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Soy protein: A fractionation and sensory study

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Soy protein: A fractionation and sensory study

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ABSTRACT

The bitter threshold concentrations for isoflavones for soy flake extract, soy protein isolate extract, and soy germ extract in water were 0.023, 0.023, and 0.039 μmol/ml. The saponin concentration of the soy flake extract, soy protein isolate extract, and soy germ extract at the threshold for milk were 0.115, 0.561, and 0.225 μmol/ml, respectively. The bitterness thresholds for isoflavones in the soy flake extract, soy protein isolate extract, and soy germ extract in milk were 0.338, 1.09, and 0.776 μmol/ml respectively, and the saponin concentrations at threshold were 0.115, 0.561, and 0.225 μmol/ml.

Control, high oleic, and high cysteine soy flakes were analyzed for storage protein, functionality, isoflavone, and saponin content. The starting materials, when analyzed for original concentration of glycinin and β-conglycinin by urea-SDS-PAGE, showed significant differences in storage protein compositions between the high oleic flakes and the other two flake varieties. The oleic flakes had 10% more glycinin, and 10% less β-conglycinin than the control and cysteine flakes. The high oleic flakes yielded > 70% more total protein in the glycinin fraction than the other two varieties.

The high cysteine flakes produced significantly higher purities of glycinin in the glycinin-rich fraction at 91% compared to the oleic at 78% and control flakes at 87% purity

The purity of the β-conglycinin in the β-conglycinin-rich fraction from the high oleic soybeans was 19%, with the major component of this fraction being glycinin. A purity of 74% for the ß-conglycinin-rich fraction from the control flakes, and a purity of 67% for high cysteine flakes were observed. The control glycinin, intermediate and β-conglycinin fractions all had higher solubilities than these fractions from the high cysteine and high oleic flakes

The glycinin-rich fraction emulsification capacity for all flake varieties was significantly lower than the emulsification capacity of the β-conglycinin-rich fraction

The high oleic flake glycinin fraction showed better emulsifying capacity compared to the glycinin fractions of the other two varieties.

In this study, in general, the control flakes contained a significantly higher concentration of isoflavones at 6.44 μmol/g than the high cysteine flakes at 5.23 μmol/g and

the high oleic flakes at 6.01μmol/g. The mass yield of isoflavones for the control, high oleic, and high cysteine beans were 374, 398, and 389 μmoles, respectively.

In general, the saponin concentration in the control, cysteine, and oleic flakes was 5.48, 5.46, and 3.74 umol/g, respectively. The mass yield of saponins for the control, high oleic, and high cysteine beans were 362, 209, and 319 μmoles, respectively.

INTRODUCTION

Soybeans

The soybean as a crop was grown in the U.S to only a limited extent prior to 1920. Processing of domestic soybeans for oil began in 1915 and this stimulated the production of soybeans so that by1920, a three-million-bushel crop was produced. Now, approximately 40 % of the world's soybean supply is produced in the United States (Liu et al., 1997a). The soy used as a food ingredient is typically in the form of flour, protein concentrate, protein isolate, or oil. Soy ingredients are highly sought after because of their functionality, nutritional properties, low cost, and abundance (Zayas et al., 1997). Further growth in the soy market is anticipated because of the 1999 Food and Drug Administration ruling that approved the use of the label claim, that soybeans can lower cholesterol. With this ruling FDA allowed food manufacturers to place a health claim (healthy heart) on the package labels of food products containing more than 6.25 g of soy protein per serving.

Health Benefits

There has been much attention given to the health effects of soy consumption. Soybean saponins and isoflavones have been considered as major active components contributing to the cholesterol-lowering effect of soy products. Soy isoflavones have been associated with lowering cancer risk, alleviating menopausal symptoms, and improving post-menopausal bone health. Soybean saponins were reported to inhibit tumor development, and have shown antihepatotoxic activity.

Importance of Study

Soybeans constitute an enormous source of proteins for human consumption. However, soybean proteins must be presented to the consumer in forms that are attractive and possess the flavor, texture, and quality desired by the consumer. Soy proteins ideally should display many versatile functional and nutritive properties that are suitable for a wide range of applications.

In this thesis, I will address the issues of flavor, and some functional qualities desired from soy producers and consumers.

LITERATURE REVIEW

The Soybean

The soybean is one of the oldest crops in the Asian world and has been used in various forms as a very important source of dietary protein. The amount of protein produced by soybeans per unit area of land is higher than that of any other crop. For this reason, soybeans can be expected to be a weapon against world hunger. The soybean as a crop was grown in the United States to only a limited extent prior to 1920. Processing of domestic soybeans for oil began in 1915 and this stimulated the production of soybeans so that by1920, a three-million-bushel crop was produced. Now, approximately 40 % of the world's soybean supply is produced in the United States (Liu et al., 1997a). The soy used as a food ingredient is typically in the form of defatted flour, soy protein concentrate, soy protein isolate, or refined oil. The soy ingredients are highly sought after because of their functionality, nutritional properties, low cost, and abundance (Zayas et al., 1997). Further growth in the soy market is anticipated because of the 1999 Food and Drug Administration ruling that approved the use of the label claim that soybeans can lower cholesterol.

Soybeans can differ in size, shape, and color depending on the variety. They range from small, round beans to large, oblong, flattened seeds with yellow, brown, green, or black seed coats. The common varieties grown in the United States are nearly spherical in shape and yellow seed coat in color. Soybeans used commercially consist of approximately, 90% cotyledon, 8% hull, and 2% hypocotyl and plumule. The hull, the outermost part of the soybean, is formed by an outer layer of palisade cells, a layer of hourglass cells, compressed parenchyma cells, aleurone cells, and layers of endosperm cells. Inside this layers of the soybean is the cotyledon. The surface of the cotyledon is covered with an epidermis, and the interior is filed with numerous elongated palisade-like cells filled with protein and oil (Wolf and Cowan, 1975). The bulk of the proteins are stored in the protein bodies. Isolated protein bodies may contain as high as 98% protein (Tombs et al., 1967). The oil is located in structures called spherosomes which are interspersed between the protein bodies. Oil and protein make up about 60% of the bean, the protein making up 40 %, and the oil, 20 %. The remaining part of the soybean consists of carbohydrates including polysaccharides (15%),

stachyose (3.8%), raffinose (1.1%), and sucrose (5.0%) (Kawamura et al., 1967). The polysaccharides include cellulose, pectins, and hemicelluloses. Other minor constituents such as phosphatides, sterols, and ash are also present in the soybean. Oil and protein contents depend on variety, soil fertility, and weather conditions (Smith and Circle, 1972).

Soy storage proteins are considered to be globulins. Globulins are insoluble in water in the region of their isoelectric points but will dissolve in the isoelectric state when salts are added. If the pH is above or below the isoelectric point, a globulin will dissolve in aqueous solutions in the absence of salts (Wolf and Cowan, 1975). Approximately 90% of the proteins in soybeans, mostly globulins, exist as dehydrated storage proteins. The remaining proteins are intracellular enzymes (lipoxygenase, urease, amylase), hemagglutinins, protein inhibitors and membrane lipoproteins (Kinsella, 1979).

The predominant storage proteins are the 2 major globulin species, β-conglycinin (7 S) and glycinin (11 S) (Gaylor and Sykes, 1981). β-Conglycinin is a trimer with a molecular weight of about 180,000. β-Conglycinin includes a heterogeneous class of glycoproteins composed of varying combinations of three possible subunits termed α', with a molecular weight of 57,000-58,000, α, with a molecular weight of 57,000, and β, with a molecular weight of 42,000 Dalton (Thanh and Shibasaki, 1976a, 1977). The α and α' subunits are composed of extension regions, and core regions. The β subunit consists of only the core region. The core regions of three subunits exhibit high absolute homologies with one another. The α and α' exhibit a homology of 90.4%. The α and β represent a homology of 76.2% and the α' and β of 75.5% . The extension regions of the α and α' subunits exhibit lower absolute homologies of 57.3%, and are rich in acidic amino acids (Maruyama et al., 1998). All subunits are N-glycosylated, meaning a carbohydrate is linked to the amide nitrogen atom or the side chain of a asparagine residue (Utsumi et al., 1997). Circular dicroism (CD) spectrascopy show that the core regions of the α and α' subunits have secondary structures similar to that of the β subunit, and the extension regions of the α and α' subunits have similar secondary structures to each other (Maruyama et al., 1998). The subunits are highly negatively charged, compactly folded glycopeptides with significant hydrophobic regions (Thanh and Shibasaki, 1978).

Glycinin (11 S) is a large oligomeric protein of approximately 360,000 Daltons. The glycinin consists of six nonidentical subunits (Plietz et al., 1984). Each subunit is composed of an acidic and a basic polypeptide chain (Croy et al., 1980, Turner et al., 1981, Weber et al., 1981). The acidic polypeptide component is linked to the basic component by a single disulfide bond. Initial translation products of glycinin subunits are single polypeptides of about 60,000 Daltons that undergo cotranslational and post-translational modification. The precursors have a short signal sequence. This is followed by the acidic component, the basic component and a short trailer peptide. Five subunit types have been purified and characterized by amino acid sequence analysis. All of the subunits are clearly synthesized by a family of homologous genes, but can be separated in two groups based on sequence homologies (Nielsen et al., 1985). Group I subunits ($A_{1a}B_2$, $A_{1b}B_{1b}$, A_2B_{1a}) are similar in size with molecular weights of 58,000. This group is relatively rich in methionine and has about 90% sequence homology among members. The group II subunits (A_3B_4, $A_5A_4B_3$) exhibit a similar level of homology among themselves, although they contain less methionine and their molecular weights are around 62,000-69,000. Sequence homology between a member of one group and a member of the other is only 60-70% (Nielsen, 1985). Wright et al. (1987, 1988) aligned the amino acid sequences to maximize the homology among the glycinin type proteins from legume and nonlegume seeds. They suggested five genetically variable regions. Variable regions are rich in hydrophilic amino acids, indication that variable regions are present on the molecular surface. This was confirmed by X-ray crystallography (Adachi et al., 2001).

Soy Glycinin and β-Conglycinin Fractionation

In the last fifty years, several soy protein fractionation processes have been published, many with the intention to fractionate a high yield of relatively pure glycinin and β-conglycinin. Among the known methods developed for fractionating soy protein, one of the first attempts was a method using low temperatures, or cryoprecipitation (Wolf, 1956). Wolf reported the recovery of a fairly pure glycinin fraction. For purity, the glycinin fraction was evaluated by ultracentrifugal analysis, which does not differentiate among different proteins of comparative mass. Koshiyama, (1965) reported a procedure for glycinin and β-

conglycinin fractionation using cryoprecipitation, salt precipitation, and isoelectric precipitation. In this work, the glycinin fraction was first cryoprecipitated, and then 25 mM $CaCl_2$ was added to remove the residual cold-insoluble proteins, or glycinin. The β-conglycinin fraction was precipitated by adjusting the pH of the supernatant to 4.5. The purities of the proteins were measured after gel filtration by ultracentrifugal analysis, which does not differentiate among different proteins of comparable mass. Later, Thanh et al. (1975,1976b) developed a straight-forward process for glycinin and β-conglycinin separation based on solubilities of the two proteins at pH 6.1-6.6. Tris (THAM) buffer at pH 8.0 containing 10 mM 2-mercaptoethanol (ME) was used to extract soy proteins. Glycinin was separated by adjusting the pH to 6.4, and centrifuging at 2-5°C. β-Conglycinin was precipitated at pH 4.8 and purified by redissolving the precipitate in the 30 mM M Tris buffer and later adjusting the pH to 6.2. The β-conglycinin fraction was kept at 3-5 °C overnight. This fraction was obtained after centrifuging to remove undissolved polymerized forms. The glycinin fraction was 79% glycinin, 6% β-conglycinin, and 15% other components. The β-conglycinin fraction was less pure than the glycinin fraction. It contained 52% β-conglycinin, 3% glycinin, and 45% other components. Purity was measured by singly radial immunodiffusion (Iwabuchi et al., 1987). Thanh and Shibasaki (1976b), further purified the glycinin and β-conglycinin fractions obtained by gel filtration on Sepharose 6B. Both O'Keefe et al. (1991) and Nagano et al. (1992b) did studies modifying the Thanh et al. (1976b) method. O'Keefe et al. (1991) modified the method of Thanh et al. (1976b) to be able to improve the purity of crude β-conglycinin fraction without the affinity chromatography step necessary in the Thanh et al. (1976b) method. O'Keefe et al. (1991) used three isoelectric precipitations instead of two at pH 6.4, 5.3, and 4.8. The purity of the β-conglycinin fraction did increase, however at the expense of yield. Nagano et al. (1992) modified the method of Thanh et al. (1976b). In the Nagano et al method, soy proteins were extracted by using water at pH 7.5 instead of Tris buffer. Sodium bisulfite was used as a reductant, a substance that chemically reduces other substances by donating an electron or electrons. Three protein fractions were precipitated at pH 6.4, 5.0, and 4.8 instead of two at pH 6.4 and 4.8. The fraction precipitated at pH 6.4 was considered the 11S fraction. The fraction precipitated at 4.8 was considered the 7S fraction, and the fraction precipitated at 5.0

was called the insoluble fraction. The Nagano et al. (1992) fractionation process produced purities of crude glycinin > 90 % in the crude glycinin fraction and β-conglycinin of > 90% in the crude β-conglycinin fraction when measured by densitometer scans of gels from sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The Nagano et al. (1992) laboratory fractionation process was successfully scaled up to pilot plant scale by Wu et al. (1999). Kilogram quantities of the individual soy storage protein fraction were produced. Three protein fractions, glycinin, an intermediate protein mixture, and β-conglycinin, were produced by using water as an extraction buffer and sodium bisulfite as a reducing agent and adjusting the pH to 6.4, 5.0, and 4.8 for precipitating the three protein fractions. The protein contents by micro-Kjeldahl of glycinin and β-conglycinin fractions were 93 and 98% , respectively. The glycinin and β-conglycinin purities were 90 and 73% of the protein which were comparable to those of the laboratory-scale process. Purity was done with urea-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel density image analysis. Wu et al. (2000) did further work on the pilot scale using a simplified method that resulted in only two fractions produced, glycinin and β-conglycinin. Glycinin was produced by precipitation at pH 6.0 and β-conglycinin was produced by precipitation at pH 7.0 and by using an ultrafiltration membrane system. The yield of glycinin fraction was 9.7% (db), and it had a protein content and purity similar to those obtained with the modified Nagano et al. (1992) method. The yield of the β-conglycinin fraction was 19.6% (db), which was twice that of the modified Nagano, et al. (1992) method. The protein content of β-conglycinin was 91.6% (db), and the purity was 62.6% of the protein content, which was 9% lower in purity than the modified Nagano et al. (1992) method. Purity was analyzed by urea-SDS-PAGE and gel density image analysis.

Saito et al. (2001) reported a novel method using phytase for separating glycinin and β-conglycinin. The glycinin fraction was obtained by adjusting the pH to 6.0, adding phytase, and centrifugation. The supernatant was adjusted to pH 5.0 and centrifuged to give the β-conglycinin fraction. From 100 g of defatted soy flakes, 22 % of total protein was in the β-conglycinin fraction and 36 % of total protein was in the glycinin fraction. The purity

of each protein obtained by this method was reported to be more than 80% by the densitometric analysis after SDS-PAGE.

Soy Protein Functionality

Soybeans constitute an enormous source of proteins for human consumption. However, soybean proteins must be presented to the consumer in forms that are attractive and possess the flavor, texture, and quality desired by the consumer. Soy proteins ideally should display many versatile properties that are suitable for a wide range of applications. These properties that determine their uses in foods are called protein functional properties. Functional properties are those physicochemical properties of food proteins, such as soy protein, that determine their behavior in foods during processing, storage, preparation, and consumption. These properties resulting from the manner in which proteins interact with other components, affect processing applications, food quality, and ultimately, food acceptance.

The use of soy proteins, because of their unique and expansive functional properties, is very extensive in foods. Soy flours are used in a wide range of foods, particularly in bakery products and cereals. Defatted soy flours are > 50% protein and are processed by dehulling, flaking, solvent extraction of oil, flash desolventizing, and milling of the flakes obtained. Enzyme-active soy flour is added to white bread and buns for bleaching functions (Rackis et al., 1977). Texturized soy flours are used as extenders in chopped meats.

Soy protein concentrates are used in food and provide improved flavor, color, and higher protein options. Soy protein concentrate is 65-70% protein and is prepared from dehulled and defatted soybeans processed like soy flours, but then are denatured by acid leaching, extraction with aqueous alcohol, or by moist heat. Soy protein concentrates are used in sausage, comminuted products, textured meat products, loaves, and meatballs due to their capacity to impart and retain structure and bind moisture.

Soy protein isolates are used in comminuted meats and dairy foods in which emulsifying, thickening, and gelling properties are of prime importance (Kinsella, 1985). Soy protein isolates are > 90% protein and are prepared from dehulled and defatted soybeans. The protein is extracted by weak alkali (pH7.5-10), followed by centrifugation to removed

insoluble fiber residue. The resulting extract is acidified whereas most of the protein precipitates as curd, separating curd by centrifugation from the soluble oligosaccharides. This is followed by multiple washing of the protein curd and spray-drying. Soy protein isolates are added to meatballs, frankfurters, and luncheon meats to improve texture and final quality.

Soy products are increasingly used in meats, meat-based products, and processed meats (Waggle et al., 1981, Nowacki et al., 1979). Soluble soy proteins are being used in hams, pork, and poultry rolls to reduce cookout and improve juiciness. There is an increasing potential for soy proteins in reformed restructured meats due to their binding and gelation functions. Thus, soy proteins perform several useful functions in meat products including water holding, emulsifying, gelling, structure forming, and adhesion.

It is important to discuss functional difference in traditional soy protein foods, such as tofu. Different ratios of glycinin to β-conglycinin and their effect on functional properties of tofu have been studied. Tofu is a soy protein gel, so the amount and possibly the ratio of soy storage proteins is critical for tofu quality (Poysa et al., 2002). Several studies found that glycinin and β-conglycinin content of the starting material had some relationships with tofu texture. The glycinin content and the glycinin to β-conglycinin protein ratio were reported to correlate positively with tofu gel hardness (Saio et al., 1969, 1979, Kang et al., 1991). In contrast, Utsumi and Kinsella, (1985) reported that the β-conglycinin protein formed harder gels than the glycinin protein. Murphy et al., (1997) reported a negative relationship between tofu hardness and the glycinin to β-conglycinin protein ratio of soybeans. Skurray et al., (1980) and Taira et al., (1990) found little correlation between the glycinin to β-conglycinin protein ratio and tofu quality. The ambiguity of the studies may show that processing differences may have a larger impact on tofu hardness than the glycinin to β-conglycinin ratio. More functionality studies on tofu and other soy products are necessary to enhance future applications of soy products.

Modified Soybeans

There are key constraints that are associated with soybean end utilization as food, including beany flavor of soyfood products, oxidative instability of soy oil, nutrition-related

constraints, lack of certain functional properties, flatus, and low consumer acceptance as food. For years, a major approach that has been used to address these problems is through plant breeding that has been coupled with new techniques like mutagenesis and genetic engineering (Liu et al., 1997). This review will discuss the plant breeding techniques.

A most important factor limiting the use of soybeans as food is the characteristic off-flavor associated with soybean products, known as beany. There are two major interrelated causes for development of such flavors. One is the high proportion of unsaturated fatty acids, in the soybean oil fraction, and the other is the abundant presence of lipoxygenases in soybeans. Lipoxygenase catalyzes the oxidation of polyunsaturated fatty acids of soy lipids. This oxidation process leads to formation of various types of volatile carbonyl compounds, including aldehydes, ketones, and alcohols. Many of these compounds have an objectionable odor or undesirable flavor, which is responsible for the beany flavor associated with various soy products (Robinson et al., 1995). Genetic elimination of the lipoxygenases from the soybean seeds has been sought to combat this problem. In general, soybeans contain three lipoxygenase isozymes, designated L-1, L-2, and L-3. Through different breeding techniques, soybean mutants lacking L-1 or L-2 of the individual lipoxygenase isozymes (Kitamura et al., 1984) and even soybeans lacking all the three isozymes (Hajika et al., 1991) have been developed. Breeding soybeans lacking lipoxigenase has been shown effective in reducing this enzyme's activities in soybean seeds and in controlling off-flavor development in the finished products. Kobayashi et al., (1995) reported that almost all the mass-spec peaks corresponding to the volatile compounds obtained from soymilk of mutants lacking L-2 and L-3 or lacking all three were markedly lower than those of a normal variety. Torres and Reitmeier, (2001) reported that soymilks made from lipoxygenase-free soybeans had a lower intensity in the attributes "raw as hexanal" aroma and "raw as hexanal" flavor than soymilks made from normal soybean lines.

Oxidative stability refers to resistance of an oil to oxidative reaction during storage and processing. The outcome of oxidation leads to off-flavor development. Therefore, an oil with high stability is desirable. The degree of unsaturation has a great impact on oil stability. The more double bonds in the fatty acid chains, the less stable the oil. Regular soy oil contains about 11% palmitic acid, 4% stearic, 23% oleic, 54% linoleic, and 8% linolenic

(Smith & Circle, 1972). Because of a large proportion of polyunsaturated fatty acids, particularly linolenic acid, soy oil often has a problem of lacking flavor stability during processing, storage, and application. Plant breeding has become an alternative approach to increase stability of soy oil. One of the initial strategies of genetic modification was to reduce the level of linolenic acid (Howell et al., 1972). A level as low as 2.2% and 2.4% of linolenic acid has been reached through conventional breeding (Fehr et al., 1992). Soybean with reduced linolenic acid was found to result in significant improvement in oxidative stability of its oil (Mounts et al., 1994).

A second strategy to improve oil stability through genetic modification is to breed soybeans with high oleic acid levels. Using genetic engineering tools, scientists at DuPont have developed high oleic soybean lines that have oleic acid of 80% or higher, with good environmental stability (Fader et al., 1995). The high oleic soy oil was reported to have greatly improved oxidative and heat stability (Knowlton et al., 1996). A third genetic strategy is to breed soybeans higher in saturated fatty acids. This includes soybeans with elevated palmitic levels and soybeans with high stearate level. Initial work at Iowa State University through chemical mutagenesis led to registration of several soybean mutants with high stearate levels (Hammond & Fehr 1989, Bubeck et al., 1989). From these germplasm, researchers at private seed companies developed their own lines with high stearate levels. Most of these high stearate lines have a stearate level of 20%, or higher, with palmitic acid level remaining unchanged or lightly decreased. At refrigerated temperatures, a typical soy oil remains liquid whereas the high stearate soy oil becomes solid. Therefore, the modified soy oils would be suitable not only for certain frying applications but also for making low to zero trans fatty acid shortening and tub margarine (List et al., 1996, Liu et al., 1997b). The palmitic acid content in normal soybean cultivars is about 11%. Several new lines were developed, with palmitic levels increased to near 20% or higher (Fehr et al., 1991). A performance study showed that high palmitic soybean oils have increased oxidative stability and higher solidification temperature compared with normal soybean oil (Shen et al., 1997), indicating that such oils, just like high stearate soy oils, would be useful for direct applications for frying as well as margarine and shortening.

The soybean is a major source of vegetable protein for human and animal nutrition in many countries today. Like proteins of other sources, the nutritional quality of soy proteins is a function of many factors, including amino acid composition, digestibility, the presence of anti-nutritional factors, and amino acid requirements of the organism fed the protein. Proteins of high quality are those fully digested, with an amino acid composition closely matching the amino acid pattern required for the animal or humans consuming the protein. Like most other leguminous proteins, soy protein is low in sulfur-containing amino acids, with methionine being considered as the most significant limiting amino acid, followed by cysteine, and threonine. The amino acid profile is adequate for humans, but not livestock. In feed for animals where soybean is the main protein source, methionine is commonly added as a supplement. Therefore, genetic modification to increase methionine content in soybean protein would improve its nutritional value for animals. Other amino acids targeted for improvement are lysine and cysteine. Progress has been made in breeding new soybean varieties with improved amino acid profile through conventional approaches. The β-conglycinin and glycinin proteins are the major storage proteins in soybeans, accounting to about 70% of total seed proteins. Since the glycinin contains significantly more methionine and cysteine per unit protein than β-conglycinin protein, any effort in increasing glycinin to β-conglycinin ratio would lead to improvements in amino acid profile of soy proteins. Therefore, mutants lacking one or two subunits of β-conglycinin protein would have higher contents of methionine and cysteine (Kitamura et al., 1995).

Molecular strategies have been seriously pursued. One example is the successful transformation of Brazil nut methionine rich protein cDNA into soybeans by Pioneer Hi-Bred International. Transgenic soybean lines showed accumulation of Brazil nut methionine rich protein of up to 8% of the total protein, equivalent to a 26% gain in methionine. Unfortunately, the transgenic soybean has been shown to contain a major Brazil-nut allergen, the 2S albumin (Nordlee et al., 1996). As a result, the program was terminated. Meanwhile, by expressing a methionine rich zein from maize, DuPont obtained soybeans with seed methionine contents up to 75% higher than typical soybeans (Kerr et al., 1996).

Protein digestibility is defined as a percentage of protein absorbed after ingestion of a certain amount of protein by humans or animals. It is a major index of protein quality. In

food, protein is mixed with other components naturally present in soybeans, such as the naturally–occuring biological active trypsin inhibitors and phytate. These two components are thought to be the major factors that affect soy protein digestibility.

The proteinase inhibitors, which have been isolated from soybeans, fall into two types: Kunitz trypsin inhibitor and Bowman-Birk inhibitor. The significance of soybean trypsin inhibitors lies in their nutritional implications towards both human and animals. Their presence reduces digestibility of proteins through decreasing or inhibiting the action of pancreatic enzymes and causing hypertrophy of the pancreas shown in some small animals, like rats and chicks (Liener et al., 1994). Efforts have been made in lowering trypsin inhibitors in soybeans through plant breeding. Mutants lacking Kunitz trypsin inhibitor (Orf and Hymowitz 1979) or Bowman-Birk trypsin inhibitor (Stahlhut and Hymowitz 1983) have been reported.

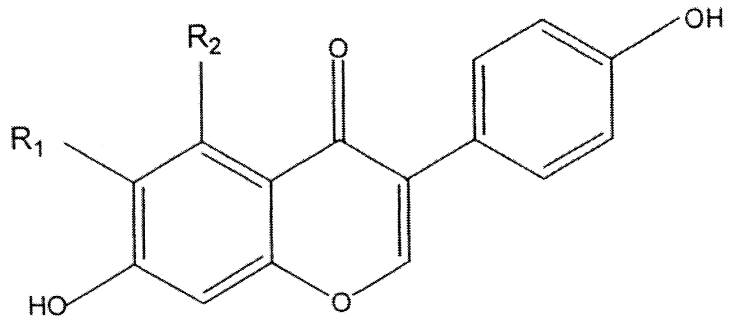
Flatulence, excessive accumulation of gas in the stomach and intestine, is another factor limiting consumption of certain types of soy foods, particularly of soy flour and cooked or roasted whole soybeans. The problem is widely believed to result from the presence of α-linked oligosaccharides, mainly raffinose and stachyose in soybeans. The two oligosaccharides are non-reducing, containing fructose, glucose, and galactose as 3 and 4 units, respectively. Human and monogastric animals lack α-1,6-galactosidase in their intestinal mucosa. When ingested, these soluble sugars remain unabsorbed, pass on to the lower intestinal tract, where they are metabolized by intestinal microflora which contain the enzyme α-1,6-galactosidase and due to the anaerobic environment, fermentation, leading to the production of gases (Liener et al., 1994). Because of their indigestibility, their presence causes reduced metabolizable energy in feed for pigs and poultry (Coon et al., 1988). An ultimate solution to the flatulence problem would be the genetic removal of oligosaccharides by plant breeding. It is known that there is a considerable variation in raffinose (0.1-0.9%) and stachyose (1.4-4.1%) among varieties of soybeans (Hymowitz et al., 1972). So, it is possible to breed lines that have low amounts of oligosaccharides through natural selection. Indeed, scientists at DuPont have successfully used two separate methods of conventional breeding, germplasm screening and chemical mutagenesis, and developed soybean strains with low raffinose saccaride contents. Soybean meal prepared from these novel varieties

display improved metabolizable energy in poultry and reduced incidence of flatulence in human subjects (Kerr et al., 1996).

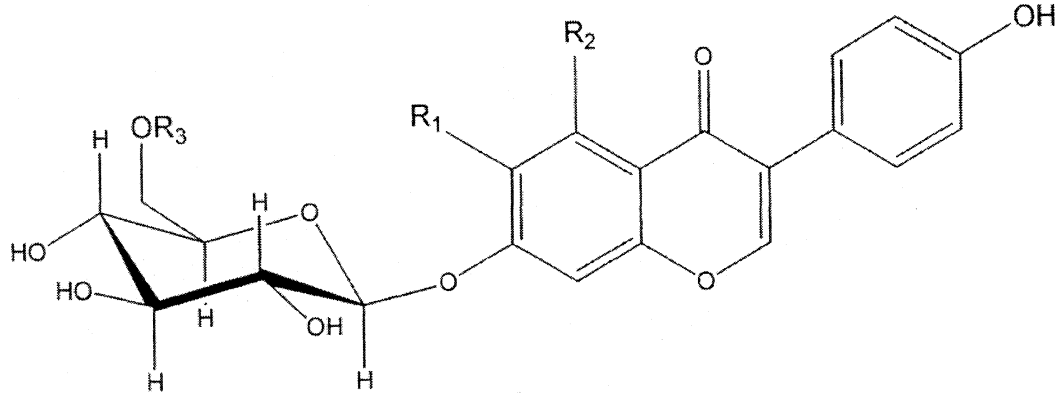
Soy Isoflavones

Isoflavones are plant compounds, which belong to a class known as phytoestrogens or plant estrogens that are particularly present in soy and soy based foods and are present in a small number of other plant foods, some including clover, alfalfa, and garbanzo beans. (Wang and Murphy, 1994). Isoflavone data from Murphy et al., (1999) reports tofu, miso and tempeh to have total isoflavone contents of 1727, 228, and 538 μg isoflavones/g product, respectively. Murphy et al., (1999) reported on isoflavone content of soy/meat analogues, including soy chicken, meatless franks, soy burgers, and soy links to be 147, 38, 82, and 38 μg total isoflavone / g product.

Genistein, daidzein, and glycitein are the aglucons of soy isoflaovones. For each of these aglucons, there are three additional major isomer forms called the β-glucosides, including genistin, daidzin, and glycitin, the 6"-*O*-malonyl-β-glucosides, and the 6"-*O*-acetyl-β-glucosides (Figure 1). Heat, moisture, and β-glucosidases are the most important factors in changing isoflavone distribution. Soybeans and soy flour are high in malonylglucosides, but heat processed soy products, such as roasted soybeans and texturized vegetable protein, have distributions shifted substantially from malonylglucosides toward acetylglucosides. Fermented foods, such as tempeh and miso, have their isoflavone distributions shifted toward the aglucons due to the action of fungal β-glucosidases Wang and Murphy (1994). Coward et al., (1998), evaluated effects of cooking on isoflavones in soyfoods. These authors found that baking processed soy products led to conversion of malonyglucosides to β-glucosides, but frying tended to promote the conversion of malonylglucosdes to acetlyglucosides, due to decarboxylation of the malonylglucosides. They indicated that as food received excessive heat treatment, aglucon concentration increased and total isoflavone extractable content decreased. They suggested that dry heat has a tendency to transform malonylglucosides to acetylglucosides, while moist heat has a tendency to transform malonylglucosides to β-glucosides, but showed no experimental data to support this hypothesis.



Isoflavone	R_1	R_2
Daidzein	H	H
Genistein	H	OH
Glycitein	OCH_3	H



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

Figure 1. Isoflavone structures

Plant estrogens have a structural and a functional similarity to the human hormone estrogen. Isoflavones, similar to the female hormone, estrogen, bind to estrogen receptors on cells of different organs in the human body. The binding affinity to the receptors of the plant

estrogens is, however, much weaker than that of the human estrogen. The relative binding affinity of genistein, an isoflavone, has been shown to be only 0.0125 compared with 1.0 for estradiol (Markiewicz et al., 1993), and the relative estrogenic potency has been calculated to be 0.0008 versus 1.0 for estradiol (Miksicek et al., 1993). Recent research has shown that cells have two types of estrogen receptors, α and β. Human estrogen has a high binding affinity for the α receptor while isoflavones have a high affinity for the β receptor (Brouns et al., 2002). It has been reported that isoflavones may have an estrogenic as well as an anti-estrogenic effect in the human body. Most probably the level of endogenous estrogen plays a role in this respect. In a situation where the amount of circulating estrogen is low, like in post-menopausal women, the binding of natural plant estrogens to cells will increase the overall estrogen effect, an agonistic action. This is assumed to have an influence in all conditions where low estrogen levels have negative effects on cell metabolism and organ function. In the case that normal estrogen levels are present, binding to cell receptors may offset the estrogen effect. This will be an anti-estrogenic action (Korach, 1998).

Isoflavones and Health-Related Issues

Since several chronic diseases of menopausal women such as breast cancer, colon cancer and arteriosclerotic cardiovascular disease in Western nations are much less prominent in Pacific Rim nations where traditional diets include substantial intake of soy foods rich in isoflavones, the explanatory hypothesis for these differences is that intake of phytochemicals accounts for these differences in disease occurrence (Hughes 1997, Adlercreutz et al., 1997, Messina et al., 1994).

Menopausal symptoms

Isoflavones do have a much weaker binding affinity than estradiol, but when consumed in high concentrations (40 mg/day), isoflavones have the potential to mimic the effects of exogenous estrogens such as in hormone replacement therapy. Evidence for this was found by Cassidy et al., (1994, 1995). Controlled intervention studies in premenopausal women provide direct evidence to suggest that diets containing isoflavones can produce estrogenic effects in women of reproductive age. A daily intake of textured vegetable protein

containing 45 mg isoflavones modified characteristics of the menstrual cycle of healthy premenopausal women and suppressed the magnitude of the normal mid-cycle surge of follicle-stimulating hormone and lutenizing hormone. This effect was not seen with an isoflavone-free soybean protein, suggesting that the isoflavone fraction exerts this endocrine modifying effect, which occurs at the level of the hypothalamic pituitary-gonadal axis. These particular studies were done monitoring only one menstral cycle. In studies examining more than one menstral cycle, results have been mixed. Nagata et al., (1998) examined three menstral cycles for folecular phase blood levels and changes in cycle length upon ingesting 68 mg/day isoflavones. The study shows a decreased level of estrone and estradiol by 23 and 27% in the blood and an increase in cycle length of two days. Wu et al., (2000) studied three menstrual cycles after feeding 32 mg/day isoflavones. The study showed a statistically significant change in reduction in serum luteal estradiol level. This studied showed no changes in folicular phase estradiol, progesterol, or menstrual cycle length. Martini et al., (1999) claims that 38mg/day of isoflavones had no effect on menstrual cycle length or serum sex hormones.

One of the most disruptive and classic symptoms of menopause is the hot flash. In Western societies it is the most prevalent symptoms of menopause, although the prevalence is much lower in Japan (Kronenberg et al., 1994). The rarity of this problem in soybean-consuming societies such as Japan, has prompted some investigations to determine whether isoflavones may be a factor for the lower prevalence of the hot flash in Western societies. Hot flashes are related to the fall in circulating estrogen, rather than absolute levels, and are associated with surges of gonadotrophins. Two studies have shown that phyto-oestrogens, lower plasma gonadotrophins after the menopause (Wilcox et al., 1990, Cassidy et al., 1997). However, two other studies showed no effects (Baird et al., 1995, Murkies et al., 1995). Several studies have concluded that hot flashes are reduced in frequency and intensity and that other symptoms such as vaginal cell maturation and dryness were improved, as well as the global sense of physical well being with the treatment of isoflavones (Adlercreutz et al., 1992, Burke et al., 1996, Washburn et al., 1996). Murkies et al., (1995) examined hot flashed directly, and reported an improvement with 45 g soybean flour/day, but an improvement occurred also with white wheat flour, which contains no phyto-estrogens.

Baird et al., (1995) investigated the effects of phytoestrogen supplements on vaginal cytology, and found an increase in cell proliferation, which is an indication of estrogenic activity with reversal of menopausal atrophy. In that particular study, subjects were given 165 mg isoflavones per day. A third study (Murkies et al., 1995) asked post-menopausal women to add 45 g soybean four daily to their diets, yet no effects were found. Following these studies, Scambia et al., (2000) supplied 50 mg isoflavones, for a period of 6 weeks after which the isoflavones were combined with conjugated equine estrogens for a period of 4 weeks. From week 10 to week 12 no isoflavones but only a combined progesteron-estrogen supplement was given. It was observed that both the number and the severity of hot flashes was significantly reduced as a result of the soy isoflavones. The placebo group experienced a decrease of 24% hot flashes after 6 weeks compared with 45% decrease in the isoflavone group. Interestingly, when continuing in combination with the estrogen supplement, both placebo and isoflavone group reduced the frequency number without differences between the two groups. Under these study conditions soy isoflavones were as effective as oral estrogens in reducing hot flashes.

Osteoporosis and Isoflavones

Osteoporosis is defined as a condition in which the amount of bone per unit volume is decreased, but the composition remains unchanged. The bone becomes porous due to an imbalance in the forming and resorbing bone cells, causing structural weakness. Osteoporosis in women is particularly associated with the menopause, since the loss of estrogen accelerates bone loss (Bingham et al., 1998). The hormonal effects of isoflavones, coupled with the comparative rarity of the disease in populations consuming soybean, such as the Japanese population, has also prompted investigation of isoflavones in the diet and the effect on osteoporosis. Post-menopausal women in particular may benefit from dietary supplementation with isoflavone-enriched soybean products to maintain bone mass. Potter et al., (1998) reported that soybean supplementation of sixty-six postmenopausal women compared bone measurements at baseline and 6 months after feeding a soybean-protein supplement enriched in genistein and daidzein with doses of approximately 56 or 990 mg of isoflavones/d, compared with treatment with a casein/non-fat dry milk supplement without

isoflavones. The higher dose resulted in significant improvements ($P<0.05$) in both bone mineral density and content of lumbar vertebrae relative to the control group, with no significant benefit in subjects receiving the lower dose. These findings, which illustrate the positive responses of vertebral bone mineral density of the postmenopausal women in the study, suggest that a threshold dose exists for any benefit of isoflavones on bone mineral density. Longer prospective studies examining the effects of isoflavones on bone parameters of postmenopausal women are needed to clarify effective doses and whether other skeletal sites will also benefit from isoflavones over a longer time frame of treatment. Some evidence is also available that consumption of soybean proteins can reduce urinary markers of bone resorption. Pansini et al., (1997) reported that non-osteroporotic, post-menopausal women who consumed 60 g isolated soybean proteins daily for 3 months had significant reductions in excretion of two urinary markers, deoxypyridinoline and N-telpeptide. Alekel et al., (2000) examined the effect of soy protein isolate with and without isoflavones on bone in peri-menopausal women. The study was done for 24 weeks with consumption of soy protein isolate with isoflavones (80 mg/day) and soy protein isolate without isoflavones. There was a control group fed whey protein. In this study, the percentage change in lumbar spine bone mineral density, and bone mineral content did not differ from zero in the two soy protein isolate groups, but loss occurred in the control group. After contrast coding using analyses of covariance with bone mineral density and bone mineral content as the outcome, it was concluded that soy isoflavones, not soy protein without isoflavones, attenuated bone loss from the lumbar spine in peri-menopausal women. There have been a few studies reporting the skeletal effects of isoflavones on rats. Arjmandi et al., (1996) reported that feeding an isoflavone-containing soybean protein-based diet to rats resulted in retention of significantly more bone than feeding a casein-based diet. Purified genistein also promoted a similar improvement in femur bone mass (Blair et al., 1996). Relatively few primate studies of the effects of isoflavones on bone mass have been published and positive effects of isoflavones on retention of bone after ovariectomy in non-human primates have been difficult to demonstrate. One study in which primates were treated with a single dose of 28 mg per animal of isoflavones for 2 years has been published (Jayo et al., 1996). No positive effects of isoflavones on bone were reported. An additional study in monkeys examined the rate of

bone formation in cortical bone (Lees and Ginn, 1998). In this study, bone was collected 7 months after feeding on either casein or soybean-based diets. The soybean-based diet consisted of 45 mg of isoflavones per day. The authors concluded that the isoflavones in the dietary soybean isolate were not effective in improving bone measurements. These published results support the concept that a threshold of isoflavones needs to be consumed for a sufficienty long period of time, months or years depending on the species, before any measurable effects on bone mass and density can be observed.

Cardiovascular Protection and Isoflavones

Evidence for an effect of isoflavones on plasma cholesterol concentrations has been demonstrated in rats, hamsters, nonhuman primates, and humans. Balmir et al., (1996), reported that rats were fed for 28 d on diets containing soybean protein without isoflavones (soy(-)), soybean protein with isoflavones (soy(+)), casein, and casein plus isoflavones (casein(+)). The soybean protein with isoflavones contained only the naturally intact isoflavone with no additional isoflavones added. For the casein(+) group, 0.36mg of isoflavone extract was added per g protein. The groups fed the casein(+), soy(-), and soy(+) had significantly lower LDL-cholesterol concentrations than the casein group. The HDL-cholesterol concentrations tended to be higher in the two groups fed the diets containing isoflavones. In a second study, Balmir et al., (1996) fed hamsters diets of either casein, casein(+), soy(+), soy plus additional isoflavones (soy(++)), and casein plus extra isoflavones (casein ++). The casein, casein(+), and soy(+) diets were the same as described earlier. Two additional groups were included in this study, a group fed on the unextracted soybean protein with 0.36 mg isoflavoness added per g protien (soy++) and a group fed on casein with 0.72 mg isoflavones added per g protein (casein ++). The hamsters were given the diet for 28 days. The soy(+), soy (++), and casein(+) groups had significantly lower LDL, and VLDL-cholesterol than for the casein group. While both soybean protein and the isoflavone-containing extract lowered LDL and VLDL-cholesterol concentrations, no additional benefit was seen for the groups receiving the higher doses of isoflavone extract. Kirk et al., (1998) reported that they fed diets containing soy protein isolate with isoflavones and soy protein isolate without isoflavones to wild-type mice and LDL receptor-deficient mice. Among the

wild-type mice, the soy with isoflavones fed group had significantly lower total and LDL and VLDL cholesterol concentrations than the mice fed the soy without isoflavones diet. Among the LDL receptor-deficient mice, no significant difference was observed in total plasma cholesterol between diet groups. The authors interpreted this to suggest that soybean isoflavones might lower plasma cholesterol concentration by increasing LDL receptor activity. In a study done with non-human primates (Anthony et al., 1996), diets containing soy protein with and without isoflavones were evaluated. Plasma LDL and VLDL-cholesterol concentrations were significantly lower with the soy protein plus isoflavone diet in both males and females. Additionally, in the females, the soy protein plus isoflavones diet consumption resulted in significantly improved GDL-cholesterol concentrations relative to when they were fed on the soy protein diet without isoflavones. In two other studies with non-human primates (Anthony et al., 1997, 1998), young monkeys were fed diets containing either casein and lactalbumin, soy protein isolate with isoflavones, or soy protein isolate without isoflavones. The was a large LDL and VLDL-cholesterol-lowering effect and HDL-cholesterol-raising effect of the isoflavone intact soybean protein relative to the soy protein isolate without isoflavones observed in both males and females. Crouse et al., (1998) reported that mildly hypercholesterolaemic men and women were treated with protein supplements that contained casein, soybean protein isolate containing 3 mg isoflavones, or soybean protein isolate containing 27 mg, 37 mg, or 62 mg isoflavones/d. The 62 mg isoflavone group had significantly lower LDL-cholesterol concentrations than the casein group, while the soy protein isolate without isoflavones group had no effect. The authors reported a dose-response relationship with progressively lower total and LDL-cholesterol concentrations with increasing isoflavone dose, also. A similar study was done by Baum et al., (1998) in postmenopausal women. This study also concluded that soy protein isolate with isoflavones intact significantly lowered LDL and VLDL-cholesterol and higher HDL-cholesterol concentrations relative to the control group. Unlike the previous study, no evidence was found of a dose-response effect on these endpoints. Studies have been done administering purified isoflavones instead of isoflavones plus soy protein to subjects. In one study (Nestel et al., 1997) menopausal and perimenopausal women were treated with either purified isoflavone pills containing about 80 mg isoflavones. These investigators found no

effect on plasma lipids. In a second study, men and postmenopausal women were treated with purified isoflavones pills containing 55 mg isoflavones. No effect was found on plasma lipid and lipoprotein concentrations (Hodgson et al., 1998).

Kirk et al., (1998) recently evaluated the effects of diets containing either intact or phytochemical extracted soybean on atherosclerosis in mice. In the mice, lesion area was significantly smaller in those fed on intact soybean protein compared with phytochemical-extracted soy protein.

Cancer and Isoflavones

Published data suggest that soy isoflavones may reduce the risk of a variety of cancers, and in particular, breast and prostate cancer. Recently, a case-control study by Lee et al., (1991) in Singapore found that the regular consumption of soybean foods was associated with a marked decreased risk of breast cancer in premenopausal women, but not in postmenopausal women. Similarly, a Japanese case-control study found that soybean intake was associated with a decreased risk of breast cancer in premenopausal women, but not postmenopausal women (Hirose et al., 1985). In contrast to those studies, a case-control study involving two different locations in China, failed to find an association between soybean intake and breast cancer risk in either pre- or postmenopausal women (Yuan et al., 1995). The only case control study thus far conducted in the USA to examine the relationship between soybean intake and breast cancer risk found that tofu consumption was protective in both pre- and postmenopausal Asian women (Wu et al., 1996). However, the overall intake of tofu among the subjects in this study was relatively low; the highest quartile of intake included women who consumed tofu as little as 50 times/year. Also, tofu was protective primarily in Asian women born in Asia who migrated to the West and not an Asian born in the USA (Wu et al., 1998). Finally, in the prospective Iowa Women's Study involving over 34,000 women, after 8 years of follow-up, tofu intake was associated with a modest decrease in postmenopausal breast cancer risk. The difference was not significantly different (Greenstein et al., 1996). In addition to the limited number of epidemiological studies that quantified soybean intake, Ingram et al., (1997), found an inverse relationship between the risk of both pre- and postmenopausal breast cancer and the urinary excretion of

isoflavonoids. Several animal studies have examined the effect of diets containing soybean isoflavones on experimental mammary cancer. In one study, Baggott et al., (1990) found that the number of dimethylbenz(a)anthracene-induced mammary tumors was decreased by approximately 30% in rats fed on a diet containing 200 g miso/kg diet. In the other study, by Gotoh et al., (1998a) a diet containing 100 g miso/kg diet reduced N-nitroso-N-methylurea (MNU)-induced mammary tumor number by about 50%. This appeared to be a function primarily of a delay in tumor appearance. In a follow-up study Gotoh et al., (1998b), a diet containing 100 g miso/kg diet was shown to be effective in reducing tumor number by about 50% in rats. An additional two studies examined the effects of isolated isoflavones on chemically-induced mammary cancer in mature animals. Constantinou et al., (1996) found that neither isolated genistein nor daidzein affected MNU-induced mammary tumor incidence. Although both isoflavones reduced tumor number by approximately 20%, this reduction was no statistically significant. Similarly, Murillo et al., (1998) found that genistein had no effect on MNU-induced mammary carcinogenesis. Several reports have shown that early exposure, during either the neonatal or prepubertal period of life, to genistein markedly inhibits the development of dimethyl-benz(a)anthracene-induced mammary tumors in rodents and increases the latency period (Brown and Lamartiniere, 1995; Lamartiniere et al., 1995; Murrill et al., 1996). Genistein appears to reduce carcinogenesis by stimulating mammary development and making this tissue less sensitive to the carcinogenic effects of dimethybenz(a)anthracene.

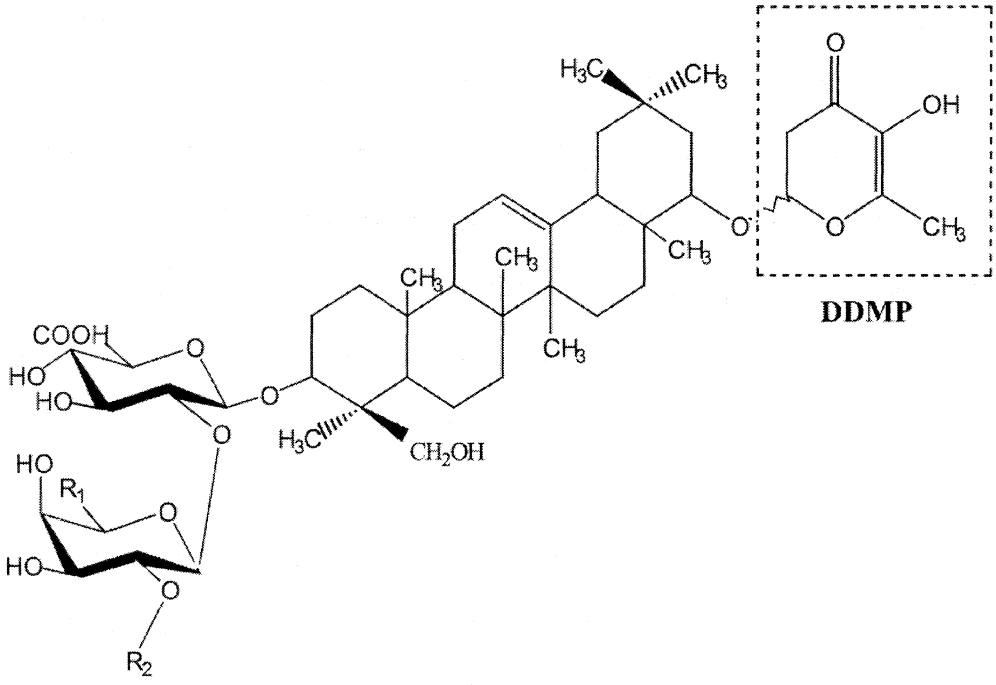
In a Japanese study, no significant association between soybean paste soup and prostate cancer risk was noted (Hirayama et al., 1979). There was not a consistent relationship for miso soup with prostate cancer evident when comparing cancer patients with patients with hyperplastic disease and control (Oishi et al., 1988). In contrast, in animal studies, Zhang et al., (1997) found that in rats fed on a diet containing soybean flour and implanted with rat prostate adencarcinoma tumors, tumor growth was significantly retarded compared with animals fed on the control diet. Schleicher et al., (1998) found that genistein given subcutaneously inhibited the development of prostate tumors in rats implanted with prostate carcinoma cells. Rats given genistein developed fewer tumors and fewer invasive tumors and no genistein-treated animals developed lung metastases. Zhou et al., (1998)

found that the addition of an isoflavone rich concentrate significantly reduced tumor volume in immunodeficient mice inoculate with human prostate adenocarcinoma cell line cells. Wang et al., (1995) found that dietary genistein inhibited the growth of human prostate adenocarcinoma cell line cells implanted into mice.

Soyasaponins

Soyasaponins are glycosidic compounds that are present in most plants (Figure 2). They are composed of a lipid-soluble aglycon (sapogenol) consisting of either a sterol or a triterpenoid and water-soluble sugar residues differing in type and amount of sugars. When the sugar are added to the sapogenol, the resulting compounds are called glycosides. The most common sugars that are added to the sapogenols to form glycosides are glucose, arabinose, glucuronic acid, and xylose. The glycosides are highly surface-active due to their amphiphilic nature (Rao et al., 1995). More than 20 saponins have been identified from soybean extract that vary in the structure of the sapogenin aglycone and their glycosides. The soy saponins are divided into three groups based on the structure of the aglycon moiety, the A, B, and E saponins (glucosides of the soyasapogenols A, B, and E) (Shiraiwa et al., 1991). The B and E group saponins are present in the soybean as conjugates with the unusual sugar 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) attached by an acetyl linkage to the C-22 position of the sapogenol moiety (Berhow et al., 2000). Kudou et al., (1992, 1993) investigated the composition and the structures of the native group B saponin in soybean seed and isolated five kinds of saponins named soyasaponins αg, βg, βa, γg, and γa, according to elution order during HPLC. The structures were characterized as having a DDMP moiety attached via labile ether linkage to the C-22 hydroxyl of soyasaponins I, II, III, IV, and V, with an absorption maximum at 292 nm. Kudou et al., (1992) reported the DDMP-conjugated soysaponins αg, βg, and βa were the genuine group B saponins present in soybeans and that their non-DDMP counterparts, soyasaponins V, I, and II, were products formed during heat treatment. The DDMP saponin bond is labile. Loss of this moiety from soy saponins βg, βa, γg, γa, and αg yields soy saponins I, II, III, IV, and V, respectively. The DDMP saponins were detected as major saponin constituents using much

milder than normal extraction conditions, while group B and E saponins, the saponins normally found when using hot solvent extraction condition, were not detected.



	R_1	R_2	DDMP
Soy saponin βg	CH_2OH	α-L-Rha	Y
Soy saponin I	CH_2OH	α-L-Rha	N
Soy saponin βa	H	α-L-Rha	Y
Soy saponin II	H	α-L-Rha	N
Soy saponin γg	CH_2OH	H	Y
Soy saponin III	CH_2OH	H	N
Soy saponin γa	H	H	Y
Soy saponin IV	H	H	N
Soy saponin αg	CH_2OH	β-D-Glc	Y
Soy saponin V	CH_2OH	β-D-Glc	N

Figure 2. Structures of group B soybean saponins (Kudou, et al., 1994)
Rha: rhamnosyl; Glc: glucosyl; DDMP: 2,3,-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one; Y: yes; N: no.

Therefore, it is strongly suggested that these DDMP saponins are genuine saponins in intact soybeans. Soybeans contain five kinds of group B saponins, soyasaponin I and II in the hypocotyls, and soyasaponin II, II, IV, and V in the cotyledon (Shimoyamada et al., 1990, 1991). There are few reports regarding group E saponin content. Kitagawa et al., (1988) suggested the interconversion of soyasapogenol B into E by photo-oxidation. Hu et al., (2002) developed a high-performance liquid chromatographic method for the isolation and quantitative determination of the group B soysaponins, including the (DDMP)-conjugated soyasaponins αg, βg, and βa, and their non-DDMP counterparts, soyasaponins V, I, and II, respectively. Hu et al., (2002) published a list of many soy ingredients and soy products analyzed for saponin concentration. Some of the soy foods and saponin contents include tofu, tempeh, soy milk and soy protein isolate at 0.59, 1.53, 0.47, and 9.51 μmole total saponins /g product.

Saponin Related Health Effects

Cancer and Saponins

First, the low incidence of cancer combined with the high intake of soybeans in Japanese living in Japan compared with Japanese who immigrated to the West suggests that saponins may play an important role in cancer protection (Dunn et al., 1975). The active component in several herbal medicines that are used as chemotherapeutic agents in Eastern countries were shown to be saponins. Yunan Bai Yao, a Chinese herbal drug, has been used as a hemostatic agent and promotes wound healing (Wu et al., 1990). Extracts of this medicine exhibited cytotoxic activity in several cancer cell lines. The cytotoxic component was later identified as the saponin formosanin-C (Ravikumar, et al. 1979). In another study, saponins extracted from *Agave cantala* and *Asparagus curillus* significantly inhibited the growth of human cervical carcinoma and leukemia cells (Sati et al., 1985). Two sapogenin components were identified as being responsible for inducing cytotoxicity against human nasopharyngeal carcinomas in culture. These saponins were extracted from the fruit of horsechesnuts, which has been used to treat mammary cancer (Konoshima and Lee, 1986). Several *in vivo* studies have shown that ginseng extract inhibits growth of different types of

tumors (Ha and Lee,1985; Kenarava et al., 1990, Lee and Huemer, 1971; Odashima et al., 1985; Yun et al., 1983). The biological activity in ginseng is largely attributed to the triterpenoid saponins, or ginsenosides. Growth inhibition and reverse transformation of melanoma cells were observed with ginsenosides treatment (Odashima et al., 1985). Tubeimoside I, a triterpenoid saponin from *Maxim franquet* was fed to mice. A significant dose-dependent inhibition of edema was observed. Also, tubeimoside I was found to significantly decrease the number of tumor bearing mice, and the number of tumors in each mouse (Yu et al., 1992). In another study, saponin extracted from *Gleditsia japonica* effectively inhibited the growth of mouse skin papilloma (Tokuda et al., 1991). Formosanin-C, a saponin extracted from *Liliaceae* has been shown to have antitumor activity that acts by modifying the immune system (Wu et al., 1990). Mice fed with the Quillaja saponin had significantly higher antibody production when they were given an oral vaccination of rabies antigen (Maharaj et al., 1986). Sapoinin feeding reportedly enhanced cell proliferation in the spleen and in the lymph node (Chavali and Campbell 1987).

In vitro, saponins were shown to form large mixed micelles with bile acids (Sidhu and Oakenfull, 1986). Similar interactions in vivo would reduce the free form of bile acids in the upper gastrointestinal tract and lowered the absorption of bile acids across the mucosa as well as the formation of secondary bile products from primary bile acids. Increases in the fecal excretion, bile acids, were observed after feeding mice diets containing 1% soybean saponins (Sidhu and Oakenfull, 1986). Rao and Sung, (1995) concluded that soybean saponins had a dose-dependent growth inhibitory effect on human carcinoma cells and that viability was also significantly reduced. This study was done in culture. Koratkar et al., (1997) did a study on mice fed with a 3% soy saponin diet, or a control diet. The mice in the study were first dosed with azoxymethane, a known colon carcinogen that causes aberrant crypt foci. After the treatment, mice were placed on the diets. Dietary intake of soy saponins significantly reduced the incidence of aberrant crypt foci at the end of the trial. A study was done by Berhow et al., (2000) on the antimutagenic activity of soybean saponins. In the study, an extract was prepared from soybean molasses and fractionated into purified chemical components. This extract was found to contain a mixture of the group B soyasaponins and the DDMP soyasaponins. The extract repressed 2-

acetoxyacetylaminofluorene induced DNA damage in Chinese hamster ovary cells. Also, a purified soyasaopgenol B fraction was used to demonstrate significant antigenotoxic activity against 2-acetoxyacetylaminofluorene.

Cholesterol and Saponins

Hypercholesterolemic effects of soybean saponins have been demonstrated by several investigators. Isolated soybean saponins reduced diet-induced hypercholesteremia in rats through an increase in bile acid excretion (Oakenfull et al., 1984). They also form complexes with bile acids and reduce their absorption in vitro (Sidhu and Oaken full 1986). Ohominami et al., (1981) found that when crude soyasaponins I, II, III, A1, and A2 were administered orally to rats with peroxidized corn oil, the crude soyasaponins were found to reduce serum cholesterol and triglyceride. Peroxidized corn oil was fed because it was found to induce elevation of rat serum transaminases indicating liver damage. It was also found that liver injury caused by peroxidized salad oil, was protected by the addition so soyasaponin A1 to the process of peroxidation. To these studies, Calvert et al., (1981) hypothesized that soybean saponins, by binding bile salts in the gastrointestinal lumen, were responsible for some of the plasma-cholesterol-lowering effect of soybean preparations. In the study, ten hypercholesterolaemic men were give 50 g of soybean flour/d, containing either 22 or 4 g saponins/kg incorporated in biscuits. Neither diet had any effect on cholesterol in any plasma lipoprotein fraction, on fasting plasma triglyceride or on fecal bile acids and neutral sterols. These results suggested that soybean saponins are not responsible the hypocholesterolaemic effect of soybean products. Potter et al., (1993) conducted a study adding saponins to either soy protein isolate or casein based diets. When saponins were added to casein, blood lipid profiles were similar to those obtained from gerbils fed the soy protein isolate-based diets. When saponins were added to soy protein isolate, no effect was observed. They reported that saponins interacted chemically with soy protein very rapidly, forming an insoluble complex, whereas the interaction with casein was much slower. Sidhu et al., (1986) fed purified saponins from soapwort, soybeans and Quillaia to rats to examine their hypocholesterolaemic activity. They found that all the purified saponins reduced the rate of absorption of the bile salt; soybean and soapwort saponins substantially so, but

Quillaia saponin to a much lesser extent. These results were explained by the formation of large mixed micelles by bile acid and saponin molecules in aqueous solution.

HIV and Saponins

Nakashima et al., (1989) investigated soybean saponins isolate from soybean seeds for their antiviral activity on HIV *in vitro*. They found that a complex of soyasaponin V and I in a ratio of 1:2, completely inhibited HIV-induced cytopathic effects and virus-specific antigen expression 6 days after infection. They found that a complex of soyasaponin V, I, and Bd from group E in a ratio of 1:2:1, inhibited HIV infection, but less potently. These results suggest that soybean saponins may have inhibitory activity against HIV infection.

Liver disease and Saponins

The antihepatotoxic activities of soyasaponin I and kaikasaponin III, isolated from *Abrus cantoniensis* were studied on liver injury induced by CCl_4 in primary cultured rat hepatocytes. Soyasaponin I showed antihepatotoxic activity equal to glycyrrhizin, the positive control except for the result at higher levels where the activity of soyasaponin I decreased. Also, compared to glycyrrhizin, soyasaponin I and kaikasaponin III were more toxic at the highest dose (Miyao et al., 1998).

Taste and Flavor Chemistry of Soy

One of the main factors limiting the increased use of soybean proteins for human consumption is flavor (Wilding et al., 1970). Kalbrener et al., (1971) did a sensory evaluation of various commercial soybean flours, soy protein concentrates, and soy protein isolates to determine the nature of the flavors limiting the wider use of soybean products in foods. The 17 member panel found 2.0% dispersions in water of the soy products to taste beany, bitter, chalky, and astringent. Several phenolic acids, such as syringic acid, have been isolated in soybeans and characterized as bitter and astringent, but their relative contribution to total bitterness is difficult to assess. This is because when these compounds are removed, the resulting product is still bitter (How et al., 1982). Other bitter components are generated from the oxidation of unsaturated fatty acids in both soy triglycerides and phospholipids.

Bitter hydroxyl-fatty acids are generated in the presence of lipoxygenase. A nonenzymatic pathway is also available to produce bitter oxo, hydroxy, ethoxy, and epoxy fatty acids (Sessa et al., 1977). Attempts to improve the solubility of soy protein through proteolytic enzyme hydrolysis have been hindered by the production of bitter peptides (Adler-Nissen et al., 1979).

Bitterness

Bitterness is commonly considered one of the four fundamental taste qualities. The binding of bitter compounds to receptor membranes must be slow to form and long lasting because the time between when a bitter compound makes contact with the tongue and the actual perception of bitterness can be as long as 2-7 seconds. This is a relatively slow response compared to other taste responses. Bitterness is often accompanied by a long aftertaste (Mayer et al., 1972). Numerous food and beverage products possess bitter substances from nature. Some of these substances include tea, coffee, cocoa, nuts, chocolate, beverages including beer, soft drinks, fruit and vegetable juices, ciders, wines, spirits, and bakery products (Roy, 1997).

The manner in which bitterness is expressed and the compounds which are responsible for bitterness are exceptionally varied. Bitterness can be expressed by a wide range of molecules, with varying sizes and functional groups. Bitterness can be found in aliphatic or aromatic compounds, straight chained or polycyclic compounds, glycosides or aglycones with just about every functional group. Unfortunately, for the systematic study of bitterness, just about any type of molecule can exhibit bitterness, yet a slight structural modification of a bitter molecule can render it non-bitter. For this phenomena, the ability to predict bitterness from molecular composition is rather limited (Rouseff 1990). The research that has been done specifically with bitter taste and compound structure has been reviewed and the following generalizations can be made. Bitter taste intensity is correlated with the degree of molecular hydrophobicity. In bitter-tasting aqueous solutions, there is an inverse relationship with surface tension (Gardner et al., 1979; Pfeilsticker et al., 1978). In the case of amino acids, it has been shown that molecular connectivity, the total possibility of each

molecule to encounter another molecule in a bimolecular interaction, appears to correlate with bitter taste thresholds (Gardner et al., 1980).

Objectionable Taste of Isoflavones

Prior to 1981, no report can be found on the relationship of isoflavones with objectionable flavor of soybean products. Huang et al., (1981) concluded that the isoflavones, the aglucon, daidzein, the β-glucosides, genestin, and particularly glycitin possess a herbal-like astringency and may contribute additively to the nonvolatile objectionable flavor of soy protein products. The scheme used to separate these particular isoflavones included solvent extraction, freeze-drying, fractional crystallization, and reverse phase HPLC. Diadzein, genestin and glycitin were identified by ultraviolet absorption, and nuclear magnetic resonance spectrometry. Preliminary testing was done with a 3-person experienced panel. No statement was made about the experience level of the panelists. The water-soluble fraction was made into a 2% solution in distilled water and evaluated by the panelists for objectionable tastes. Water was used as a rinse before and after tasting each sample. Matsuura et al., (1989) reported daidzein and genistein to have an objectionable aftertaste. They reported that the degree of the objectionable aftertaste of soy milk increased with the increase in the amounts of the daidzein and genistein in the soy milk. Soy milk with different concentrations of daidzein and genistein were made by soaking the soybeans in water with and without the addition of glucono-δ-lactone, which is a highly specific competitive inhibitor of β-glucosidases. β-glucosidases hydrolyze the isoflavone glucosides and therefore, increase the amount of daidzein and genistein. Sensory evaluation was performed by a 12-member experienced panel. No further statement was made about the experience of the panelists. Sensory evaluation was made with 20 ml of each of the five types of soymilk. The isoflavone content of the soymilks ranged from 0.3-1.6 mg daidzein per 100 ml soy milk, and 0.4-1.9 mg genistein per 100 ml of soymilk. Panelists evaluated each sample of soy milk on a five-point scale for objectionable aftertaste. Panelists were given a sugar-water solution to take away any aftertaste between samples.

Okubo et al., (1992) reported isoflavones to be bitter by measuring first by sensory and second by electrophysiological methods. The isoflavones for the study were ethanol

extracted from ground soybeans, and the resulting residue filtered. The filtrate was evaporated, and the residue dissolved in methanol. The methanol solution was put on a Sephadex LH-20 column, eluted with methanol, fractionated, and examined by thin layer chromatography. First, the isoflavone solution was measured for threshold by panelists. The threshold value of daidzin for astringency and bitterness was 10μmol / L – 1μmol / L and that of daidzein, its aglucon, for astringency and lingering was 1μmol / L. The value of genistin (10 μmol / L) for astringency, biting, and sweet was lower than that of genistein (100 μmol / L) for astringency and bitterness. The threshold study concluded that the isoflavone aglucons were more bitter than the glucoside forms. There is no mention to the size or experience of the sensory panel. Second, the response intensities of isoflavone glucoside were done by measurements of dry mouth feel activities by the electrical response of the glossopharyngeal nerve of the frog. The dry mouth feel activity of genistin was stronger than that of the daidzin fraction, and the threshold value of genistin was also lower than that of daidzin. These results were not consistent with the findings of the human sensory test.

Objectionable Taste of Saponins

Price et al., (1984) identified one of the compounds contributing to the bitter and astringent characteristics of some cultivars of dried pea to be soyasaponin I. In fact, the authors concluded that the compound possessing the undesirable taste characteristics of astringency and bitterness in pea is soyasaponin I. The sensory panel for this sudy consisted of eight trained and experienced assessors. In this experiment, the sensory panel tasted the soysaponin I extracts at different levels along the purification scheme. There is no mention of the medium used for the saponins that were tasted. First, pea flour was stirred with water, centrifuged, the precipitate freeze–dried, and stirred in methanol. Sensory evaluation established the methanol-insoluble fraction as containing the undesirable flavor characteristics. This fraction was then filtered, dissolved in water and subjected to reversed phase flash chromatography. The sensory panel found this fraction to be bitter and astringent. This is the last sensory evaluation done for that particular paper. The paper reports that further sensory analysis, which would be reported elsewhere, demonstrated very

similar sensory properties for the compound isolated from pea four and soyasaponin I isolated from soy. Further fractionation was done in the experiment to further purify the soyasaponin I, and analyze it. The mixture was subsequently fractionated by rechromatography and normal-phase flash chromatography. This saponin fraction was analyzed by mass spectrometry and ^{13}C-NMR.

Price et al, (1985) described the sensory properties of isolated soyasaponin I as bitter, astringent, and metallic. In this study, soyasaponin I was isolated from both pea and soy flours. The relationship between the concentration of soyasaponin I and sensory response was positively correlated. The purified saponin was assessed at a range of concentrations for .03-.1% purified soyasaponin I in Malvern water. Evaluations were performed by 60 panelists in one replication. Evaluations were performed on 5 mL samples which were swirled in the mouth for 10 s and expectorated. Soysaponin I was purified as in the previously reported by Price et al., (1984). Okubo et al. (1992) reported that soybean saponins, particularly saponin A contribute to the bitterness and astringency of soybeans by using sensory and electrophysiological methods. A 70% ethanol extract of the whole soybean meal was partitioned between water and n-butanol to begin saponin purification. The other layer was dried and dissolved in methanol. Ether was added and the precipitate was collected by centrifugation and further fractionated by gel filtration. A portion of each fraction was hydrolyzed and examined by TLC. Soybean saponins Aa, Ab, V, and I were isolated by preparative HPLC. Threshold was done on the soybean saponin A group. The values of the threshold were 0.1μmol / L – 0.001 μmol / L. The taste characteristic was undesirable with intense bitterness. The threshold value of the soyasapongenol A aglycon was higher than those of saponin A group, and a similar relationship was found for other saponins and their aglycons. Secondly, measurements of dry mouth feel activities by the electrical response of the chorda tympany nerve of the rat were done. In contrast to the threshold information, when a measurement of dry mouth feel activity of soybean saponin A was doneby electrical response, there was not a membrane potential seen. It is known that the membrane potential change of the nueroblastoma cell is induced by bitter substances. From these data, it was concluded that the bitterness of soybean saponin may be different from those of other bitter substances. Also, when measuring dry mouth feel activities by the

electrical response of the chorda tympany nerve of the rat, no electrical response was found when adding soy saponin. Measurements of dry mouth feel activities by the electrical response of the glossopharyngeal nerve of the frog where done. After the application of soybean saponin solution to the tongue of the frog, a specific response from the glossopharyngeal nerve was observed. The effects of the isolated saponins Aa, Ab, V, and I on the intensity of the response were examined, and their threshold values measured. The threshold values of A group were lower than that of B group. These results are consistent with the results of human organoleptic tests. Furthermore, the dry mouth feel activity caused by the saponin A group was more intense than that of the B group, especially at the higher concentration. Saponins Aa and Ab were found to have almost equivalent to the threshold values and response magnitude. There is no mention to the size or experience of the sensory panel.

Research Objective

The objectives of this research were to gain a better understanding of (1) soy storage protein fractionation, (2) fractionation products, (3)functionality of the fractionation products, and (4)sensory attributes of soy isoflavones and saponins. The objective of this research was completed by a bench scale soy protein fractionation study which was completed for three soybean varieties. The fractions were compared for protein yield, composition, solubility, emulsification capacity, and isoflavone and saponin yields, and profiles. A separate novel fractionation method using phytase was evaluated. A threshold and descriptive analysis study was done on isoflavones and saponins extracted from three different soy products. Threshold was measured for bitterness in water and milk. Descriptive analysis was done for bitterness, astringency, and off-flavor of soy isoflavones and saponins in water.

MATERIALS AND METHODS

Project I: Sensory Characteristics of Soy Isoflavones and Soy Saponins, a Threshold and Descriptive Analysis Study.

Materials

Defatted white soy flakes were obtained from Cargill (Minneapolis, MN) in 2000. Soy protein isolate was produced and provided by the Center for Crops Utilization and Research, Iowa State University (Ames, IA). The isolate was produced in 1999. Soy germ was generously donated by Acatris USA, Inc., (Minneapolis, MN). The white flakes, soy protein isolate, and soy germ were stored at 4°C. Food grade ethanol, 200 proof, was purchased from Aaper Alcohol and Chemical Co., (Shelbyville, KY). HPLC grade acetonitrile, methanol, and trifluoroacetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all of the mobile phases for HPLC analysis. Microcrystalline cellulose NF was purchased from FMC Corp., (Philadelphia, PA). Food grade citrus acid and caffeine were purchased from Tate and Lyle (Decatur, IL). Food grade alum was purchased from Tones Brothers, Inc., (Ankeny, IA). Granulated sugar was purchased from United Sugars Corporation, (Minneapolis, MN). Sodium chloride was purchased from Mortan Inernational, Inc., (Chicago, IL). HyVee (Des Moines, IA) skim milk was purchased at HyVee (Ames, IA). The chemicals for the phosphorus analysis included hydrochloric acid, zinc oxide, ascorbic acid, sulfuric acid, sodium molybdate, potassium hydroxide and potassium dihydrogen phosphate, all from Fisher Scientific (Fair Lawn, NJ).

Methods

Isoflavones and saponins were extracted for a sensory study from 40 g of ground, defatted white flakes, 40 g of soy protein isolate, and 4 g of defatted soy germ by stirring each in 2 L 70% ethanol for two hours at 25°C. The extracts were filtered and dried using a rotary evaporator (Buchner, Brinkman, R-114, Switzerland) at 30°C. The residues were suspended in 100 mL of water and loaded on a C-18 High Capacity Sep-pak from the Waters

Corporation (Milford, Massachussets). The Sep-pak was equilibrated with deionized water, and fractionated with 80% ethanol. The fractions were dried in a rotary evaporator at 30°C and re-suspended in 100 mL of deionized water. A total of 480 g of soy flakes, 480 g of soy protein isolate, and 48 g of soy germ were extracted through the preceding process to obtain 36 extracts, including 12 extracts from soy flake, 12 extracts from soy protein isolate, and 12 extracts from soy germ. The twelve extracts within the same soy matrix were pooled to form on extract. All procedures were done in a food grade laboratory with food safe equipment and chemicals.

To analyze for isoflavones, an aliquot of each of the 36 extracts produced was filtered through a 0.45-μm poly(tetrafluoroethylene) filter (Alltech Associates, Deerfield, IL) and analyzed by HPLC within 3 h of extraction. The HPLC method of Murphy et al (1999) was used to separate and quantify the individual isoflavones. Peak areas were evaluated using molar extinction coefficients for the 12 isoflavone forms. Soyasaponins V, I, II, αg, βg, and βa were separated and quantified by HPLC using the method described by Hu, et al. (2002). Aliquots of each extract were measured for total phosphorus (AOAC 995.11), as a seregate for phospholipids. Briefly, the samples were dry ashed to remove organic material in a muffle furnace from Barnstead ThermoLyne 1400 (Dubuque, IA). The acid-soluble inorganic residue is used for color reaction based on formation of a blue complex between phosphate and sodium molybdate in the presence of ascorbic acid as reducing agent. Intensity of blue color was measured spectrophotometrically at 823 nm in a Genesys 2 Spectrophotometer from Thermo Electon Corporation (Madison, WI).

Panel Training

Eleven judges from Iowa State University students and staff with previous experience evaluating soy products were selected on the basis of interest, availability, and ability to articulate sensory characteristics. A consent form identifying the project, objectives, and possible safety concerns was agreed to and signed by all panelists (Appendix A). Pre-screening questionnaires for aroma and flavor were initially used to screen panelists (Meilgaard, et al. 1987) (Appendix B). A basic taste test and an intensity ranking test were used. The basic taste test included recognition of sweet, salty, bitter, sour, and astringent

flavors (Appendix C). A 10% solution of sucrose in water was used for the sweet standard. A 1.0% sodium chloride solution in water was used as the salty standard. The bitter standard was 0.15% caffeine in water, and the sour standard was 0.15% citric acid in water. The astringent standard was 0.15% alum in water. The intensity ranking test included ranking the previously stated flavors, each at different concentrations (Appendix D). Sucrose was presented at 2.0, 5.0, and 10% in water. Sodium chloride was presented as 0.2, 0.5, and 1.0% in water. Caffeine, citric acid, and alum were presented at 0.05, 0.08, and 0.15% in water.

Panelists were trained for forced-choice ascending concentration series method of limits (ASTM E679-79) and descriptive analysis using the Flavor Profile Method (Caul, et al. 1957). During descriptive analysis training, panelists were given foods representing sour, bitter, and astringent tastes. The foods representing sour were apple cider and grapefruit juice. Brewed cold coffee and semisweet chocolate represented bitter flavor, and brewed cold tea and walnuts represented astringency. Panelists were asked to evaluate sensory differences among samples, and mark their response on a 10 cm intensity scale line (Appendix E) and discuss a list of descriptors for the samples. To train for the forced choice ascending concentration series method of limits, panelists ranked the bitter, astringent, and sour standards at three different concentrations each from least bitter, astringent, sour to most bitter, astringent, sour. A form similar to the form in Appendix D was used, without salty and sweet. Panelists used this form to evaluate the isoflavone/saponin samples at different concentrations and ranked the samples for intensity of bitter, astringent, sour. It was decided by the panelists that sour was not a flavor of the isoflavone/saponin samples. The panelists did notice an "off-flavor" which was described as herbal and beany for the soy flake and soy germ extracts. The "off-flavor" was described as plastic and cardboard for the soy protein isolate extract. Panelists were trained for 3 weeks in 1-hour sessions 2 times a week. As soon as the panel was trained, sensory evaluation was conducted for 8 weeks, 2 sessions per week. The first six weeks consisted of the threshold study of isoflavones and saponins in milk and water, and the final two weeks consisted of descriptive analysis of isoflavones and saponins in water.

Bitter Threshold Evaluation of Isoflavones and Soyasaponins in Water and Milk

A modification of the ASTM procedure E679-79 was used. Two samples, of 10 ml each, one water or milk reference, and one isoflavone/saponin sample in water or milk were presented. Five concentration levels in ascending concentration were presented per panel session. The extracts were presented in 3 oz. plastic cups labeled with randomly selected 3-digit numbers. Samples were presented in partitioned booths under red light. Red lighting was used to mask the color difference between the extract samples and the water or milk samples. Panelists were provided crackers and water to rinse their mouths. Panelists received a rinse solution of 0.55% microcrystalline cellulose NF (MCC) suspended in water to remove bitter flavor (Brannan, et al. 2001). Because MCC is insoluble in water, panelists were instructed to mix the MCC and water before using as a rinse. Subjects were asked to choose the item with the most intense bitter taste, and circle the sample number with the most intense bitter taste (Form F). Each extract was evaluated in skim milk and de-ionized water in three replications. Each replication was a separate session. The individual best estimate threshold was calculated as the geometric mean of the last concentration of isoflavones with an incorrect response and the first concentration with a correct response. Group thresholds were calculated as the geometric mean of the individual best estimate thresholds (Vinas, et al. 1998). Saponin concentration was calculated based on the threshold levels for isoflavone concentration.

Descriptive Analysis of Isoflavones and Soyasaponins in Water

Three samples were used for the evaluation by descriptive analysis. The samples were isoflavone/saponin extracts from defatted soy flakes, soy protein isolate, and soy germ at 4 μmol/ml isoflavones in water. Bitterness, astringency, and "off-flavor" of the three extracts were evaluated in three replications. The three replications were completed during three separate panel sessions during three different days. Judges recorded their responses on separate 10-cm intensity line scales for bitterness astringency and "off-flavor". The form used was similar to Form E, only did not include sour. Beyond marking an intensity for the "off flavor", panelists were asked to name the "off-flavor" because there was no complete consensus of a name for the "off-flavor" taste during panel training. The 3 samples were

presented in 4 oz. plastic cups in a different random order for each judge during each replication. Water, crackers, and MCC were given to rinse the mouth as in the threshold panels.

Statistical Analysis of Sensory Data

Three replications were completed for threshold in water, threshold in milk, and descriptive analysis. Analysis of variance at $P<0.05$ (SAS Institute, Inc. 1996) was conducted (general linear model, PROC ANOVA) to determine the differences in bitterness, astringency, and off-flavor intensity for the descriptive analysis study, and the differences in the isoflavone profiles.

Project II: Altered Yield and Functionality of Glycinin and β-conglycinin in Different Soybean Varieties

Materials

Soybeans were obtained from Cargill (Minneapolis, MN) in 2000, to be used as the processing control. Prolina A-1191 variety, also known as high cysteine soybeans, were obtained in 1999 from the North Carolina Crop Improvement Association. A Dupont A233HO variety, or high oleic soybeans, were obtained in 1999 from Dupont (St Louis, MO). The soybeans were defatted and made into flakes at the Center for Crops Utilization and Research at Iowa State University (Ames, IA). Flakes were stored in sealed containers at 4 ^{0}C until use. Chemicals used in the protein fractionation methods were sodium bisulfite, sodium chloride, hydrochloric acid, and sodium hydroxide from Fisher Scientific (Fair Lawn, NJ). Crude phytase from *Aspergillus ficuum* was purchased from Sigma-Aldrich (St. Louis, MO). Additional chemicals necessary to do the urea-SDS-PAGE were urea, glycerol, sodium dodecyl sulfate, bromophenol blue, 40% stock acrylamide, ammonium persulfate, acetic acid, methanol, and glycine, purchased from Fisher Scientific (Fair Lawn, NJ). N,N,N',N'-tetramethyl-ethylenediamine was purchased from Sigma Chemicals (St. Louis, MO). Coomassie brilliant blue G-250 was purchased from Acros Organics (Morris Plains,NJ). Affinity chromotography was used to further purify glycinin and β-conglycinin using Concanavalin A Sepharose 4B purchased from Sigma (St. Louis, MO). Other chemicals needed for purification included THAM, potassium dihydrogen phosphate, dipotassium hydrogen phosphate from Fisher Scientific (Fair Lawn, NJ) and methyl-α-D-mannopyranoside, and β-mercaptoethanol from Sigma (St. Louis, MO). Sodium azide was purchased from Eastman Organic Chemicals (Rochester, NY).

For isoflavone extraction and analysis, HPLC grade acetonitrile, and methanol, were purchased from Fisher Scientific (Fair Lawn, NJ). For saponin analysis, HPLC grade trifluoroacitic acid was purchased from Fisher Scientific (Fair Lawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all of the mobile phases. Additional chemicals for functionality tests were necessary including sodium potassium tartrate and copper sulfate from Fisher Scientific (Fair Lawn, NJ), and bovine

serum albumin and Sudan Red 7B were purchased from Sigma, (St. Louis, MO). Soybean oil was purchased from Sam's Club (Ames, IA).

Methods

Fractionation Study

Glycinin and β-conglycinin were fractionated according to the method reported by Wu et al (1999) that is a modified version of Nagano et al, (1992). In this process, 80 g of flakes were extracted in distilled water at 15:1 water to flake ratio at 45°C. This slurry was adjusted to pH 8.5 with 2N NaOH and stirred using a Fisher Scientific Isotemp Stir/Hot Plate (Fair Lawn, NJ) for 1 h, adjusting the pH every 15 min. The insoluble fraction was separated using a nylon 40 mesh (Fisher Scientific, Fair Lawn, NJ) and then by centrifugation at 9000 g for 30 min at room temperature using a Sorvall RC 5B Plus S centrifuge and SLA-3000 rotor from Sorvall Products, L.P. (Newton, CT). The supernatant was brought to 10 mM of SO_2 with sodium bisulfite and the pH was adjusted to 6.4 with 2N hydrochloric acid and stored at 4°C overnight. The glycinin was separated by centrifugation at 6500 g for 20min at 4°C. The supernatant was brought to 0.25 M NaCl, and the pH was adjusted to 5.0. This solution was stirred for 1 h on a stir plate and centrifuged at 9000 g for 30 minutes at 4°C. The resulting precipitate yielded an intermediate fraction composed of a mixture of glycinin, β-conglycinin, and other proteins. The supernatant was diluted 2-fold with distilled water and adjusted to pH 4.8 with 2 N hydrochloric acid. The β-conglycinin fraction was removed by centrifugation at 6500 g for 20 min at 4°C. One deviation was made from the Nagano, et al. (1992) procedure. Nagano, et al. (1992) extracted the protein with 15-fold water at pH 7.5. My study was done at 15-fold water at pH 8.5 for a better protein extraction. All fractions were freeze dried and stored in a dessicator at room temperature. All extractions were done in triplicate for each flake variety.

A separate fractionation method using phytase was evaluated using a modified method of Saito, et al. (2001). One hundred g of defatted soy flakes were stirred at pH 8.5 in 1.5 L distilled water. The pH was adjusted with 2N sodium hydroxide every 15 min. After 1 h of stirring, the solids were removed by a nylon 40 mesh. The liquid was centrifuged at

9,000 g with the SLA-3000 rotor for 30 minutes at room temperature. The precipitate was discarded. The supernatant was adjusted to pH 6.0 with 2 N HCl and measured for total protein by the biuret method (Gornall, et al 1949). An equivalent to 10 g protein of the supernatant was allocated into three separate beakers. Phytase was added to the supernatant at 0, 25, and 100 FYT phytase/10g soy protein. The resulting mixtures were incubated at 40°C for 1 h. The solutions were then centrifuged at 3,500 g for ten minutes to separate the precipitate at room temperature. Total soluble protein by biuret was analyzed in the supernatant. The precipitate was the glycinin-rich fraction. Purity was evaluated for the glycinin-rich fraction, and the supernatant by a urea-SDS-PAGE gel electrophoresis as described by Wu et al. (1999). In different trials, with and without the addition of sodium bisulfite to achieve a 10 mM concentration of SO_2,and with and without a 14 h cryoprecipitation at 4°C were used after the phytase incubation step. This was done to examine effects of these modifications because the purity of the glycinin was not high as expected. Sodium bisulfite and cryoprecipitation were used in the modified Nagano et al. method. It was hypothesized that purity may rise in the phytase method upon addition of sodium bisulfite or a cryoprecipitation step. Total soluble protein by the biuret method and purity by urea-SDS-PAGE were analyzed in these samples.

Purification of glycinin and β-conglycin for use as standards

Glycinin and β-conglycinin produced by the modified Nagano fractionation process were further purified by affinity chromotography to use as reference standards on urea-SDS-PAGE. Ten g of the glycinin curd was dissolved in 80 ml of Wolf's buffer (potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium cholide, sodium azide) with 0.78 mL β-mercaptoethanol /L buffer. Six g of the β-conglycinin fraction were dissolved in 60 mL of Wolf's buffer with 0.78 mL β-mercaptoethanol /L buffer. Both solutions were adjusted to pH 7.0 and stirred for 30 minutes, then centrifuged at 14,500 g with a Sorvall SA-600 rotor for 10 minutes. The precipitates were discarded. To purify glycinin, 20 mL of the glycinin solution was added to the Con-A Sepharose 4B column. The column was developed with Wolf's buffer including β-mercaptoethanol at 0.78 mL/L buffer. Fractions were collected, and the absorbence of all fractions were read at 280 nm. Any fraction with an

absorbance greater than 1.0 was pooled. The mg/mL soluble protein of the pooled tubes was measured using the biuret method. The protein was evaluated for purity on urea-SDS-PAGE. The purified glycinin solution can be kept at 4°C for up to one month with the addition of NaN_3. After eluting 300 mL of the Wolf's buffer plus β-mercaptoethanol through the Con A Sepharose 4B column, the buffer was changed to Wolf's buffer plus 19.41 g methyl α-D-mannopyranoside /L buffer to wash the column, or remove all β-conglycinin left on the column until the absorbance of the eluting buffer returned to < 0.1. To purify β-conglycinin, Wolf's buffer plus β-mercaptoethanol and 15 mL of the β-conglycinin solution was loaded onto the column. Fractions were collected and measured at 280 nm at until absorbance was < 0.1. The buffer was then changed to Wolf's buffer plus methyl α-D-mannopyranoside at 19.41 g/L buffer. Fractions were collected and the absorbance read at 280 nm. Fractions with an absorbance greater 0.4 were pooled and concentrated using a disc membrane filtration system with 50,000 dalton cut-off membrane filter (Altech Associates, Deerfield, IL). The solution was filtered until approximately 25 mL remained. The mg/mL concentration of soluble protein was measured using the biuret method. Purity was evaluated by urea-SDS-PAGE. The purified β-conglycinin solution can be kept in the refrigerator at 4°C for up to 1 month with the addition of NaN_3.

Analysis of protein Fractions of Modified Nagano Fractionation Process

The nitrogen content of the insoluble, glycinin, intermediate, β-conglycinin, and whey fractions were measured using the Nitrogen combustion method or Dumas method (AOAC 992.23) with a Rapid N-III analyzer (Elemantar Americas, Inc., Mt Laurel, NJ). A correction factor of 6.25 was used to convert from percent nitrogen to protein (Jung et al 2003). Moisture was determined using a forced air oven (AACC method 44-15A). Urea-SDS-PAGE was performed using the method described by Wu et al (1999) to describe purity. A 25 mg protein sample was dissolved in 10 ml of protein extraction buffer (50mM THAM, pH 8.0, 5.0 M urea, 0.2% SDS, and 2% 2-mercaptoethanol). This protein solution was diluted 1:1 with 2X sample buffer (125mM THAM, pH 6.8, 5.0 M urea, 0.2% SDS, 20% glycerol, and 0.01% bromophenol blue). The column purified proteins were treated with this same solubilization method. Protein was loaded at 45 μg per well. An 8-18%

polyacrylamide gradient was used. Electrophoresis was performed at 130 V for 6 h. Gels were stained with a methanol/acetic acid/water (50:10:40) solution containing 0.22% Coomassie Blue for 5 h and then destained in the same solution without Coomassie Blue for 20 h. Densiometry was determined using Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech Image Scanner (Amersham Parmacia Piscataway, NJ). SDS-PAGE results were calculated as follows. Absorbence values for lipoxygenase, β-conglycinin subunits, and glycinin subunit bands were summed. This sum was the denominator for a corresponding numerator comprised of a sum of subunit bands for a given protein. When multiplied by 100, this provided a percentage composition for that protein with respect to the protein bands measured by densiometry analysis.

Functionality of the glycinin, intermediate, and β-conglycinin fractions

The solubility of the glycinin, intermediate, and β-conglycinin fractions from the modified Nagano method was measured according to a modification of a method described by Bian, et al. (2003). A 1% w/w dry basis sample dispersion was prepared in deionized water and the pH was adjusted to 7 using 2N NaOH. The dispersions were stirred for 1 h. Twenty-five gram aliquots of the dispersions were portioned into 50 mL centrifuge tubes and centrifuged at 10,000 g on a SA-600 rotor for 10 minutes at 20°C. The supernatant was measured for soluble protein content using the biuret method. Solubility at pH 7.0 was calculated as a percent by dividing the protein in the supernatant by the initial protein content (determined by the Nitrogen combustion method) and multiplying by 100. Samples were run in triplicate.

Emulsification capacity was measured using the method of Bian, et al. (2003). Twenty-five g of a 2% w/w dry basis sample dispersion adjusted to pH 7 with 2N NaOH was transferred to a 400 mL plastic beaker, yielding 0.5 g sample in the beaker. Soybean oil, containing Sudan Red 7B (Sigma, St. Louis, MO), was continuously blended into the dispersion at a rate of 37 ml oil/min using a Bamix wand mixer (ESGE AG Model 120, Mettlen, Switzerland) at the low setting until phase inversion was observed. The emulsification capacity (gram oil/gram sample) was calculated as g of oil used to cause inversion multiplied by 2. Samples were run in triplicate.

The HPLC method of Murphy et al. (1999) was used to separate and quantify the individual isoflavones. Peak areas were evaluated using molar extinction coefficients for the 12 isoflavone forms. Soyasaponins V, I, II, αg, βg, and βa were separated and quantified by HPLC using the method described by Hu et al (2002). Isoflavones and saponins were extracted from all the insoluble, glycinin, intermediate, β-conglycinin, and whey fractions of all three soy flake varieties, and starting defatted flake materials by stirring each in 20 mL acetonitrile and 14 mL distilled water for two hours at 25°C. The extracts were filtered and dried using a rotary evaporator (Buchner, Brinkman, R-114, Switzerland) at 30°C. The residues were dissolved in 10 mL of 80% methanol. An aliquot of each extract produced was filtered through a 0.45-μm poly(tetrafluoroethylene) filter (Alltech Associates, Deerfield, IL) and analyzed by HPLC within 3 h of extraction.

Statistics of Protein Data

All treatments were done in triplicate. Analysis of Variance (SAS Institute, Inc. 1996) was done using the general linear model of PROC ANOVA to test the effect on different soy flake varieties on protein yields, purity, solublity, emulsification capacity, isoflavone content, isoflavone profiles, saponin content, and saponin profiles.

RESULTS AND DISCUSSION

Project I : Sensory Evaluation of Isoflavones and Saponins, a Threshold and Descriptive Analysis Study

The soy matrices selected for this study varied greatly from one another in their isoflavone form distributions (Table 1). This was reflected in the 70% ethanol extract concentrates of isoflavones, which were derived from the three soy matrices. The concentration of isoflavones in the soy flake extract concentrate, soy protein isolate concentrate, and soy germ extract concentrate were 134.6, 92.6, and 1050.4 μmol/ml, respectively. The concentrates contained 10X more isoflavones and saponins than the original extracts. This was done by diluting the isoflavone residue in the round bottom flask to 10 mL with water, instead of 100 mL, which is called for in the method for isoflavone and saponin extractions and analysis. The soy flour extract concentrate contained predominantly malonyl-β-glucosides or about 60 mole % isoflavone mass, about 30 mole % as the β-glucoside, and small amounts of acetyl-β-glucosides, and aglucons (Table 1). Soy protein isolate extract concentrate contained 35 mole % malonyl-β-glucosides, about 35 mole % as the β-glucoside, 20 mole % of acetyl-β-glucosides, and approximately 12 mole % aglucons (Table 1). We see a difference in the soy flake and soy protein isolate profile probably due to heat and/or processing of the two products. The soy flour typically receives less heat processing than the soy protein isolate. Therefore, we see more malonyl-β-glucoside forms. (Coward, et al. 1998). Wang and Murphy (1996) reported that during the alkaline extraction step, in making soy protein isolate from soy flour, principal glycosides in the initial soy flour decreased in concentration and as a consequence, the soy protein isolate contained higher mole percentages of isoflavone aglycons compared to soy flour. Soy germ extract concentrate contained 50 mole % β-glucosides, and 40 mole % acetyl β-glucosides (Table 1). The soy germ extract concentrate contained very small amounts of malonyl-β-glucosides and aglucons (Table 1). Beside differences in the soy matrice isoflavone distribution, there were differences between the three matrices in isoflavone concentration.

The soy matrices selected for this study varied greatly from one another in their soyasaponin form distributions (Table 2). This was reflected in the ethanol extract

concentrates of soyasaponins, which were derived from the three soy matrices. The concentration of soyasaponin in the soy flake extract concentrate, the soy protein isolate extract concentrate, and the soy germ extract concentrate were 45.9, 47.3, and 331.2

Table 1. Isoflavone profile composition of the different matrixes in mole (%) of their 70% ethanol extracts[a]

Compound	Flakes	Isolate	Germ
β-glucosides			
Daidzin	10.64[a]	9.05[a]	28.97[b]
Glycitin	4.67[a]	2.48[a]	15.86[b]
Genistin	15.97[a]	19.52[b]	7.59[c]
Malonyl-β-glucosides			
Malonyldaidzin	24.60[a]	13.57[b]	1.67[c]
Malonylglycitin	4.77[a]	2.42[b]	2.18[b]
Malonylgenistin	31.92[a]	21.72[b]	0.75[c]
Acetyl-β-glucosides			
Acetyldaidzin	1.11[a]	5.67[b]	22.00[c]
Acetylglycitin	2.30[a]	3.26[a]	11.03[b]
Acetylgenistin	1.32[a]	9.11[b]	6.25[c]
Aglucons			
Daidzein	0.97[a]	4.84[b]	0.92[a]
Glycitein	0.75[a]	1.51[a]	2.19[a]
Genistein	1.00[a]	6.87[b]	0.60[a]
TOTAL	100.00	100.00	100.00
Total (μmol/mL)	134.60	92.60	1050.40

[a] n = 10 (1 assay on 10 different extracts). Means in the same row with different letters are significantly different at $p < 0.05$

μmol/ml, respectively. The soy flake extract concentrate, soy protein isolate extract concentrate, and the soy germ extract concentrate had very different soyasaponin form distributions. Soy flake extract concentrate contained almost 60% of the soyasaponin mass as 2,3-dihydro-2, 5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)-conjugated soyasaponins (Table 2). Approximately 40% of the saponins were the non-DDMP counterparts to the DDMP-conjugated forms. The soy flake extract concentrate was the only extract examined to contain more than 10% of saponins in the DDMP form. Of the DDMP forms in the soy flake extract concentrate, 40% were βg. Another 16% were βa. Soy protein isolate contained 90% non-DDMP forms and 10% DDMP-conjugated forms of soyasaponins (Table 2). Of the 90% non-DDMP forms, 55% was soyasaponin I, and 25% was soyasaponin II.

Soy germ extract concentrate contained almost undetectable amounts of DDMP conjugated soyasaponins, while containing almost 100% non-DDMP soyasaponins (Table 2). Fifty percent of the soyasaponins was soyasaponin I and 40% was soyasaponin V. Beside differences in the soy matrices soyasaponin distribution, there were differences between the three matrices in soyasaponin concentration.

Table 2. Soyasaponin profile composition the different matrixes in mole (%) of their 70% ethanol extracts[a]

Compound	Flakes	Isolate	Germ
Non-DDMP[b]			
V	5.63[a]	10.11[b]	39.02[c]
I	20.48[a]	54.62[b]	51.18[c]
II	12.07[a]	25.12[b]	2.67[c]
DDMP			
Ag	2.64[a]	0.97[b]	5.92[c]
Bg	42.30[a]	6.33[b]	0.00[c]
Ba	16.89[a]	2.87[b]	1.22[c]
Total	100.00	100.00	100.00
Total (μmol/mL)	45.90	47.30	331.20

[a] n = 10. (1 assay on 10 different extracts) Means in the same row with different letters are significantly different at $p < 0.05$

[b] DDMP = 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one

Threshold study

Different dilutions of the concentrated solutions were prepared using water and milk as the medium for the threshold study. The dilutions presented to the panelists are shown in Table 3 for water as the medium, and Table 4 for milk as the medium. The dilutions were made in function of isoflavone concentration. Each concentrate solution was serially diluted. The concentration of saponins was calculated using the dilution factor previously applied. Five different concentrations of isoflavones and saponins for each extract were presented to each panelist at a given panel session. Evaluation of more than five different levels of concentrations were necessary due to the difference in tasting acuity of bitterness among the panelists. About half of the panelists received the extracts at the lower concentrations (0.004-0.064 μmol/mL), while the other half at the higher concentrations (0.128-2.048 μmol/mL). It is known that the sensitivity to bitter taste is a heritable trait (Drewnowski et al., (1990). Two substances, phenylthiocarbamide (PTC) and 6-*n*-propylthiouracil (Prop)

taste bitter to some people but are tasteless to others (Fox et al., 1932). The genetically mediated ability to taste PTC and Prop has long been associated with enhanced sensitivity to other bitter compounds (Bartoshuk et al., 1980).

The bitter threshold values obtained from the panels from the extracts in water and milk are listed in Table 5. The bitter threshold concentrations for isoflavones for soy flake extract, soy protein isolate extract, and soy germ extract in water were 0.023, 0.023, and 0.039 μmol/ml. The saponin concentration of the soy flake extract, soy protein isolate

Table 3. Isoflavone and saponin extract concentration used to evaluate bitter taste detection in water

Ethanol Extracts	Isoflavone Concentration (μmol/mL)	Saponin Concentration (μmol/mL)
Flake	0.004	0.001
	0.008	0.003
	0.016	0.006
	0.032	0.011
	0.064	0.022
	0.128	0.045
	0.256	0.090
	0.512	0.178
	1.024	0.356
	2.048	0.712
Isolate	0.004	0.002
	0.008	0.004
	0.016	0.008
	0.032	0.015
	0.064	0.031
	0.128	0.061
	0.256	0.122
	0.512	0.245
	1.024	0.490
Germ	0.004	0.001
	0.008	0.002
	0.016	0.005
	0.032	0.010
	0.064	0.020
	0.128	0.040
	0.256	0.080
	0.512	0.156
	1.024	0.315

Table 4. Isoflavone and saponin extract concentrations used to evaluate bitter taste detection in milk

Ethanol extracts	Isoflavone Concentration (μmol/mL)	Saponin Concentration (μmol/mL)
Flake	0.064	0.022
	0.128	0.045
	0.256	0.089
	0.512	0.178
	1.024	0.356
	2.048	0.712
	4.096	1.425
Isolate	0.128	0.061
	0.256	0.122
	0.512	0.245
	1.024	0.490
	2.048	0.979
	4.096	1.959
Germ	0.128	0.040
	0.256	0.079
	0.512	0.158
	1.024	0.315
	2.048	0.630
	4.096	1.260

Table 5. Bitter threshold concentrations of isoflavones and saponins in skim milk and water[a]

	Isoflavones μmol/ml	Isoflavones μmol/mL	Saponins μmol/mL	Saponins μmol/ml
	Water	Skim Milk	Water	Skim Milk
Flakes	0.023	0.338	0.008	0.115
Isolate	0.023	1.090	0.011	0.561
Germ	0.039	0.776	0.012	0.225

[a] Group threshold was calculated from the geometric mean of individual thresholds n=11 panelists

extract, and soy germ extract at the threshold for milk were 0.115, 0.561, and 0.225 μmol/ml, respectively. It is interesting to note that the isoflavone bitter threshold of the soy germ extract was almost twice as high as the soy flake extract, and soy protein isolate extract, indicating that the bitterness of the soy germ extract was much harder to detect. The indices that made the soy germ extract different from the other extracts were that its isoflavone profile contained a much higher percentage of the β-glucosides, and acetyl-β-glucoside forms of the isoflavones. The soy germ extract contained a high percentage of soyasaponin V and a

low percentage of soyasaponin II in comparison to the soy flake extract and soy protein isolate extract. This could indicate that the bitter taste of soy does not reside in the β-glucoside or acetyl-β-glucoside forms of the isoflavones, or soyasaponin V of the soyasaponins because the soy germ contains high concentrations of these compounds, yet the panelists did not perceive the extracts as bitter.

The thresholds for the three extracts in skim milk were much higher than the thresholds for the extracts in water, indicating that the bitterness of the extracts was less detectable in milk. The bitterness thresholds for isoflavones in the soy flake extract, soy protein isolate extract, and soy germ extract in milk were 0.338, 1.09, and 0.776 μmol/ml respectively, and the saponin concentrations at threshold were 0.115, 0.561, and 0.225 μmol/ml. The bitterness thresholds for the extracts in milk are probably higher due to the higher protein and sugar content, and higher viscosity of milk compared to the extracts in water. Sugar is a commonly used masking agent for bitterants. Sweetness suppression of bitterness has been repeatedly demonstrated for sucrose and quinine mixtures (Bartoshuk, 1975, Lawless, 1979), sucrose and caffeine mixtures (Pangborn, 1960) and in more complex systems (Guadagni et al., 1974). The magnitude of the bitterness suppression is directly related to the sweetness intensity. Moskowitz and Arabie (1970) observed that increasing solution viscosity decreased quinine bitterness intensity. Burns and Noble et al., (1985) found that at constant sucrose levels, perceived bitterness was reduced with increasing viscosity.

The milk extract threshold results differ from the water extract threshold results in that the soy protein isolate extract in milk had the highest threshold value, opposite that of the threshold result in water. This may indicate that the malonyl-β-glucoside forms of the isoflavones and the non-DDMP soysaponins, particularly soyasaponin I are easily masked. One way these compounds may be masked is by binding to the milk protein, so that their bitter flavor moieties are not exposed.

Overall, the extract from soy flakes had the lowest bitter threshold in both water and milk when compared to the thresholds of extract from soy protein isolate and soy germ. The soy flake extract contained a higher percentage isoflavones in the malonyl β glucoside form. Very interestingly, the soy flake extract contains a much higher concentration of the DDMP-

conjugated saponins than the other two extracts. These may indicate that the bitter flavor originates from the malonyl-β-forms of isoflavones, and/or the DDMP-conjugated forms of the saponins.

Only one other threshold study has been reported for bitterness of isoflavones and saponins (Okubo et al., 1992). For the isoflavone threshold study, water was used as the medium for isoflavones. The extracts in Okubo et al., (1992) differed from our study in that only one isoflavone was represented per extract. Our extracts contained all twelve soy isoflavones. Okubo et al., (1992) examined four different extracts by panelists for bitterness. The four extracts included an extract of daidzin, another of daidzein, another of genestin, and finally one of genistein. Okubo et al., (1992) reported that the isoflavones for the study were ethanol extracted from ground soybeans, and the resulting residue filtered. The filtrate was evaporated, and the residue dissolved in methanol. The methanol solution was loaded on a Sephadex LH-20 column, fractionated by elution with methanol, and examined by thin layer chromatography. The isoflavones were said to be in purified form. For the daidzin, daidzein, genistin and genestein extracts, the thresholds were reported as, daidzin at 10μM – 1μM, daidzein at 1μM, genistin at 10μM, and genestein at 100μM, respectively. The thresholds reported by Okubo et al (1992) for daidzin and genistin, both β-glucoside forms of isoflavones were within range of our results. The threshold reported for daidzein was approximately 10-fold lower than our results, and the threshold for genestein was 10-fold higher than our results. Genestein and daidzein are aglucons. Our extracts had predominantlyhigher concentrations of β-glucolsides than aglucons which may be a reason that our thresholds matched the thresholds reported by Okubu et al., (1992) for daidzin and genistin, and not for genestein and daidzein.

Descriptive Analysis

Panelists were asked to do a descriptive analysis of the soy flake extract, soy protein isolate extract, and the soy germ extract at 2 μmol isoflavones /ml extract in water. The saponin concentrations were 0.7, 0.9, and 0.6 μmol/L for the soy flake, soy protein isolate, and soy germ extracts, respectively. The concentration 2 μmol/ml was chosen because it was much higher than the calculated bitterness threshold level and was the concentration used

during panel training. Panelists evaluated the extracts for bitterness, astringency, and off-flavor by marking intensity on a 15 cm line. Panelists were asked to name the off-flavor noted when marking the intensity. Panelists were asked to do this because during training, panelists were unable to come to agreement regarding what the "off-flavor" should be named. The panelists noticed a different "off-flavor" for each of the extracts during training. The panelists used terms such as herbal, plastic, beany, cardboard, grassy, and floral to describe the extracts. In general, the "off-flavor" for the soy flake extract, soy protein isolate extract, and soy germ extract was characterized as herbal, cardboard and plastic, and beany, respectively during the actual panel sessions.

The panelists found soy isolate extract to be significantly less astringent and less off-flavored than the soy flake and soy germ extracts. Table 6 shows that panelists marked significantly lower intensities for the soy protein isolate extract for astringency, and off-flavor, but not bitterness at concentrations of 2 μmol/ml each. This was an interesting finding because the isolate contained about 20 mole % more saponins than the soy flake extract, and about 30 mole % more saponin than the soy germ extract. The majority of the soyasaponins in the isolate extract were in the non-DDMP forms. This may demonstrate that soyasaponins, particularly the non-DDMP forms, do not cause the astringency and off-flavor of soy. The soy protein isolate extract had the highest percentage of the aglucon isoflavones, compared to the extracts from flakes and germ. This may indicate that the aglucon forms of the isoflavones have more astringent, and off-flavors.

Table 6. Descriptive analysis on a 15cm line scale of 10 panelists on the taste of the water dispersed matrixes[a]

Treatment	Bitter	Astringent	Off-flavor
Flake	5.8[a]	6.5[a]	8.9[a]
Isolate	6.5[a]	5.1[b]	6.4[b]
Germ	6.5[a]	6.9[a]	8.6[a]

[a] n = 10. Means in the same column with different letters are significantly different at $p < 0.05$

No significant difference was found for bitterness intensity of the three extracts at the isoflavone concentration of 2 μmol/ml, as they were presented. In general, for the three extracts, the off-flavor was perceived as more intense than the bitterness or astringency of the extracts.

There are two published studies reporting on a descriptive analysis of isoflavones, both reporting on the off-flavor of isoflavones (Huang et al., 1981, and Matsuura et al., 1989). Huang et al., (1981) reported that the isoflavone aglucon daidzein, and the β-glucosides, genestin and particularly glycitin, possess a herbal-like astringency. Matsuura et al., (1989) reported daidzein and genistein to have an objectionable aftertaste. They reported that the degree of objectionable aftertaste of soymilk increased with the increase in amounts of daidzein and genistein in the soy milk. Interesting, the extract in our study with more daidzein and genistein was found by the panelists to have less off-flavor and astringency than extracts with less daidzein and genistein. The samples reported by Huang et al., (1981) probably contained more than just daidzein and genistein because they were using soymilk as the matrix. Soymilk contains a high concentration of glucosides (Wang and Murphy, 1996), however this was not considered by Huang et al., (1981).

Two studies reported descriptive analysis of saponins (Price et al., 1984, 1985). Price et al., (1984) identified one of the compounds contributing to the bitter and astringent characteristics of some cultivars of the dried pea to be soyasaponin I. In fact, the authors concluded that the compound possessing the undesirable taste characteristics of astringency and bitterness in dry peas is soyasaponin I. Price et al., (1985) described the sensory properties of isoated soyasaponin I as bitter, astringent, and metallic. In their study, soyasaponin I was isolated from both pea and soy flours. Price et al., (1984) reported that the relationship between the concentration of soyasaponin I and sensory response was positively correlated. The purified saponin was assessed for bitterness, astringency, and metallic taste at range of concentrations from 0.32-1.06 μmol/g purified soyasaponin I in Malvern water or spring water. In our extracts, soyasaponin I was found in high concentration in the soy protein isolate extract and the soy germ extract. However, our descriptive analysis these two particular extracts did not show a tendency of more bitter or astringent characteristics than the soy flake extract, which has a much lower concentration of soyasaponin I.

Project II: A soy protein fractionation study of different soybean varieties, their protein yields, purities, functional properties, and isoflavone and saponin concentrations

Three sources of defatted soy flakes were selected for this study. These flakes where from three different soybean varieties. Cargill 2000, with a PDI (protein dispersibility index) of 84.0, a protein content of 49.0 ± 0.7%, a concentration of total isoflavones of 6.44 μmol/g, and a concentration of total saponins of 5.36 μmol/g, which will be referred to as control. Prolina A-1191 variety from North Carolina Corp Improvement Association, that are documented to be high in cysteine with a PDI of 96.1, a protein content of 54.6 ± .04%, a concentration of total isoflavones of 5.22 μmol/g, and a concentration of total saponins of 5.43 μmol/g, which will be referred to as high cysteine. A233HO from Dupont Protein Technologies, that produce an oil that is high in oleic acid with a PDI of 62.4, a protein content of 53.5 ± 1%, a concentration of total isoflavones of 6.01 μmol/g, and a concentration of total saponins of 3.74 μmol/g, which will be referred to as high oleic. PDI analyses were performed by Silliker Laboratories (Minnetonka, MN).

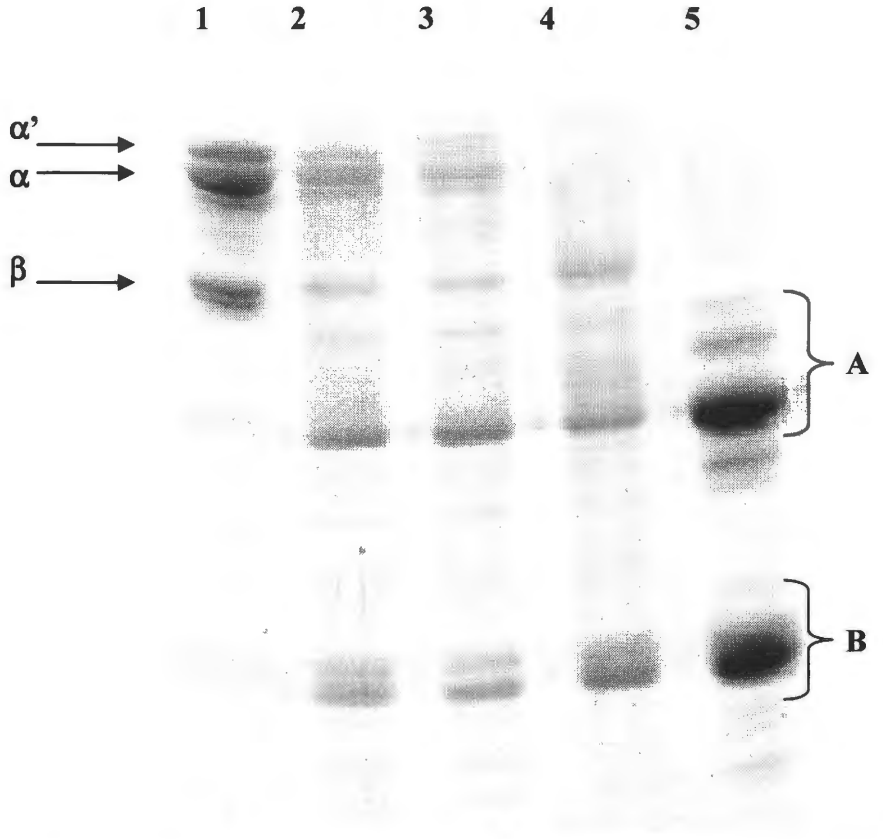
The starting materials, when analyzed for original concentration of glycinin and β-conglycinin by urea-SDS-PAGE, the starting materials showed significant differences in storage protein compositions between the high oleic flakes and the other two flake varieties (Table 7). The oleic flakes had 10% more glycinin, and 10% less β-conglycinin than the control and cysteine flakes. Figure 2 shows a urea-SDS-PAGE gel of the three flake varieties. The β-conglycinin subunits of the high oleic flakes show less intense staining, which I interpret to mean a lower concentration of β-conglycinin in comparison with the control and high cysteine flakes. Densiometry data for the SDS-PAGE gel report that the oleic flakes contained less total β-conglycinin subunit concentrations than the control and high cysteine flakes. There were significant differences among the α, α', and β subunit concentrations of the β-conglycinin from the three varieties. The profile for the control and high cysteine flakes subunit composition was similar. The control flakes had a relative

Table 7. Starting soybean flake storage protein composition %[b] by urea-SDS-PAGE for fractionation methods utilized[a]

Starting Material	Glycinin (%)	β-conglycinin (%)
Control flakes	58.90[a]	37.60[a]
High Cysteine flakes	60.60[a]	39.30[a]
High Oleic flakes	70.20[b]	29.70[b]

[a] n = 3. Means in the same column with different letters are significantly different at $p < 0.05$
[b] Percentace composition of each fraction= (sum of bands corresponding to sub-units for a given protein)/ (sum of total bands measured)

Figure 3. urea-SDS-PAGE of control, high cysteine, and high oleic flakes



Lane 1 is β-conglycinin standard, lane 2 represents the control flakes, lane 3 is high cysteine flakes, lane 4 is high oleic flakes, and line 5 is a glycinin standard. α', α, and β are the subunits of β-conglycinin. A and B stands for acidic and basic subunits of glycinin. Amount of protein loaded to each lane was 45 μg.

proportion of 30.3 ± 0.1 % α', 34.3 ± 0.1 % α, and 35.3 ± 0.1% β, β-conglycinin subunit composition, calculated as optical density of each band divided by the sum of the bands for

this particular protein. The high cysteine flakes had 30.3 ± 4.1 % α', 37.9 ± 2.4 % α, and 31.8 ± 1.8% β, β-conglycinin subunit composition calculated in the same way as for the control flakes. The only statistically significant difference between the control flakes and the high cysteine flakes was in the β subunit concentration, where the control flakes had a higher percentage of the β subunit, than the high cysteine flakes. For the high oleic flakes, the subunit profile of the β-conglycinin fraction was statistically different for each of the subunits with 20.6 ± 1.8 % for α', 19.9 ± 2.8 % for α, and 57.9 ± 1.3% for the β subunit, calculated as for the control flakes.

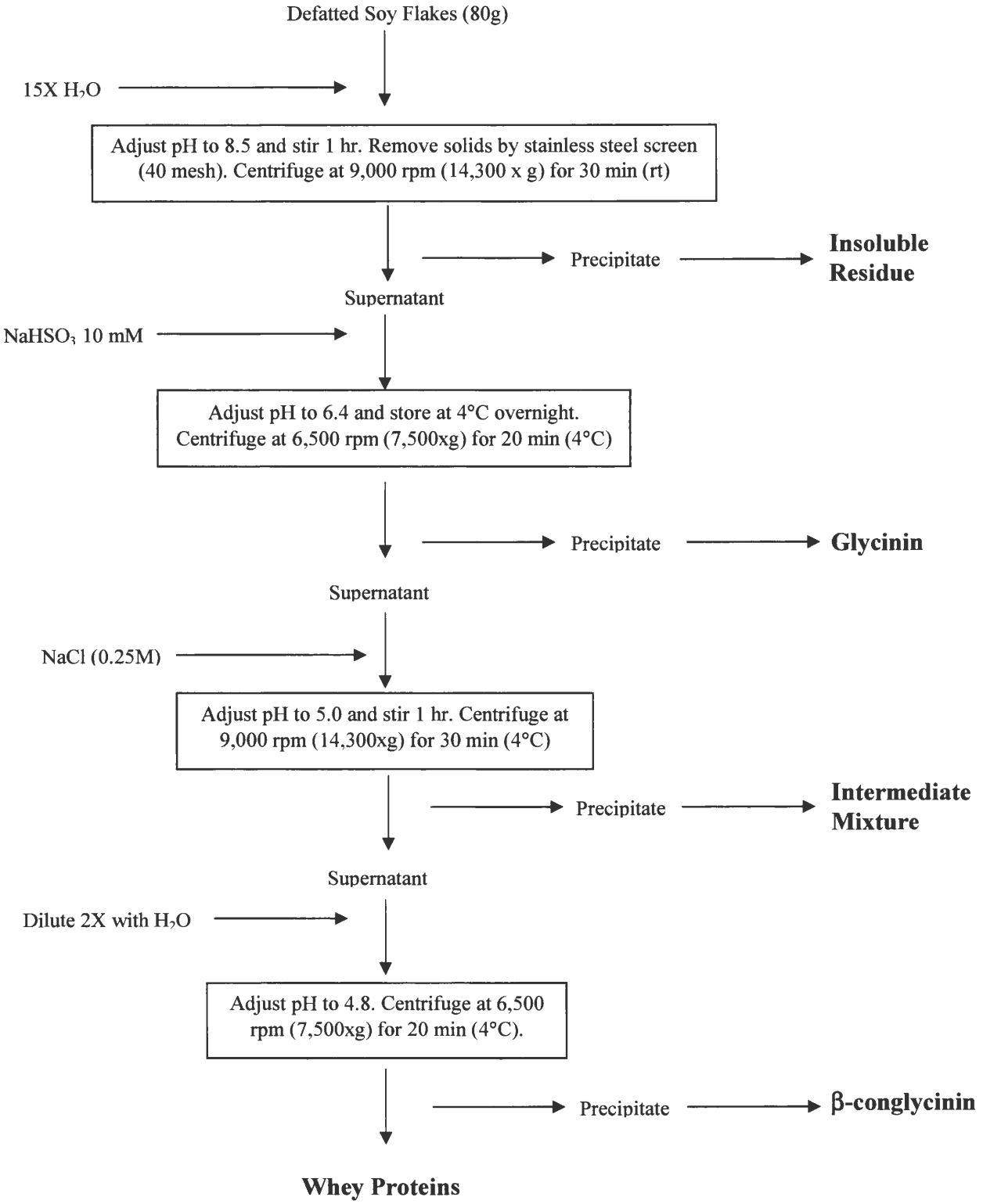
A well-known and published soybean protein fractionation method was selected for this study, which will be referred to as modified Nagano (Wu et al., 1999). The modified Nagano method renders five fractions including an insoluble residue, a glycinin rich, an intermediate, a β-conglycinin rich, and a whey fraction (Figure 4). The fractionation method was used to produce these five fractions from the three varieties of soybeans mentioned before, and to compare their protein yields, purities, functional properties, and isoflavone and saponin concentrations. A second fractionation method, using phytase as the fractionating agent, was studied for yields and purities of soybean storage proteins. This method will be referred to as the phytase method.

In my study the insoluble fraction consisted of 44.16 ± 0.21 % protein. The glycinin fraction was 98.36 ± 3.14% and the β-conglycinin fraction consisted of 96.77 ± 1.03% protein. The intermediate fraction consisted of 82.55 ± 1.72% protein. The whey fraction consisted of about 16.61 ± 0.69% protein. These percentages are given for soy flakes obtained from Cargill in 2000, the flakes that we used as control and are reported on a dry basis, using N X 6.25 as the nitrogen to protein conversion factor. The protein percentages of the different fractions may differ in soybeans from different years, environments and locations, and varieties.

Protein Yields

Protein yield was evaluated for the insoluble, glycinin, intermediate, β-conglycinin and whey fractions produced in the modified Nagano fractionation process for the three

Figure 4. Modified Nagano Protein Fractionation Flowchart (Wu et al 1999)



soybean flake varieties. The mass yields of total protein of the different fractions, except for the insoluble and whey fractions, differed greatly among soybean varieties (Table 8). The control, high cysteine, and high oleic flakes yielded different total protein amounts for the glycinin fraction. The high oleic flakes yielded > 70% more total protein in the glycinin fraction than the other two varieties. The high cysteine flakes yielded significantly more total protein yield than the control flakes, but the yields were similar in total protein yield. The total protein yield for the intermediate fractions of the three different varieties were similar yielding approximately 5-7 g of total protein each. The total protein yields of the β-conglycinin fraction were different for each variety. In general, the control and high cysteine flakes produced > 5 fold total protein yield in the β-conglycinin fraction.

Table 8. Protein yield of the fractions obtained by modified Nagano method[a]

Soybean flake variety and fraction	Total protein yield (g)
Starting material	
Control	39.24[a]
High cysteine	43.68[b]
High oleic	42.80[b]
Insoluble fraction	
Control	12.38[a]
High cysteine	12.76[a]
High oleic	12.73[a]
Glycinin fraction	
Control	7.18[a]
High cysteine	9.55[b]
High oleic	17.13[c]
Intermediate fraction	
Control	6.27[a,b]
High cysteine	6.79[a]
High oleic	5.64[b]
β-conglycinin fraction	
Control	5.27[a]
High cysteine	6.89[b]
High oleic	0.984[c]
Whey fraction	
Control	4.95[a]
High cysteine	5.34[a]
High oleic	4.91[a]

[a] n = 3. Means in the same fraction with different letters are significantly different at $p < 0.05$

Protein Purity

Protein purity was analyzed by urea-SDS-PAGE on the insoluble, glycinin, intermediate, β-conglycinin, and whey fractions produced in the modified Nagano process for the three soybean flake varieties. The high cysteine flakes produced significantly higher purities of glycinin in the glycinin-rich fraction at 91% compared to the oleic at 78% and control flakes at 87% purity (Table 9). The glycinin-rich fraction purity of the control flakes was significantly higher than the purity of the high oleic glycinin-rich fraction. All three glycinin rich fraction purities were significantly different from each other. The control and high cysteine glycinin-rich fractions yielded similar amounts of protein and similar purities whereas the high oleic had much higher yield and lower purity, this might be due to the genetic variations or the different PDI of the starting materials. To have a better understanding of why these products fractionate differently we would need to start with materials with similar PDIs so we could eliminate any additional influence beyond the genetic variation. The glycinin-rich fraction purity followed the PDI trend (high cysteine > control > high oleic). This is in accordance with what Wu et al. (1999) reported. The purity of the β-conglycinin in the β-conglycinin-rich fraction from the high oleic soybeans was 19%, with the major component of this fraction being glycinin. A purity of 74% for the ß-conglycinin-rich fraction from the control flakes, and a purity of 67% for high cysteine flakes were observed (Table 9). All three β-conglycinin-rich fraction's composition was found to be significantly different. The purity of the ß-conglycinin in the β-conglycinin-rich fraction from the control flakes was the highest at 74%, compared to the other two varieties. The lower yield and poor purity of the β-conglycinin-rich fraction of the high oleic flakes might be due to genetic variation, as the oleic flakes were the starting material with the lowest β-conglycinin content that was mostly composed by the β-peptide. The poor partitioning of the two storage proteins in this material could be due to the low PDI. The high cysteine β-conglycinin-rich fraction yielded more protein, but with less purity than the control flakes. The insoluble, intermediate, and whey protein fractions were mixtures of the two storage proteins (Table 9). The control and high cysteine intermediate protein fractions averaged 53% glycinin, and 38% β-conglycinin. The oleic intermediate protein fraction was different, consisting of 61% glycinin, and 24% β-conglycinin. The control and oleic insoluble protein

fractions were 72% glycinin, while the cysteine insoluble protein fraction was 59% glycinin. The insoluble fraction is the only fraction where the control and high cysteine products differ so greatly. The control and high cysteine variety insoluble protein fractions were 37% β-conglycinin, and the oleic insoluble protein fraction was 23% β-conglycinin, which can be explained by the lower β-conglycinin content of the high oleic starting material. Surprisingly the protein yield in this fraction was the same regardless the starting material. The high cysteine and high oleic whey fractions averaged 72% glycinin and 16% β-conglycinin. In contrast, the control whey fraction was 78% glycinin, and 13% β-conglycinin. For all varieties the amount of glycinin in the whey fractions was much higher than the amount of β-conglycinin in the whey fractions.

Table 9. Fraction composition by urea-SDS-PAGE for purity of products for modified Nagano process[a]

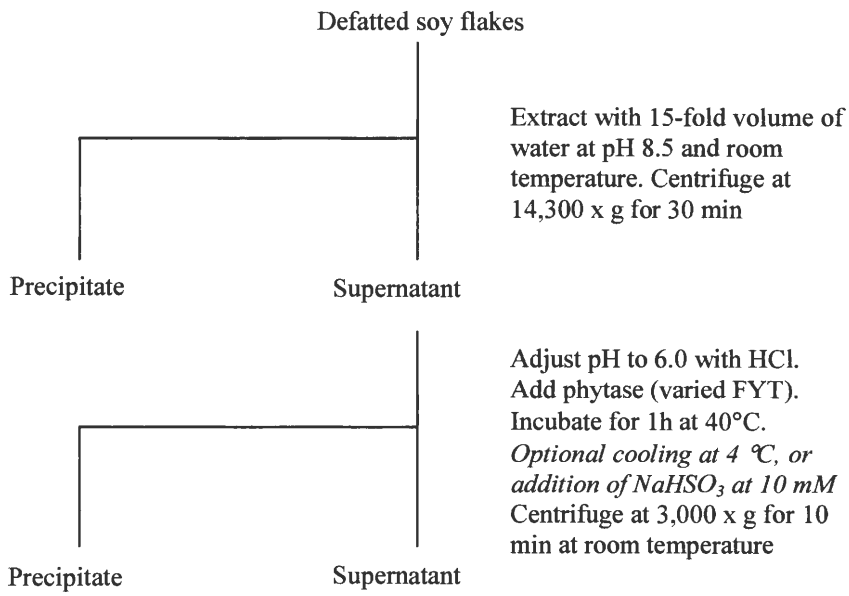
Flake variety and fraction	Glycinin (% of protein)	β-conglycinin (% of protein)
Insoluble fraction		
Control	72.06[a]	37.81[a]
High cysteine	59.46[b]	36.40[a]
High oleic	71.46[a]	23.01[b]
Glycinin fraction		
Control	87.04[a]	12.96[a]
High cysteine	90.73[b]	9.26[b]
High oleic	78.24[c]	21.76[c]
Intermediate fraction		
Control	52.45[a]	38.89[a]
High cysteine	54.07[a]	38.65[a]
High oleic	61.53[b]	24.31[b]
β-conglycinin fraction		
Control	23.11[a]	74.38[a]
High cysteine	33.21[b]	66.79[b]
High oleic	69.48[c]	19.31[c]
Whey fraction		
Control	78.62[a]	12.76[a]
High cysteine	71.35[b]	16.89[b]
High oleic	72.13[b]	16.21[b]

[a] n = 3. Means in the same fraction with different letters are significantly different at $p < 0.05$

Phytase Fractionation

Phytase was used to fractionate glycinin and β-conglycinin from the Cargill control flakes using the method described by Saito et al., (2001). The phytase fractionation scheme can be seen in Figure 5. First, a trial study was done by adding different concentrations of phytase at 0, 250, and 1000 FTY/100 g of soy protein, according to Saito et al., (2001), to determine how much protein was precipitated at the different phytase concentrations. FTY is an enzyme activity unit that is defined as the amount of enzyme that releases 1 μmol of inorganic orthophosphate per minute at pH 5.5, 37 °C and 5 mM sodium phytate (Saito et al., 2001). The addition of phytase was done after alkali extraction and centrifugation of the insoluble fiber-rich residue, as can be seen in Figure 5. The trial study was important to

Figure 5. Modified phytase method flowchart (Saito et al 2001)



determine if the experiment described by Saito et al., (2001) was reproducible. The amounts of protein precipitated obtained were significantly higher than those obtained by Saito and are shown in Table 10. With no phytase present, 39.2 % of the total proteins in the protein extract were precipitated. At 250 and 1000 FTY phytase, 47% and 65% of total proteins were precipitated, respectively. For the same phytase concentrations, Saito et al (2001)

reports a protein precipitation of 27% with no phytase addition, and 55% protein precipitated for 1000 FYT. These differences would suggest that the type and quality of starting material and/or enzyme used were not the same. My second approach for the phytase method was to chill the protein extract after addition and incubation with phytase, since glycinin is known to be insoluble at refrigeration temperatures, 10% more protein was precipitated from the protein extract containing a concentration of 250 FTY phytase. My third approach was to add sodium bisulfite after the addition and incubation with phytase. The same result was observed as with the chilling process (Table 10). Chilling or adding sodium bisulfite had no

Table 10. Yield of protein in precipitate for phytase method (protein precipitated, %)[a]

Phytase added (FTY/100g protein)	Phytase method	Phytase and chilling	Phytase and sodium bisulfite
0 FTY	39.2[a]	42.0[a]	44.0[a]
250 FTY	47.4[b]	58.0[c]	60.0[c]
1000 FTY	65.0[d]	64.0[c,d]	66.0[d]

[a]n = 3. Means with different letters are significantly different at $p < 0.05$
FTY = phytase activity units.

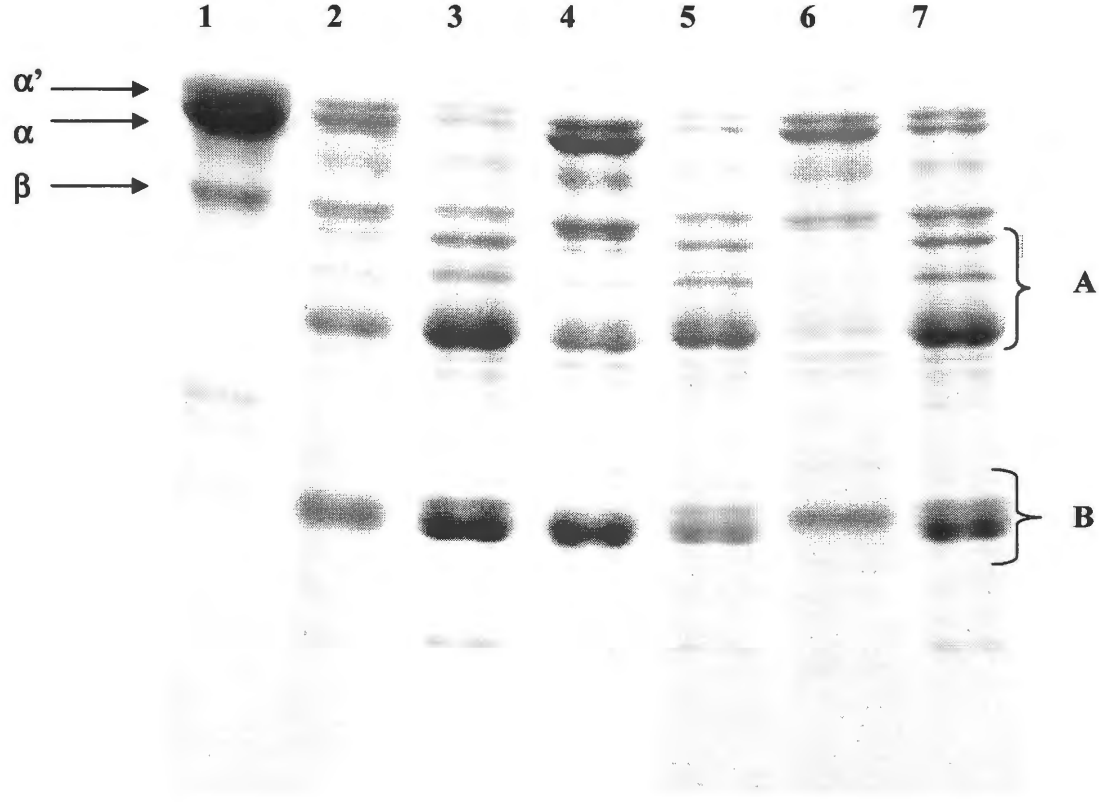
Table 11. Protein fraction composition for phytase treatment by urea-SDS-PAGE

Treatment and Fraction	Glycinin (% of protein)	β-conglycinin (% of protein)
Precipitate		
Control	77.6[a]	18.5[a]
250 FYT	70.6[b]	24.0[b]
1000 FYT	68.8[b]	27.8[b]
250 FYT and chilling	65.7[c]	34.3[c]
1000 FYT and chilling	59.2[d]	35.3[c]
250 FYT and bisulfite	64.5[c]	30.1[d]
1000 FYT and bisulfite	60.8[d]	33.6[c,d]
Supernatant		
Control	61.3[a]	34.4[a]
250 FYT	62.0[a]	34.3[a]
1000 FYT	42.9[b]	49.7[b]
250 FYT and chilling	18.3[c]	71.2[c]
1000 FYT and chilling	15.0[d]	72.7[c]
250 FYT and bisulfite	16.8[c,d]	73.9[c]
1000 FYT and bisulfite	17.4[c]	73.5[c]

[a]n = 3. Means in the same fraction and for each storage protein, with different letters are significantly different at $p < 0.05$
FTY = phytase activity units.

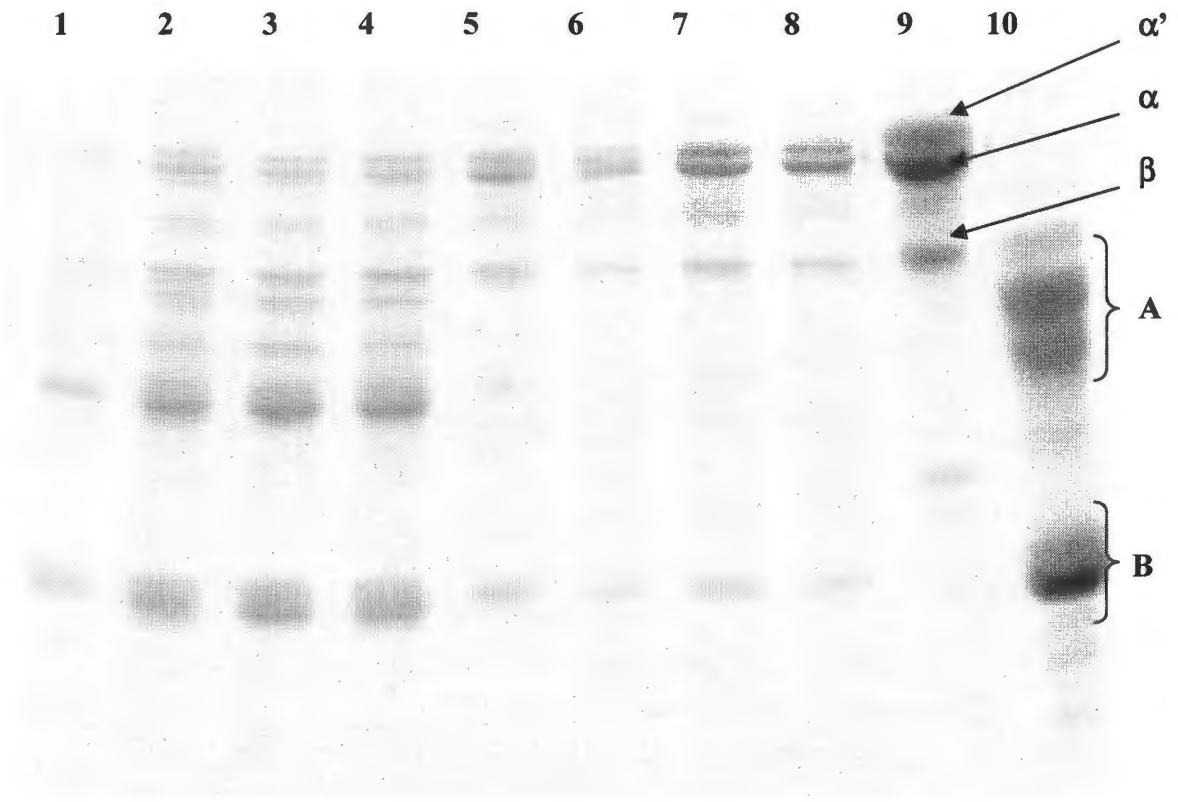
statistically significant effect on the percentage of protein precipitated in either the protein extract that had no enzyme added or the protein extract that was treated with 1000 FTY. As seen in the Table 10, the addition of 1000 FTY alone precipitated the highest percentage of protein. This is an improvement upon the modified Nagano method which requires a chilling step and addition of sodium bisulfite. Another benefit of the phytase method is that it does not produce an intermediate fraction. However, these protein yielding benefits, had negative purity implications. The results of purity analyzed by urea-SDS-PAGE are shown in Table 11. The elimination of the intermediate fraction causes a decrease in purity.

Figure 6a. Urea-SDS-PAGE of phytase method supernatants and precipitates



Lane 1 represents β-conglycinin standard, lane 2 is the supernatant resulting from the control (no phytase addition), lane 3 is the precipitate from the control, lane 4 is the supernatant from 250 FYT treatment, lane 5 is the precipitate from 250 FYT treatment, lane 6 is the supernatant from the 1000 FYT treatment, and lane 7 is the precipitate from the 1000 FYT treatment. α', α, and β are the subunits of β-conglycinin. A and B stands for acidic and basic subunits of glycinin. Each lane was loaded with 45 μg of protein.

Figure 6b. Urea-SDS-PAGE of modifications of the phytase method supernatants and precipitates



Lane 1 is the precipitate from 250 FYT and 4°C treatment, lane 2 is the precipitate from the 1000 FYT and 4°C treatment, lane 3 is the precipitate from 250 FYT and 10mM $NaHSO_3$ treatment, lane 4 is the precipitate from 1000 FYT and 10mM $NaHSO_3$ treatment, lane 5 is the supernatant from 250 FYT and 4°C treatment, lane 6 is the supernatant from 1000 FYT and 4°C treatment, lane 7 is the supernatant from 250 FYT and 10mM $NaHSO_3$ treatment, lane 8 is the supernatant from the 1000 FYT and 10mM $NaHSO_3$ treatment, lane 9 is β-conglycinin standard, and lane 10 is glycinin standard. α', α, and β are the subunits of β-conglycinin. A and B stands for acidic and basic subunits of glycinin. Each lane was loaded with 45 μg of protein.

The purity results for the precipitate or glycinin-rich fraction at 250 FTY and 1000 FTY were 70 and 68% glycinin, respectively. With addition of chilling, the glycinin-rich fraction purity at 250 FTY and 1000 FTY decreased to 65 and 60% glycinin, respectively. Similar results were observed with addition of sodium bisulfite. However, the supernatant, or β-conglycinin-rich fraction, showed a statistically significant difference by adding a chilling step, or by addition of sodium bisulfite. Without these extra steps, the β-

conglycinin-rich fraction was 34-40% β-conglycinin, depending on the concentration of phytase added. With either chilling, or sodium bisulfite addition, the percent β-conglycinin increased to more than 70% in the β-conglycinin-rich fraction, suggesting an obvious improvement from the former. This method seems to be useful to produce glycinin and β-conglycinin enriched fractions, but purities may depend on the quality of the enzyme and starting material in addition to a rigorous process control.

Functional properties

Functional properties are the basis for the industrial utilization of soybean protein ingredients in the food industry. The future use and applicability of the products in this study will depend on their functional properties. The functional properties tested in this study were solubility and emulsification capacity. The principal functional property that will determine the use of a functional food ingredient, such as any of these soy protein fractionation products, will be the product's solubility. This functional property is defined as the most important by several authors (Kinsella, 1979, 1985, Kalbrener et al., 1971, Saio, et al., 1969) and it is also suggested in these studies to be the functional property that might predict the behavior of an ingredient in a given food system. Emulsification capacity is other very important functional property of food ingredients. This functional property tests the ability of a food ingredient to act in water/oil interfaces. The ability of emulsifying oil will determine the usefulness of these ingredients in water/oil food systems such as salad dressings and mayonnaise.

Solubility

Liu et al., (1997) and Wolf (1970) recommended the use of low solubility soy protein for beverages and bakery products such as crackers and cereals, and high solubility for foamed and gelled products. When incorporated into food and beverage, soy protein must form stable dispersions. A stable dispersion is usually correlated to proteins with good solubility (Wang and Johnson, 2001).

In my study, the protein solubility was evaluated at pH 7.0 for the glycinin, intermediate, and β-conglycinin fractions. The results of this functional property test can be

seen in Table 12. The pH 7 was selected because isolated soy proteinates are sold after being neutralized. Solubility was not analyzed for the oleic β-conglycinin fraction because there was not enough sample. In general, the glycinin fractions and the β-conglycinin fractions have high solubilities because these proteins are in native state (Wu et al., 1999). The more denatured proteins, found in the intermediate fractions, are less soluble (Wu et al., 1999). The control glycinin, intermediate and β-conglycinin fractions all had higher solubilities than these fractions from the high cysteine and high oleic flakes (Table 12). The high oleic flakes registered the lowest solubilities for each of the fractions tested. This might be a reflection of the PDI of the flakes which is the lowest for the three flake varieties tested. The high cysteine fractions were consistently lower in solubility when compared to the control fractions. This cannot be due to the PDI, since the PDI of the control flakes was lower that that of the high cysteine flakes. These differences could be due to genetic variation of the two flake varieties or because there are differences in purities of fractions tested in this study.

Table 12. Solubility of modified Nagano process protein fractions[a]

Flake variety and fraction	Solubility (%)
Glycinin fraction	
Control	94.52[a]
High cysteine	84.15[b]
High oleic	78.11[c]
Intermediate fraction	
Control	48.22[a]
High cysteine	38.44[b]
High oleic	35.53[b]
β-conglycinin fraction	
Control	82.72[a]
High cysteine	63.25[b]
High oleic	N/A

[a] n = 3. Means in the same fraction with different letters are significantly different at $p < 0.05$

It was not unexpected to see such different results in different flake varieties and different fractionation products. Storage protein composition of soybeans, that is glycinin to β-conglycinin ratios, and native state conformation are responsible for protein product functionality (Petruccelli et al., 1995). This is in accordance with Kwanyuen et al., (1998) in that compositional differences that may alter functionality include the ratio of storage

proteins, and variations in subunit concentrations within these individual storage proteins. Influences in functional properties might be determined by differences in amino acid profiles (Serretti et al., 1994).

Emulsification Capacity

The ability of protein to aid the formation and stabilization of water and oil emulsions is necessary for many food applications. In food products, such as comminuted meats, cake batters, milks, and salad dressings, varying emulsifying capacities are required due to the differing composition and stresses to which the products are subjected (Kinsella, 1979). In my study, emulsification capacity was evaluated for the glycinin, intermediate and β-conglycinin fractions of all three varieties. Emulsification capacity was not done on the oleic β-conglycinin fraction because not enough of that fraction was available. The emulsification capacity of the glycinin was quite different than the emulsification capacity of β-conglycinin for all varieties (Table 13). The glycinin-rich fraction emulsification capacity for all flake

Table 13. Emulsification Capacity of modified Nagano process protein fractions[a]

Flake variety and fraction	Emulsification Capacity (g oil/g product)
Glycinin fraction	
Control	143[a]
High cysteine	218[b]
High oleic	368[c]
Intermediate fraction	
Control	225[a]
High cysteine	246[b]
High oleic	299[c]
β-conglycinin fraction	
Control	890[a]
High cysteine	679[b]
High oleic	N/A

[a] n = 3. Means in the same fraction with different letters are significantly different at $p < 0.05$

varieties was significantly lower than the emulsification capacity of the β-conglycinin-rich fraction. This finding is in accordance with Yamaguchi et al., (1991) that β-conglycinin has shown to possess greater emulsifying properties than glycinin, probably due to that it is a more hydrophobic protein when compared to glycinin. Control β-conglycinin had a much

larger emulsification capacity than the high cysteine β-conglycinin. The high oleic flake glycinin fraction showed better emulsifying capacity compared to the glycinin fractions of the other two varieties, possibly due to its lower purity. The high oleic variety had about 10% less glycinin in its glycinin-rich fraction, and a higher percentage of β-conglycinin than the other two varieties tested in this study. Unfortunately, there was not enough sample for testing emulsification capacity on the β-conglycinin-rich fraction of the high oleic flakes. The high oleic intermediate fraction showed a significantly better emulsifying capacity compared to the intermediate fractions of the other two varieties. This observation is interesting because all of the intermediate fractions from the different varieties have similar glycinin and β-conglycinin profiles and protein yields. The better emulsification capacity on the high oleic intermediate fraction may be do to the low PDI of the high oleic flakes. A low PDI is associated with higher denaturation that, would in turn, result in a higher surface hydrophobicity because of unfolding of the proteins, thus improving the emulsification capacity (Kinsella 1979).

Isoflavones

In this study, isoflavones were quantified for the insoluble, glycinin, intermediate, β-conglycinin, and whey fractions. Isoflavones were not measured in the high oleic β-conglycinin fraction because there was not enough β-conglycinin produced from the high oleic flakes to measure isoflavones. In this study, in general, the control flakes contained a significantly higher concentration of isoflavones at 6.44 μmol/g than the high cysteine flakes at 5.23 μmol/g and the high oleic flakes at 6.01μmol/g. High concentrations of isoflavones were found in the glycinin fractions compared to the ß-conglycinin fractions (Table 14). The highest concentration of isoflavones was found in the intermediate fractions of all flakes. The cysteine intermediate fraction contained a significantly lower concentration of isoflavones than the intermediate fraction of the oleic and control intermediate fraction. The mass yield of isoflavones for the control, high oleic, and high cysteine beans were 374, 398, and 389 μmoles, respectively.

Table 14. Total isoflavone contents of modified Nagano process protein fractions[a]

Flake variety and fraction	Total Isoflavones (μmol/g)
Starting material	
Control	6.44[a]
High cysteine	5.23[b]
High oleic	6.01[c]
Insoluble fraction	
Control	3.83[a]
High cysteine	3.41[b]
High oleic	3.88[a]
Glycinin fraction	
Control	7.04[a]
High cysteine	6.27[b]
High oleic	6.41[a,b]
Intermediate fraction	
Control	11.59[a]
High cysteine	9.93[b]
High oleic	11.42[a]
β-conglycinin fraction	
Control	2.46[a]
High cysteine	1.94[a]
High oleic	N/A
Whey fraction	
Control	3.85[a]
High cysteine	4.49[b]
High oleic	3.36[c]

[a] n = 3. Means in the same fraction with different letters are significantly different at $p < 0.05$

Rickert et al., (2003) reported similar isoflavone concentrations for the insoluble, glycinin, intermediate, β-conglycinin, and whey fractions. The isoflavone concentrations in the Rickert et al., (2003) study were reported slightly higher in all fractions than the isoflavone concentrations in my study, but follow the same trend as my concentration values. We both find the intermediate fraction to have the highest concentration of isoflavones (μmol/g, dry basis), followed by the glycinin, insoluble and whey fractions, and the lowest concentration of isoflavones found in the β-conglycinin fractions. I would expect to have similar results as Rickert et al., (2003) for the control flakes because both studies used the Cargill 2000 flakes as the starting material

Table 15. Isoflavone profile of fractionation products for modified Nagano process (μmol/g).[a]

Product	Control	High Cysteine	High Oleic
Starting Flakes			
β-glucosides			
Daidzin	0.79[a]	0.18[b]	0.78[a]
Glycitin	0.18[a]	0.17[a]	0.33[b]
Genistin	1.29[a]	0.36[b]	1.17[a]
Malonyl-β-glucosides			
Malonyldaidzin	1.14[a]	1.57[b]	1.22[a]
Malonylglycitin	0.30[a]	0.46[a]	0.30[a]
Malonylgenistin	2.49[a]	2.33[a]	1.44[b]
Acetyl-β-glucosides			
Acetyldaidzin	0.06[a]	0.06[a]	0.05[a]
Acetylglycitin	0.16[a]	0.09[a]	0.11[a]
Acetylgenistin	0.11[a]	0.11[a]	0.08[a]
Aglucons			
Daidzein	0.02[a]	0.02[a]	0.20[b]
Glycitein	0.02[a]	0.02[a]	0.05[a]
Genistein	0.01[a]	0.01[a]	0.31[b]
Insoluble residue			
β-glucosides			
Daidzin	0.49[a]	0.45a	0.61[b]
Glycitin	0.15[a]	0.19[a]	0.22[a]
Genistin	0.73[a]	0.95[b]	0.99[b]
Malonyl-β-glucosides			
Malonyldaidzin	0.74[a]	0.45[b]	0.34[b]
Malonylglycitin	0.12[a]	0.16[a]	0.10[a]
Malonylgenistin	0.90[a]	0.80[a]	0.47[b]
Acetyl-β-glucosides			
Acetyldaidzin	0.01[a]	0.01[a]	0.01[a]
Acetylglycitin	0.06[a]	0.02[a]	0.02[a]
Acetylgenistin	0.03[a]	0.03[a]	0.03[a]
Aglucons			
Daidzein	0.21[a,b]	0.11[a]	0.34[b]
Glycitein	0.05[a]	0.05[a]	0.09[a]
Genistein	0.32[a]	0.21[a]	0.66[b]

Table 15. Continued

Product	Control	High Cysteine	High Oleic
Glycinin rich fraction			
β-glucosides			
Daidzin	0.06[a]	0.05[a]	0.04[a]
Glycitin	0.05[a]	0.31[b]	0.02[a]
Genistin	0.15[a]	0.16[a]	0.10[a]
Malonyl-β-glucosides			
Malonyldaidzin	1.47[a]	0.72[b]	0.70[b]
Malonylglycitin	0.23[a]	0.23[a]	0.17[a]
Malonylgenistin	2.72[a]	2.18[b]	1.35[c]
Acetyl-β-glucosides			
Acetyldaidzin	0.07[a]	0.04[a]	0.01[b]
Acetylglycitin	0.03[a]	0.15[b]	0.02[a]
Acetylgenistin	0.17[a]	0.12[a]	0.03[b]
Aglucons			
Daidzein	0.75[a]	0.64[a]	1.25[b]
Glycitein	0.20[a]	0.25[a]	0.37[b]
Genistein	1.14[a]	1.41[b]	2.37[c]
Intermediate fraction			
β-glucosides			
Daidzin	0.39[a]	0.23[b]	0.43[a]
Glycitin	0.16[a]	0.16[a]	0.24[b]
Genistin	0.98[a]	0.86[a]	1.16[b]
Malonyl-β-glucosides			
Malonyldaidzin	2.18[a]	0.99[b]	0.74[b]
Malonylglycitin	0.32[a]	0.30[a]	0.21[b]
Malonylgenistin	3.70[a]	2.54[b]	1.47[c]
Acetyl-β-glucosides			
Acetyldaidzin	0.06[a]	0.01[b]	0.01[b]
Acetylglycitin	0.08[a]	0.31[b]	0.16[a,b]
Acetylgenistin	0.13[a]	0.07[b]	0.03[b]
Aglucons			
Daidzein	1.38[a]	1.30[a]	2.27[b]
Glycitein	0.23[a]	0.34[a,b]	0.48[b]
Genistein	1.99[a]	2.81[b]	4.21[c]

Table 15. Continued

Product	Control	High Cysteine	High Oleic
β-conglycinin fraction			
β-glucosides			
Daidzin	0.03[a]	0.04[a]	N/A
Glycitin	0.02[a]	0.04[a]	N/A
Genistin	0.15[a]	0.11[a]	N/A
Malonyl-β-glucosides			
Malonyldaidzin	0.44[a]	0.16_b	N/A
Malonylglycitin	0.07[a]	0.06[a]	N/A
Malonylgenistin	0.66[a]	0.35[b]	N/A
Acetyl-β-glucosides			
Acetyldaidzin	0.02[a]	0.01[a]	N/A
Acetylglycitin	0.02[a]	0.06[a]	N/A
Acetylgenistin	0.03[a]	0.03[a]	N/A
Aglucons			
Daidzein	0.37[a]	0.32[a]	N/A
Glycitein	0.06[a]	0.02[a]	N/A
Genistein	0.59[a]	0.75[b]	N/A
Whey			
β-glucosides			
Daidzin	0.36[a,b]	0.30[a]	0.45[b]
Glycitin	0.15[a]	0.21[a.b]	0.25[b]
Genistin	0.36[a]	0.39[a,b]	0.44[a,b]
Malonyl-β-glucosides			
Malonyldaidzin	1.37[a]	1.13[b]	0.63[c]
Malonylglycitin	0.20[a]	0.37[b]	0.17[a]
Malonylgenistin	1.02[a]	1.22[b]	0.54[a]
Acetyl-β-glucosides			
Acetyldaidzin	0.01[a]	0.11[b]	0.01[a]
Acetylglycitin	0.02[a]	0.12[b]	0.02[a]
Acetylgenistin	0.03[a]	0.06[b]	0.03[a]
Aglucons			
Daidzein	0.18[a]	0.26[b]	0.43[c]
Glycitein	0.02[a]	0.04[b]	0.02[a]
Genistein	0.13[a]	0.28[b]	0.37[c]

[a] n = 3. Means in the same row with different letters are significantly different at $p < 0.05$

Among the insoluble, glycinin, intermediate, β-conglycinin, and whey fractions, the Malonyl-β-glucosides predominate for all flake varieties (Table 15). Malonyl forms predominate in less processed soy products (Wang and Murphy, 1994). In my study, there was one exception, the intermediate fraction of the high oleic and high cysteine flakes, where

the aglucons predominate. Coward et al., (1998) reported that a rise in aglucon concentration was seen in foods receiving higher heat treatment when compared to foods with less heat treatment. The high oleic flakes did have a higher concentration of aglucons measured in the starting material. We know that the high oleic flakes have a lower PDI than the two other varieties and probably received more heat treatment, this could be a possible factor in the higher concentration of aglucons measured in the high oleic flakes. The date in Table 15, it is notable that all flake varieties have a higher concentration of aglucons in their intermediate fractions, than any other fractions. This is probably due to the intermediate fraction having the highest concentration of total isoflavones of all the fractions.

Saponins

In this study, saponins were quantified in the insoluble, glycinin, intermediate, β-conglycinin, and whey fractions. Saponins were not measured in the high oleic β-conglycinin fraction because there was not enough β-conglycinin product to do the extraction. In general, the saponin concentration in the control, cysteine, and oleic flakes was 5.48, 5.46, and 3.74 umol/g, respectively. In contrast with isoflavones, saponins were found in higher concentrations in the ß-conglycinin fractions of the process (Table 16), but at highest concentrations in the intermediate fractions. The mass yield of saponins for the control, high oleic, and high cysteine beans were 362, 209, and 319 μmoles, respectively. The smaller mass yield of saponins from the high oleic flakes is reflected in the intermediate fraction, where the high oleic intermediate fraction had about a 40% decreased concentration of saponinins when compared to the control and high cysteine intermediate fractions.
My results compare well with results from Rickert et al., (2003), except for concentration of total saponin in the β-conglycinin fraction. My results were higher, making the β-conglycinin fraction a richer source of saponins than the glycinin fraction. Rickert et al., (2003) showed the glycinin and β-conglycinin fractions to be similar in total saponin concentration. The data from the two studies did reflect that saponin concentration is the highest in the intermediate fraction, then the insoluble, glycinin and β-conglycinin fractions, and there are almost undetectable amounts of saponins in the whey fraction.

Table 16. Total saponin contents of modified Nagano process protein fractions[a]

Flake variety and fraction	Total Saponins (μmol/g)
Insoluble fraction	
Control	7.56[a]
High cysteine	4.85[b]
High oleic	3.81[c]
Glycinin fraction	
Control	0.782[a]
High cysteine	1.38[b]
High oleic	1.42[b]
Intermediate fraction	
Control	15.31[a]
High cysteine	15.29[a]
High oleic	9.30[b]
β-conglycinin fraction	
Control	3.56[a]
High cysteine	3.17[a]
High oleic	N/A
Whey fraction	
Control	0.295[a]
High cysteine	0.425[b]
High oleic	0.371[b]

[a] n = 3. Means in the same fraction with different letters are significantly different at $p < 0.05$

Saponin I and its DDMP-form conjugate βg are the most prevalent saponins measured in most of the fractions for all three varieties as shown in Table 17. Hu, et al. (2002) measured soysaponins V, I, II, αg, βg, and βa in foods on a μmol/g basis, and reported saponins I and βg to be the most prevalent saponins in soy products such as soybean flour, tofu, tempeh, soymilk, and soy protein isolate.

Table 17. Saponin profile of fractionation products for modified Nagano process (μmol/g).[a]

Product	Control	High Cysteine	High Oleic
Starting Flakes			
Non-DDMP[b]			
V	0.232[a]	0.394[a]	0.427[a]
I	1.230[a]	0.411[b]	0.724[c]
II	0.351[a]	0.563[a,b]	0.745[b]
DDMP			
Ag	0.190[a]	0.199[a]	0.126[a]
Bg	2.760[a]	2.954[a]	1.381[b]
Ba	0.891[a]	1.237[b]	0.448[c]
Insoluble residue			
Non-DDMP[b]			
V	0.336[a]	0.230[a]	0.483[a]
I	2.804[a]	2.713[a]	0.917[b]
II	1.571[a]	1.937[a]	0.808[b]
DDMP			
Ag	0.172[a]	0.061[a]	0.128[a]
Bg	1.875[a]	0.974[b]	1.156[c]
Ba	0.701[a]	0.406[a]	0.322[a]
Glycinin fraction			
Non-DDMP[b]			
V	0.255[a]	0.262[a]	0.299[a]
I	0.270[a]	0.613[b]	0.504[a,b]
II	0.087[a]	0.307[a]	0.218[a]
DDMP			
Ag	0.011[a]	0.037[a]	0.037[a]
Bg	0.067[a]	0.151[a]	0.242[a]
Ba	0.093[a]	0.011[a]	0.121[a]
Intermediate fraction			
Non-DDMP[b]			
V	1.047[a]	0.669[b]	0.519[b]
I	5.803[a]	6.989[b]	2.813[c]
II	2.635[a]	3.481[b]	0.951[c]
DDMP			
Ag	0.266[a]	0.175[a]	0.275[a]
Bg	3.995[a]	3.072[b]	3.652[a]
Ba	1.564[a]	0.904[b]	1.102[b]

Table 17. Continued

β-conglycinin fraction			
Non-DDMP[b]			
V	0.344[a]	0.360[a]	N/A
I	1.254[a]	0.971[a]	N/A
II	0.828[a]	0.886[a]	N/A
DDMP			
Ag	0.078[a]	0.073[a]	N/A
Bg	0.717[a]	0.593[a]	N/A
Ba	0.343[a]	0.829[b]	N/A
Whey			
Non-DDMP[b]			
V	0.183[a]	0.112[a]	0.242[a]
I	0.000[a]	0.094[b]	0.000[a]
II	0.037[a]	0.015[a]	0.069[a]
DDMP			
Ag	0.026[a]	0.017[a]	0.032[a]
Bg	0.000[a]	0.041[b]	0.000[a]
Ba	0.049[a]	0.052[a]	0.036[a]

[a] n = 3. Means in the same row with different letters are significantly different at $p < 0.05$

GENERAL CONCLUSIONS

A threshold and descriptive analysis sensory evaluation were completed for bitterness of isoflavones and saponins. From the results, it can be concluded that the DDMP-conjugated saponins and the malonyl-β-glucoside isoflavones have more off-flavor and/or are more bitter than other saponins and isoflavones found in soy. Furthermore, it would seem that less processed or heat treated soy products would have more off flavor or bitter flavor caused by isoflavones and saponins.

Further work could be done to look at saponins or isoflavones individually and assessing them to bitter flavor. There is a need for threshold values for other suspected off-flavor components in soy, so that our bitter thresholds can be compared to other bitter compounds.

The modified Nagano fractionation method was successfully executed at bench scale for three soybean varieties, and several conclusions can be drawn out of my findings. First, the genetic background and thermal history of the starting materials significantly influences protein partitioning during this process, protein purity, functionality of fractions, and isoflavone and saponin content and distribution. Further studies are to be conducted since very little is known about the behavior of different genetically modified soybeans during processing. If there would be an interest in producing these protein fractions on a larger scale, I would recommend the high cysteine flakes as a starting material. The high oleic flakes are not recommended for this fractionation process if significant amounts of a β-conglycinin enriched fraction are desired. A second problem encountered is that these flakes were difficult to centrifuge since they did not give a compact curd that allows an effective separation. As new varieties become available, it would be a good idea to fractionate them on lab scale, now that we know different varieties behave differently in the same process.

The phytase method seems promising, but further development is needed to better understand which is the mechanism of action and if different flakes with different phytic acid contents can be treated in the same way. I do not recommend immediate scaling up of this process, since it requires energy intensive steps of incubation and cooling that would be

economically impractical at this stage. The use of enzymes is a drawback for considering this process suitable for industrial purposes.

APPENDIX A

INFORMED CONSENT

Novel Approaches to New Soybean Protein Ingredients with Enhanced Health Benefits
October 2002

This research is being conducted by Dr. Patricia Murphy and Dr. Cheryll Reitmeier, Department of Food Science and Human Nutrition, Iowa State University. The evaluation involves the sensory evaluation of soy protein products containing isoflavones and saponins. These compounds may be of benefit nutritionally, but are bitter and astringent. The objective of this work is to determine the flavor thresholds for isolated soy isoflavones and saponins. Panelists will be asked to evaluate solutions containing various concentrations of soy compounds as well as foods containing the soy protein products.

The soy protein fractions were produced in the processing facility of the Center for Crops Utilization Research, ISU, under Good Manufacturing Practices. The protein and fractions are of food-grade quality. Samples for sensory evaluation will be prepared in the Test Kitchen, 2951 FSB, ISU, under sanitary conditions.

Panelists will be screened for soy allergies and not allowed to participate if allergic. Panelists will be trained in 6 hourly sessions to recognize bitter and astringent flavor characteristics. Training is tentatively scheduled for October and November 2002, with evaluation sessions to begin in November 2002. Panelists will evaluate 3 solutions of samples at each of 12 sessions.

Responses to the sensory evaluation will be used only in coded statistical analysis without reference to the respondent. Benefits include a reward of food at each session, a gift of $20.00 at the end of the sessions, and a significant contribution to improving the health benefits of soy foods. Dr. Reitmeier (294-4325) will be available throughout the study to answer questions associated with the evaluation.

Emergency treatment of any injuries that may occur as a direct result of participation in this research is available at the Iowa State University Student Health Services, and/or referred to Mary Greenley Hospital or another physician or medical facility at the location of the research activity. Compensation for any injuries will be paid if it is determined under the Iowa Tory Claims Act, Chapter 669 Iowa Code. Claims for compensation should be submitted on approved forms to the State Appeals Board and are available from the Iowa State University Office of Risk Management and Insurance.

***** I understand the research being conducted and agree to evaluate soy protein products. I understand that I should be present for all evaluation sessions. I understand that I can withdraw at any time. I will notify the investigator if I can no longer participate. *****

_____ **Name**

_____ **Date**

APPENDIX B

Panel Questionnaire

History:
Name:
E-mail:

Health:

1. Do you take any medications which affect your senses, especially taste and smell?

2. Do you have any allergies to soy foods?

Daily Living Habits:

1. How often do you eat out in a week?_____
2. What are your favorite foods?_____
3. What foods do you not like to eat?_____
4. What foods can you not eat?_____

Flavor Quiz:

1. How would you describe the difference between flavor and aroma?_____
_____
2. How would you describe the difference between flavor and texture?_____
_____
3. Describe some of the noticeable flavors in cola_____
_____

APPENDIX C

Name:_____
Date:_____

Please taste the samples and indicate whether they are sweet, sour, salty, bitter, or astringent by marking an X on the corresponding line. Please rinse with water between samples. You do not need to swallow the samples.

Sample	Sweet	Sour	Salty	Bitter	Astringent
_____	____	____	____	____	____
_____	____	____	____	____	____
_____	____	____	____	____	____
_____	____	____	____	____	____
_____	____	____	____	____	____

Comments:

APPENDIX D

Name:_____
Date:_____

Please rank each coded solution in ascending order of intensity/strength of the solution.

	Code
Least salty	_____
	_____
Most salty	_____

	Code
Least sweet	_____
	_____
Most sweet	_____

	Code
Least bitter	_____
	_____
Most bitter	_____

	Code
Least sour	_____
	_____
Most sour	_____

	Code
Least astringent	_____
	_____
Most astringent	_____

Comments:

APPENDIX E

Name:_____
Date:_____

Instructions: Please taste each sample and evaluate sample for intensity of sour, astringent, and bitter flavors, by marking a line on the intensity scale. Hold the sample in your mouth for at least 10 seconds and swallow samples if possible. You may use the special rinse between samples if it helps you. Remember to swirl the rinse before using. Please open your window if you have any problems. Please push your score sheet under the window when finished. Thank you

Sour

_____

none intense

Bitter

_____

none intense

Astringent

_____

none intense

Off Flavor

_____

none intense

Comments

APPENDIX F

NAME _____
DATE _____

DIRECTIONS: Taste each pair of samples in order, as appears on this scorecard. Indicate which sample is more **bitter** by circling the sample number which corresponds to the more bitter sample. Hold the sample in your mouth for at least ten seconds and swallow if possible. You may use the special rinse between samples if it helps you. Remember to swirl the rinse before using. **You may take a break and leave the panel room if you wish.** Please open your window if you have any problems. Please push your scorecard under your window when finished. Thank you.

Milk samples

1. _____ _____

2. _____ _____

3. _____ _____

4. _____ _____

5. _____ _____

6. _____ _____

Comments:

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