T. Enthalpy, Sol, So, EC, EA, ESI, FC, K, Vi, and PA; N = 12, for V k, and V n, and Iso. * denotes correlation coefficients significant at $p<0.05$ and ** significances at $p<0.01$.

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