IOWA STATE UNIVERSITY
Digital Repository

Retrospective Theses and Dissertations

Iowa State University Capstones, Theses and Dissertations

2003

Effects of processing on soybean glycinin, Î²-conglycinin, isoflavones, and saponins

David Alan Rickert
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Agriculture Commons, and the Food Science Commons

Recommended Citation
Rickert, David Alan, "Effects of processing on soybean glycinin, Î²-conglycinin, isoflavones, and saponins " (2003). *Retrospective Theses and Dissertations*. 743.
https://lib.dr.iastate.edu/rtd/743

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

المنارة للاستشارات

www.manaraa.com

Effects of processing on soybean glycinin, β-conglycinin, isoflavones, and saponins

by

David Alan Rickert

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Program of Study Committee:
Patricia A. Murphy, Major Professor
Lawrence A. Johnson
Anthony L. Pometto III
Suzanne Hendrich
Charles E. Glatz

Iowa State University

Ames, Iowa

2003

Copyright © David Alan Rickert, 2003. All rights reserved.

UMI Number: 3118257

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform 3118257

Copyright 2004 by ProQuest Information and Learning Company.

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

David Alan Rickert

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

TABLE OF CONTENTS

ABSTRACT vi

CHAPTER 1. GENERAL INTRODUCTION 1
Introduction 1
Dissertation Organization 4
Literature Review 4
A. Soybean Production, Soy Food Sales, and Perception of Soy 4
B. Composition of Soybeans and Soy Products, Advances in Soybean Composition 7
C. Soy Foods, Soy Ingredient Production, and Cost of Some Soy Ingredients 9
D. Soy Consumption: Concerns and Potential Benefits 13
Soy Allergen 13
Protease Inhibitors 13
Dehydroalanine and Formation of Lysinoalanine 15
Oligosaccharides 16
Phytate 17
Saponins 18
Isoflavones 27
Isoflavones, Soy, and Menopausal Symptoms 38
Isoflavones, Soy, and Cardiovascular Health 39
Isoflavones, Soy, and Osteoporosis 40
Isoflavones, Soy, and Cancer 41
E. Quantification of Isoflavones and Saponins and Preparation of Enriched Fractions 42
Isoflavone Quantification 42
Saponin Quantification 44
Preparation of Enriched Fractions 45
F. Soy Protein Structure 47
G. Fractionation of Soy Proteins 48
H. Soy Protein Functionality 56
Solubility 57
Gelation 58
Emulsification Capacity 61
Emulsification Activity and Stability 64
Foaming 67
Surface Hydrophobicity in Relation to Protein Functionality 69
Viscosity 73
Conclusions 76
I. Conclusions 77
References 77

CHAPTER 2. SOY PROTEIN, ISOFLAVONE, AND SAPONIN PARTITIONING DURING PILOT-SCALE FRACTIONATION OF GLYCININ AND β-CONGLYCININ 91
Abstract 91
Introduction 91
Materials and Methods 93
Results and Discussion 98
Acknowledgments 116
References 116

CHAPTER 3. ISOFLAVONE AND SAPONIN PARTITIONING DURING BENCH-SCALE SOY ISOLATE PRODUCTION: EFFECTS OF TEMPERATURE AND pH 119
Abstract 119
Introduction 119
Materials and Methods 121
Results and Discussion 124
Conclusions 146
Acknowledgments 148
References 148

CHAPTER 4. PILOT-SCALE FRACTIONATION OF GLYCININ AND β-CONGLYCININ: PROCESS IMPROVEMENT 151
Abstract 151
Introduction 151
Materials and Methods 153
Results and Discussion 162
Acknowledgments 195
Literature Cited 195

CHAPTER 5. PILOT-SCALE FRACTIONATION OF SOY STORAGE PROTEINS: FUNCTIONAL PROPERTIES 198
Abstract 198
Introduction 198
Materials and Methods 201
Results and Discussion 208
Conclusions 232
Acknowledgment 232
References 232

CHAPTER 6. CONCLUSIONS 236

APPENDIX A. ANOVA TABLES FOR CHAPTER 3 TOTAL ISOFLAVONE AND SAPONIN CONCENTRATION DATA 239

APPENDIX B. MICROBIAL LOAD OF DEFATTED SOYBEAN FLAKES AND PILOT-SCALE PROCESS FRACTIONS 243

ABSTRACT

Processing effects on soy storage proteins, glycinin and β-conglycinin, as well as isoflavones and group B soyasaponins, protein-associated phytochemicals, were evaluated. It was hypothesized that the physicochemical characteristics of these molecules, such as protein native state, hydrophobicity, or ionization, would affect their partitioning during processing and, for the proteins, affect functional properties.

A pilot-scale soy protein fractionation process, yielding glycinin, β-conglycinin, and an intermediate fraction comprised of a mixture of these proteins, was scaled up from 15 kg to 50 kg of defatted soy white flake starting material in order to produce larger quantities of protein products. The scaled-up process recovered 12% less of the initial protein in the product fractions compared to the 15-kg process, but 2.4 times more total protein were recovered in the scaled-up process product fractions. The insoluble fraction, a waste stream, retained 59, 67, and 75% of the protein, isoflavones, and saponins, respectively. The whey contained 6% of the protein and 12% of the isoflavones, but almost no saponins. The saponin to isoflavone ratio was about 2:1 in the β-conglycinin, but about 1:1 in the glycinin. These data demonstrate differences in phytochemical partitioning, possibly due to physicochemical characteristics such as hydrophobicity and ionization. The isoflavone profile shifted from malonylglucosides toward aglucon forms, possibly due to the action of native β-glucosidases.

Solubilization temperature and pH influenced protein and phytochemical recovery and partitioning during bench-scale soy protein isolate production. Increasing temperature from 25 to 60 °C and pH from 8.5 to 10.5 increased extraction of phytochemicals from the soy flakes. Saponin concentration increased in the isolate. Neutralizing samples prior to phytochemical extraction significantly increased measured saponin concentrations in the isolate and measured isoflavone concentrations in the insoluble fraction. Increasing temperature and pH shifted isoflavone profile from malonylglucosides to glucosides and saponin profile from DDMP saponins to non-DDMP saponins.

Extraction pH, ethanol concentration, water-to-soy flake ratio, and temperature were evaluated during bench-scale protein fractionation for ability to improve protein and phytochemical solubilization and recovery. A 10:1 water-to-flake ratio and 45 °C were selected as optimized solubilization conditions. When compared to the control (15:1 water-to-flake ratio and 20 °C) under pilot-scale conditions, the optimized method produced more β-conglycinin, but at the expense of purity. The optimized method displayed increased saponin extraction from the soy flakes. A portion of the malonylglucoside isoflavones were converted to β-glucoside and aglucon forms.

Protein products from the optimized and control pilot-scale fractionation processes were compared for functionality. Optimized process intermediate and β-conglycinin fractions, which had higher surface hydrophobicity than the corresponding control fractions, exhibited significantly better emulsification activity compared to those for the control process. Because the β-conglycinin fractions had a lower denaturation temperature than the glycinin fractions, and denaturation of the protein is required as a first step in gel formation, the β-conglycinin fractions gelled at a lower temperature than did glycinin. The β-conglycinin fractions made firmer gels than did glycinin fractions at pH 3 and 7. The intermediate fractions, which contained little native-state protein, demonstrated higher apparent viscosities than the soy protein isolate and other process fractions, probably due to the increased molecular interaction of the unfolded, non-native state protein.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Soybeans are an extremely valuable agricultural resource, providing oil with both food and industrial applications and a concentrated source of high-quality food protein with a myriad of functional applications in food systems. Some functional aspects of soy ingredients are foaming, emulsification, solubility, hydrophobicity, water binding, fat binding, and gelling. While soybeans have certain anti-nutritional aspects, such as trypsin inhibitors and oligosaccharides, mounting evidence suggests that several components, such as isoflavones, saponins, and even the soy proteins, acting alone or in a synergistic fashion, may be important factors providing positive health consequences when consumed as part of a healthy diet.

Developing an in-depth understanding of the soy proteins is critically important. Physicochemical properties of glycinin and β-conglycinin, the major storage proteins in soy and major components of the 11S and 7S soy protein fractions, respectively, influence the quality and characteristics of traditional soy foods, such as tofu, as well as the functionality of commercially-produced soy ingredients, such as soy concentrates and soy protein isolates. A considerable body of research has been directed toward isolating and characterizing these proteins to better understand how each of these proteins influences food systems and to evaluate how each protein or combinations of the proteins, whether modified or unmodified, could provide useful, unique functionalities in food systems.

One early study (Briggs and Mann 1950) characterized soy storage proteins using electrophoretic analysis. The authors found evidence that the globulin termed "glycinin" by Osborne and Campbell (1898), commonly considered to be the principle soy storage protein, was actually a mixture of components and the composition of the components differed depending on method of isolation. Wolf and Sly (1967), Eldridge and Wolf (1967), and Wolf and Briggs (1959) have described bench-scale methods to isolate 11S proteins. Roberts

and Briggs (1965) have described what they termed a "large-scale preparation", starting with 1300 g of defatted soy flakes, to isolate 7S proteins. These methods did permit the isolation of enriched or purified fractions and provided information concerning protein characterization, but the methods were not convenient for sequential fractionation of the 11S and 7S proteins. However, Thanh and others (1975), Thanh and Shibasaki (1976), O'Keefe and others (1991), and Nagano and others (1992) have all described bench-scale methods for the sequential fractionation of 11S and 7S proteins and include some protein characterization information. Pilot-scale methods for sequential fractionation of these proteins have been disclosed in U.S. patents awarded to Davidson and others (1979), Lehnhart and others (1983), and Howard and others (1983). The advantage of pilot-scale fractionation is that production of kilogram quantities of material permits more exhaustive evaluation of protein functionality characteristics, nutritional aspects, and product applications of the protein fractions.

Significant advances have been made toward developing a commercially feasible glycinin and β-conglycinin fractionation process at Iowa State University. Previously, Dr. Shaowen Wu developed a bench-scale process, a modification of the process used by Nagano and others (1992), for fractionation of glycinin and β-conglycinin from defatted soy flakes. Wu's process was scaled up for use in the Iowa State University Center for Crops Utilization Research Pilot Plant (Wu and others 1999), using 15 to 20 kilograms of starting material. Subsequent scale-up, from 20 kg of starting material to 50 kg of starting material, was performed in the same facility to produce a sufficient quantity of β-conglycinin such that the protein could be used in a human feeding study to evaluate the effects of β-conglycinin on serum cholesterol and lipid levels.

These pilot-scale fractionation runs have provided topics for further research. One obvious shortcoming of the pilot-scale fractionation processes was the poor initial solubilization of protein from the starting material and low yield of protein end products, so improving initial protein solubilization has been a priority. Since soybean isoflavones and saponins, soy protein-associated phytochemicals, may have significant, positive influences

on human health, there is interest in determining the concentrations of these valuable isoflavones and saponins being partitioned into the fractionation process streams and end products, and how the concentrations in end products differ from that in commercially-available products, such as soy protein isolate. Of course functionality aspects of the protein fractions produced in the pilot plant are of interest: 1) from a research point of view to develop additional understanding how glycinin and β-conglycinin may each affect or contribute to food systems and 2) from an industrial point of view to identify end-products with potential as new, unique ingredients.

A central hypothesis dealing with the above issues can be stated as follows. Physicochemical properties of the soy storage proteins, glycinin and β-conglycinin, and the protein-associated soy phytochemicals, isoflavones and saponins, will influence the solubility and recovery of these co-products during soy protein fractionation and soy protein isolate production, and changes in protein structure due to process parameters will affect partitioning and functionality of the protein products.

Four main research questions will be addressed in this body of work. First, do isoflavones and saponins partition differently during bench-scale isolate production, bench-scale protein fractionation, and pilot-scale protein fractionation processes, and how can partitioning be influenced or directed? Secondly, can the bench-scale protein fractionation process be optimized with respect to initial protein recovery, protein end-product partitioning, and phytochemical recovery? Third, how does scale up affect the partitioning of the soy proteins and the protein-associated phytochemicals? And finally, how do pilot-scale protein fractionation process end-products produced by the original and optimized methods, as well as soy protein isolate produced in the pilot plant from the same starting material, differ among one another in terms of protein functionality?

Dissertation Organization

This dissertation consists of a literature review, in the format of the *Journal of Food Science*, and four papers, each paper being presented as a chapter. The first paper, "Soy Protein, Isoflavone, and Saponin Partitioning During Pilot-Scale Fractionation of Glycinin and β-Conglycinin," will be submitted to the *Journal of the American Oil Chemists' Society*. The second paper, "Isoflavone and Saponin Partitioning During Bench-Scale Soy Isolate Production: Effects of Temperature and pH," will be submitted to the *Journal of Food Science*. The third paper, to be submitted to the *Journal of Agricultural and Food Chemistry*, is "Pilot-Scale Fractionation of Glycinin and β-Conglycinin: Process Improvement." Desired process improvement outcomes included increased recoveries of total protein and phytochemicals in product fractions without sacrificing purity of the glycinin- and β-conglycinin-rich protein fractions. The fourth paper, "Pilot-Scale Fractionation of Soy Storage Proteins: Functional Properties," evaluates the functionality of protein products from a control fractionation process, an optimized fractionation process, and a soy protein isolate process, all produced from the same starting material. The fourth paper will be submitted to the *Journal of Food Science*. A general conclusion follows the four papers. Appendix A displays ANOVA tables for isoflavone and saponin concentration comparisons for Chapter 3. Appendix B shares data about the microbial load of the protein products prepared in the pilot plant as described in Chapter 4.

Literature Review

A. Soybean Production, Soy Food Sales, and Perception of Soy

World soybean production has increased from about 132 million metric tons in 1996-97 to about 184 million metric tons in 2001-02 (Soya & Oilseed Bluebook 2003). During this same time frame, U.S. production increased from about 65 to 79 million metric tons. In

2000, the U.S. produced 45% of the world soybean crop, with Brazil, Argentina, and China producing 21, 15, and 9%, respectively (United Soybean Board 2001). Approximately 98% of the 43 million metric tons of soybean meal produced each year is primarily used for animal feed. The other two percent receives additional processing to prepare soy food ingredients and soy products.

Consumption of traditional soy foods and foods prepared with soy ingredients has increased dramatically in the U.S., with U.S. soyfood sales totaling $2.77 billion in 2000 and increasing by 16.8% in 2001 to $3.2 billion according to a study by SPINS and Soyatech, Inc. (2002). *Soyfoods: Trends and Developments,* a study by Business Communications Co. Inc., Norwalk, Conn. (2001), predicts that soy food sales will top $6.9 billion in 2005. They also project that the soy ingredient market, which includes soy protein, soy fiber, and soy isoflavones, will grow from $523.3 million in 2000 to $659.2 million in 2005. Soymilk sales exceeded $550 million in 2001 and meat alternative sales experienced about $440 million in retail sales in 2000 (Wrick 2003). LMC International (1999) projected that the soy sports supplements and powders market segment will grow 20% per year from 2000 to 2005. LMC also reports that sales of nutritional beverages, nutraceuticals and supplements, nutrition bars, soymilks, and meat analogs are all growing at over 10% per year.

Clearly, there is an enthusiastic market for soy foods and soy ingredients. There are several reasons for this popularity. First, soy has long been recognized as an abundant source of high-quality protein that is generally cost efficient on a protein basis, promoting its use in foods needing protein fortification or in regions whose populations have chronic dietary protein deficiency. The protein digestibility corrected amino acid score (PDCAAS), the official assay of the World Health Organization and the United States Department of Agriculture for evaluating protein quality, for soy protein is 1.0. A food with a score of 1.0 or higher indicates that it is capable of meeting the amino acid requirements for 2- to 5-year-old children, the segment of the population having the highest requirement. This means that soy protein can meet the protein needs of adults when it is consumed at the recommended intake of 0.6 g/kg body wt. (Young 1991). Friedman and Brandon (2001) do express

concerns, however, that methionine and lysine levels may be marginal in some instances. Methionine availability in some plant proteins is low, probably due to lower digestibility (Begbie and Pusztai 1989). Another factor that may affect methionine availability is chemical modification during storage and processing (Oste 1991). Fortification of a soymilk formula with methionine increased nitrogen retention in malnourished children (de Oliviera and others 1998). Lysine availability may also be lost during storage and processing due to Maillard reactions (Friedman 1982) and during processing at high pH due to formation of lysinoalanine (Mao and others 1993).

Second, a wide variety of soy ingredients is available, and they have useful functional properties that have permitted food manufacturers to select a soy ingredient for a specific functional purpose or as an inexpensive replacement for other food proteins. Functional properties of proteins in food were defined by Kinsella (1976) as "those physical and chemical properties that affect the behavior of proteins in food systems during processing, storage, preparation and consumption." Functional roles of food proteins include foaming, emulsification, solubility, viscosity, cohesion, adhesion, water binding, fat binding, flavor binding, and gelling.

Third, traditional soy foods are more available in grocery stores and consumers are now more accepting of soy ingredients in foods. Factors influencing this availability and acceptance may be the increase in popularity of Asian foods in the U.S., increase of Asians comprising the U.S. population, and the increase in people choosing plant-based foods. Soyfoods.com (2003) reported data from consumer surveys showing that 55% of participants in 1991 said they were aware of soy protein in foods, compared with 79% in 1996, while other data from that website stated that 25.4% of consumers surveyed plan to use more soy foods in the future than they now use.

Possibly the most significant reason consumers are choosing soy is that soy has become recognized as a nutraceutical or functional food. A functional food is a food or food ingredient that may provide a health benefit beyond simple nutrition, whereas a nutraceutical may be defined as a food or food component that provides medical or health benefits, such as

the prevention or treatment of a disease (ISU University Extension 2000). Such components in soy may be isoflavones and saponins, phytochemicals found in soy, or even the soy proteins themselves. Aging baby boomers, a significant portion of the population, may be more interested in living a healthy lifestyle than previous generations. Ten percent of shoppers surveyed in 1998, versus three percent in 1996, were including soy as part of a healthy diet because they felt it might reduce the risk of disease (Henkel 2000).

B. Composition of Soybeans and Soy Products, Advances in Soybean Composition

This section provides basic information about the chemical composition of soybeans and a few major soybean products. Liu (1997) provides more detailed information. Tables 1-4 contain soybean composition information.

Table 1. Soybean and soybean component proximate analyses presented on a dry weight basis

Component	Yield (%)	Protein (%)	Fat (%)	Ash (%)	Carbohydrates (%)
Whole soybeans	100.0	40.3	21.0	4.9	33.9
Cotyledon	90.3	42.8	22.8	5.0	29.4
Hull	7.3	8.8	1.0	4.3	85.9
Hypocotyl	2.4	40.8	11.4	4.4	43.4

Source: Perkins (1995)

It is important to note that plant breeders have been making progress toward altering the compositional analysis of soybeans. Liu (1997) includes a chapter titled "Soybean Improvements Through Plant Breeding and Genetic Engineering," which provides references and comments on breeding to increase oil content and profile; to increase protein content; to alter 11S/7S storage protein ratio; to alter amino acid composition for protein quality; to

reduce the concentration of trypsin inhibitors, oligosaccharides, phytates, and lipoxygenase; and to increase isoflavone content.

Table 2. Proximate analyses of soybeans and soybean products

Material	Moisture (%)	Protein (%)	Fat (%)	Fiber (%)	Ash (%)
Soybeans	11.0	37.9	17.8	4.7	4.5
Defatted meal dehulled	10.7	47.5	0.5	3.5	6.0
Full-fat soy flour	5.0	44.3	21.0	2.0	4.9
Soy protein concentrate	7.5	66.6	-	3.5	5.5
Soy protein isolate	5.0	93.1	-	0.2	4.0

Adapted from Perkins (1995)

Table 3. Carbohydrate content of dehulled, defatted soybean flakes from cotyledons

Carbohydrate	% wt
Monosaccharides	
Glucose	trace
Oligosaccharides	
Sucrose	5.7
Raffinose	4.1
Stachyose	4.6
Polysaccharides	
Arabinan	1.0
Arabinogalactan	8-10
Acidic polysaccharides	5-7

Adapted from Smith (1972)

Table 4. Primary inorganic components of soybeans: ranges from 12 selected varieties

Potassium	1.5 to 1.92%
Sodium	0.4 to 0.61%
Phosphorous	0.352 to 0.733%
Magnesium	0.094 to 0.208%
Calcium	0.024 to 0.063%
Iron	0.0044 to 0.0163%

Source: Perkins (1995)

High sucrose beans are an example of a potentially successful and economically beneficial alteration of soybean composition. High sucrose beans contain 40% more sucrose and 90% less raffinose and stachyose than regular soybeans. Drastically reduced concentrations of these oligosaccharides that cause flatulence and intestinal discomfort in humans could revolutionize soy protein ingredient manufacturing. There were 4500 acres of high sucrose beans contracted for production in Iowa for the 2003 season by Protein Technologies International (Illinois Specialty Farm Products 2003). These beans are reported to yield 85-90% of normal soybean yields, but farmers receive a $0.90/bu premium.

New soybean compositions, produced by traditional and genetically engineered plant breeding, will undoubtedly open niche markets for specialty soybeans that will further increase the demand for soy products and soy ingredients.

C. Soy Foods, Soy Ingredient Production, and Cost of Some Soy Ingredients

Wang and Murphy (1994) divided soy foods into three categories: soy ingredients, traditional soy foods, and second-generation soy foods. Murphy and Hendrich (2001) suggest a fourth category, foods with soy as a functional ingredient. Examples of traditional soy foods are soymilk, tofu, miso, natto, and tempeh. These products are generally made from whole soybeans, but some soymilk and tofu processes are exceptions to this and may

use soy flakes as the starting material. Unprocessed soybeans, soy flours, soy protein concentrates, soy protein isolates, and textured soy protein are examples of soy ingredients. Second-generation soy foods may be described as those food items containing significant amounts of soy and manufactured to resemble the traditional food item in appearance, texture, taste, and flavor. Examples are soy-based hot dogs and hamburgers, other soy-based meats, and soy-based cheese. The final category, foods with soy as a functional ingredient, covers applications where soy protein is used to replace other food proteins, such as replacing milk protein in infant formula and wheat protein in baked goods.

The starting material for soy ingredients, second-generation soy foods, and in some cases functional ingredients is defatted soy flakes. The flakes are a by-product of the soy oil recovery process. In brief, soybeans are cleaned, conditioned to about 10-11% moisture, cracked to remove the hulls, milled to produce grits, and then the grits are rolled into flakes. The crude oil is then extracted from the flakes, usually using an organic solvent. Removal of the solvent from the flakes is a critical step and greatly influences the usefulness of the desolventized flakes. Flakes that will be used for animal feeds are toasted to remove residual solvent. However, this denatures the proteins, greatly reducing protein solubility, and imparts undesirable odor, flavor, and appearance characteristics. White flakes, the usual starting material for defatted soy flours, soy protein concentrates, and soy protein isolates, are produced by employing super-heated streams of hexane vapors and vacuum to quickly remove the solvent. This rapid removal of the solvent limits the exposure of the flakes to heat and produces flakes with a higher protein dispersibility index (PDI), meaning that the proteins in the flakes can be more easily solubilized during additional processing of the flakes. Flakes having a PDI as high as 90-95 (high protein solubility) and as low as 20 (low protein solubility) are commercially available. High PDI white flakes are used where high solubility of the protein is desired, such as in the preparation of soy protein isolate. Low PDI flakes will have low protein solubility, but the protein is still able to bind fat and water. These characteristics are useful in some meat applications. The object is to produce soy ingredients with the desired functional characteristics. Examples of functional characteristics

are solubility, foaming capacity, foaming stability, emulsification capacity, emulsification stability, water and fat binding capacities, gel strength, and viscosity.

Soy flour is generally produced by grinding the defatted flakes, and it is used as a protein supplement and functional ingredient. High enzyme flours with viable lipoxygenase, produced from defatted flakes with a minimum heat treatment, are used in baking applications to increase dough mixing tolerance and promote bleaching in bread products. On a moisture-free basis defatted soy flour is about 52-54% protein. Defatted flours receive varying moist heat treatments to provide products with a range of solubility, depending on desired application. Soy flour may also be made from dehulled soybeans, producing a full-fat flour. The protein content of these flours is generally about 40% on a moisture-free basis, and they are used primarily in the baking industry and for the production of soy milks.

Soy protein concentrate is produced by removing soluble carbohydrates, such as raffinose and stachyose, from defatted soy flakes. The carbohydrates are leached from the flakes using a weak acid solution at about pH 4.5, a 70-90% aqueous alcohol solution, or moist heat followed by hot water. Each process provides a concentrate with different functional properties and flavor profile. Concentrates must be at least 65% protein (6.25 correction factor multiplied by nitrogen content) on a moisture-free basis. They are used in systems requiring emulsifiers, emulsion stabilizers, and water and fat binders. Food system examples are meat systems, gravies, and baked goods.

Soy protein isolates are also made from defatted soy flakes. Isolates are the highest protein soy ingredient product, produced by the removal of most of the fiber, soluble carbohydrates, and ash. The protein is solubilized from flakes as they are mixed in a mild alkali solution at pH 8 to 9 at up to 80 °C. Insoluble material is removed by centrifugation. The soluble soy protein is then precipitated by lowering the pH of the solution to approximately 4.5, near the isoelectric point of most soy proteins. The resulting precipitate, or curd, is removed by centrifugation, eliminating soluble carbohydrates. The curd may then be neutralized with NaOH or KOH to enhance solubility and functionality after spray drying. Soy protein isolates must be at least 90% protein on a moisture free basis and using a 6.25

correction factor. They are used in a wide variety of food systems requiring emulsifiers, emulsion stabilizers, water and fat absorption, and fiber-forming properties.

Textured vegetable proteins (TVP) are produced by thermoplastic extrusion or steam texturization of soy flours or soy protein concentrates; extrusion of concentrates yielding different sized and shaped pieces; or extrusion of isolate into an acid or salt bath that coagulates protein into fibers. If binders are present in the acid or salt baths the fibers can be formed into fiber bundles or structured isolates. TVP must be 50% protein on an "as is" basis and contain no more than 10% moisture. TVP may be used as an ingredient in ground meat products, providing texture as well as water and fat binding capability. The structured isolates are often used as food analogs.

When a soy protein product can fill the functional and nutritional role of another protein source, such as egg whites, meat, milk, or wheat proteins, cost also becomes a driving factor for the replacement. Whereas wheat flour costs only $228 per ton compared to $289 per ton for defatted soy flour (American Soybean Association 2003a), the cost on a price per pound of protein basis is $1.00 vs. $0.25, respectively. Price per ton and price per pound of protein for soy protein concentrate, soy isolate, and soy TVP are $2150 and $1.39 (American Soybean Association 2003b), $2750 and $1.36 (American Soybean Association 2003c), and $396 and $0.30 (American Soybean Association 2003d), respectively. This should not imply that all soy products are inexpensive. Soymilk may be as much as 2-3 times as expensive as dairy milk, and many of the meat analogs are quite expensive compared to the conventional products with which they are competing.

The large variety of soy foods and soy ingredients described above display a means by which soy has been incorporated into the U.S. diet. However, the reported health benefits of soy have been an influential component of the recent enthusiasm for consumers to seek out soy foods or foods containing soy ingredients and for food manufacturers to gladly meet the resulting increased demand for soy by incorporating soy ingredients into their product formulations. The nutraceutical, functional food, and phytochemical aspects of soy, all related to the potential health benefits of soy, are covered in the next section.

D. Soy Consumption: Concerns and Potential Benefits

This section will provide a very brief overview of the potentially harmful and beneficial aspects of soy components in the human diet.

Soy Allergen

Soybeans are included in the list of major food allergens, as are cow's milk, egg, fish, crustacean shellfish, tree nuts, wheat, and peanuts. Glycinin and β-conglycinin, as well as nearly all of the subunits that comprise these soy proteins, have been implicated in eliciting an allergenic response in some humans. Use of infant soy formula is quite common in the U.S. Concern has been expressed that exposure to soy at an early age could lead to an increase in soy allergies. However, at least one study has shown that soy formulas rarely elicit allergic reactions in infants (Cantani and Lucenti 1997). Inducement of allergic response in children can occur, but cost and use for those infants suffering from cow's milk allergenic response drive the popularity of soy-based infant formulas as a breast-milk alternative. As mentioned above, soy is becoming more prevalent as an ingredient in foods such as bread, muffins, cake mixes, cookies and bars, cereals, cooking spray, frozen desserts, meat and meat substitutes, pancake mixes, sauces and toppings, and soups, making it increasingly difficult for someone with a soy allergy to avoid soy. Perkin (1990) offers a short list of examples of common, brand name food items, which contain soy proteins.

Protease Inhibitors

The Kunitz (MW 20 kDa) and Bowman-Birk (MW 6–10 kDa) protease inhibitors bind to and inactivate trypsin and trypsin plus chymotrypsin, respectively. Table 5 shows the concentration of Kunitz and Bowman-Birk inhibitors in a few soy ingredients and products. Since trypsin and chymotrypsin are bound by the inhibitors and inactivated, exogenous dietary protein absorption declines. Rats fed diets with soy protease inhibitors showed poor weight gain and hyperplasia of the pancreas, in some cases leading to pancreatic cancer. For

rats, binding of trypsin leads to excessive excretion of cholecystokinin (CCK) by the intestinal mucosa, stimulating the pancreas to release even more proteolytic enzymes. These enzymes are high in sulfur amino acids and are not readily re-absorbed, potentially leading to sulfur amino acid deficiency. Schneeman and Gallaher (1986) showed that rats, chickens, and growing guinea pigs display such pancreatic hyperplasia, but dogs, pigs, calves, and monkeys do not.

Table 5. Kunitz and Bowman-Birk inhibitors in selected soy protein products.

Sample	Kunitz Inhibitor (mg/g sample)	Bowman-Birk Inhibitor (mg/g sample)
Flours	1.1-19.6	<0.2-4.9
Concentrates	<0.5-6.1	<0.2-1.0
Isolates	<0.5-3.6	0.3-2.0
Dehydrated Soymilk	11.3	1.9
Wheat-soy pancake mix	1.0	0.4

Source: DiPietro and Liener (1989). Quantification of Kunitz and Bowman-Birk inhibitors is by rocket immunoelectrophoresis and chymotrypsin inhibition, respectively.

Heat treatment, especially moist heat, inactivates the Kunitz inhibitor (KI) and substantially inactivates Bowman-Birk inhibitor (BBI). Rackis and Gumbmann (1982) indicate that about 80% of the inhibitor activity is lost during the processing and preparation of soy foods. However, Liener (1986) warns that excessive heat treatment and processing may damage the nutritional value and functionality characteristics of the other soy proteins. Human gastric juice has a greater inactivation effect on KI than BBI (Liener 1986). Liener (1986) states that there is minimal evidence for concern from protease inhibitors for adults consuming soy, although there may be some concern for infants consuming soy-based infant formulas, adults consuming vegetarian diets very high in legumes, and those replacing meat protein with soy protein to help control hyperlipidemia. Kennedy (1995) reviews a body of evidence that suggests that hyperplasia, hypertrophy, adenoma, or cancer development in

humans from soy and other dietary sources of trypsin inhibitors in the diet is unlikely and points out that populations with high intakes of trypsin inhibitors have not displayed an increased risk of pancreatic cancer.

A growing body of information suggests that BBI may have anticarcinogenic properties. Kennedy (1995) reviews some of this research and contends that the most important index of tumorigenicity is whether an organism develops tumors or cancers. She presents a table reviewing the success of isolated soybean constituents, including BBI, phytic acid, and β-sitosterol, toward having a suppressive effect on carcinogenesis. Of these three constituents, BBI had the most dramatic results. Three of the four studies concerning BBI resulted in greater than 60% suppression of benign and malignant tumors. Kennedy shares additional data supporting the hypothesis that BBI can suppress malignant transformation in the liver and mammary tissue, and in animal models using 125I-PBBI (purified BBI) Kennedy showed that up to 40% of the BBI is taken up in the gastrointestinal tract and transported to liver and mammary tissues. Friedman and Brandon (2001) review research suggesting that the cancer preventative action of this protein is due to the trapping of free radicals by the cystine-rich BBI or BBI-protease complexes in tissues or by adsorbing carcinogens in the digestive tract like a dietary fiber, preventing colon cancer. In evaluating the results of these studies, as with any other studies evaluating the health effects of soybean components, it is important to consider the concentration of other soy components in the diets or treatments that may provide health benefits, such as isoflavones, saponins, and soy storage proteins.

As will be clear with other components of soy, the protease inhibitors are able to exert positive and negative effects on animal and, possibly, human health. It is the responsibility of the scientific community and the consumer to consider available research data and make reasonable dietary recommendations or choices.

Dehydroalanine and Formation of Lysinoalanine

Alkaline solubilization (pH >8.5-9) of soy proteins in the presence of heat can react cysteine with serine phosphate, forming dehydroalanine. Dehydroalanine can then react with

water, ammonia, L-lysine (via the ε-amino group), cysteine, or histidine forming D, L-serine, β-aminoalanine, L-lysino-D, L-alanine, lanthionine, and histidinoalanine, respectively (Finot 1997). For lysinoalanine this leads to a loss of lysine, though usually negligible, e.g. 0.1% loss of total lysine in casein-based milk products rich in serine phosphate and levels of lysinoalanine near 1200 ppm. Reactions with dehydroalanine also create cross-linking that can lead to decreased digestibility. Friedman (1999) reviewed publications that demonstrated that free and protein-bound lysinoalanine could cause nephrocytomegaly in rats. Studies by De Weck-Gaudard and others (1988) and Langhendries and others (1992) diminished toxicological concerns about lysinoalanine by showing that protein-bound lysinoalanine was not well absorbed and that infants fed commercially processed formulas containing up to 1000 ppm lysinoalanine experienced no abnormal kidney functioning. This again demonstrates the importance of thoroughly considering and evaluating results from studies concerning the intake of a dietary substance or component.

Oligosaccharides

Soybean oligosaccharides of importance are sucrose, raffinose, and stachyose, and their usual percentage by weight in soybeans is 5.7, 4.1, and 4.6%, respectively (Perkins 1995). The concentration of raffinose and stachyose is about 60 mg/g of defatted soybean meal (Kuo and others 1988). Raffinose and stachyose are not digested in the human gut due to the absence of endogenously produced α-galactosidase. They are however fermented in the gut by microbes, producing flatulence and intestinal discomfort. Consequently, the goal of most soy ingredient manufacturing processes is the removal of these oligosaccharides.

A growing body of evidence is suggesting that maintenance of high numbers of fermentative organisms in the human gut is desirable. In the large intestine, these fermentative organisms may out compete and displace pathogenic organisms, thought to be at least partially responsible for ulcerative colitis, bowel cancer and irritable bowel syndrome. Gibson and Macfarlane (1995) address such health issues. The oligosaccharides in soybeans may be considered prebiotics, non-digestible food ingredients that beneficially

affect the host by improving the growth or activity of one or more types of bacteria in the host and in turn improve the health of the host (Gibson 2002). Hayakawa and others (1990) showed that oligosaccharides promoted the growth of bifidobacteria, nonpathogenic, fermentative gut microorganisms thought to be beneficial for human gut health. Healthy populations of bifidobacteria may reduce the incidence of colon cancer (Koo and Rao 1991). So, raffinose and stachyose may be undesirable at high levels in soy foods and soy food products, but at low concentrations they may have a beneficial effect on human health.

Phytate

Phytate, or inositol hexaphosphate, binds iron, zinc, calcium, and other minerals. In this bound state the minerals are poorly absorbed. Table 6 shows the phytic acid content of soybeans, selected processed soy protein products, and selected soy foods. Phytic acid is stable at food preparation conditions and is probably not degraded by extrusion during preparation of texturized vegetable protein. Washing soy precipitates at pH 5.0 has been shown to remove up to 75% of the phytates (Rhee and Choi 1981).

Phytate may have beneficial health implications. Hirose and others (1991) used a rat model to demonstrate that a 2% supplement of phytic acid inhibited development of neoplasmatic lesions in the pancreas and liver. Shamsuddin and others (1992 and 1996) used *in vitro* models to show that phytic acid reduced cell proliferation rate in erythroleukemia and human mammary cancer cell lines. Graf and Eaton (1993) showed that phytic acid may play a role in colon cancer suppression. Thompson and Zhang (1991) proposed that the anticarcinogenic properties of phytic acid may be due to its antioxidant capabilities. However, Rimbach and Pallauf (1999) indicated that phytic acid did not have any significant effect on liver oxidant or antioxidant status in growing rats. Other evidence suggests that phytic acid may be able to reduce serum cholesterol levels (Jariwalla and others 1990).

The mechanisms for the observed beneficial effects of phytic acid are not well understood. More research will need to be done to build a better understanding about the

actions of phytic acid. For most human populations the diet is varied enough that the dietary phytic acid encountered in some foods will not cause mineral deficiency.

Table 6. Phytic acid in soybeans, soy protein products, and traditional soy foods

Sample	Phytic acid content (g/100g, dry basis)
Soybeans	1.00-2.30
Soybean Hulls	0.12-0.50
Hypocotyl	0.88
Cotyledons	1.58
Full-fat Flour	1.51-1.81
Defatted Flour	1.30-1.85
Textured Flour	1.10-2.02
Texture Soy Protein Concentrate	1.25-2.17
Soy Protein Isolate	0.97-2.00
Spun Isolate Fiber	1.48
Soymilk	1.68-1.83
Tofu	1.5-2.88
Okara	0.5-1.2
Tempeh	0.69-0.73

Adapted from Anderson and Wolf (1995)

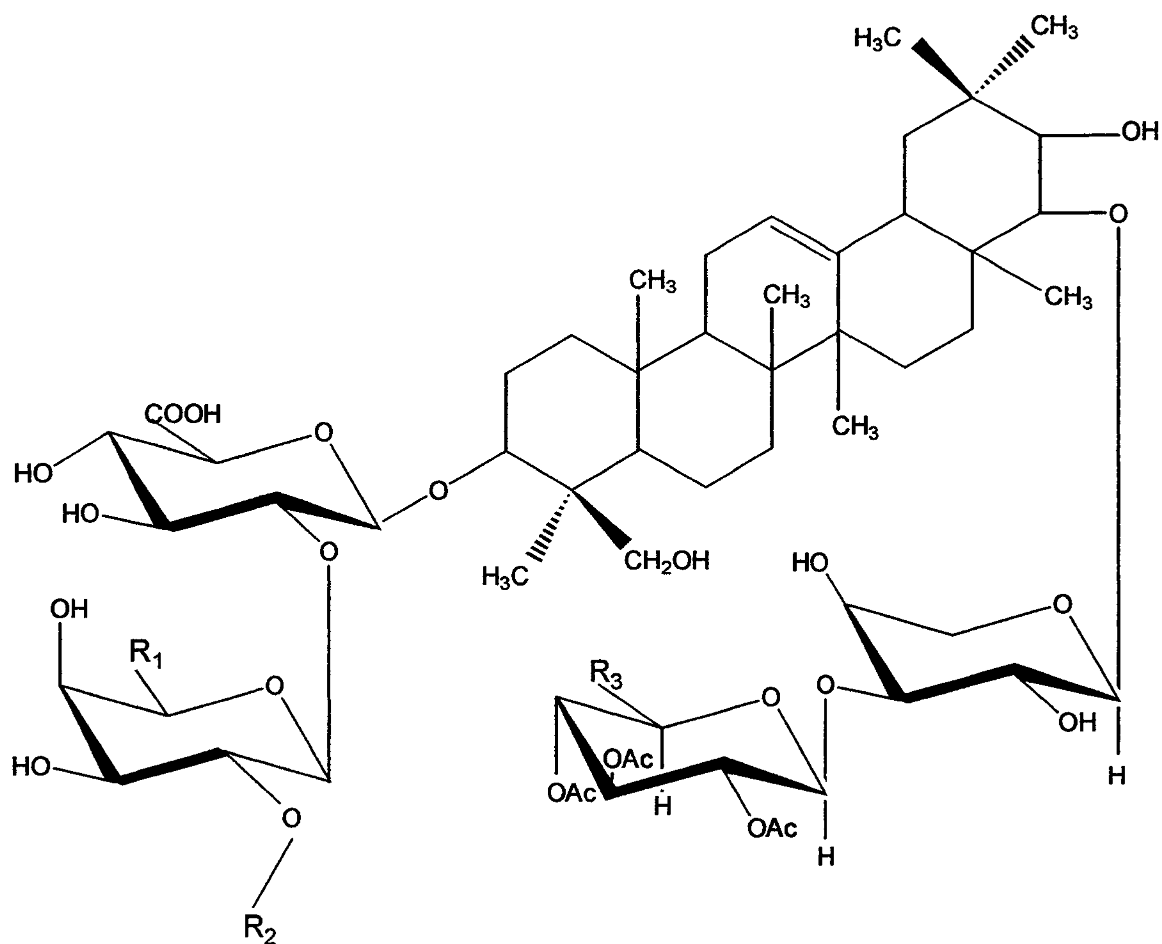
Saponins

Saponins are present in a wide variety of plants, and the chemical structures of the saponins number in the thousands. However, there are two central building block structures for saponins, triterpenoid or steroid aglucons. Saponin aglucons are referred to as sapogenins. When sugars or sugar chains are added to the sapogenins, the resulting compounds are called glycosides. There are three classes of saponin glycosides: triterpene, steroid, and steroid alkaloid. The most common sugars that are added to the sapogenins to

form glycosides are glucose, arabinose, glucuronic acid, and xylose. Soybean saponins are oleanene-type triterpenoid glycosides and can be further categorized into sapogenols A, B, and E, each category having their respective glycosides. On a dry weight basis soybean saponin concentration is about 0.2% (Tsukamoto and others 1995), while soy ingredients may contain up to 0.76% (Ireland and others 1986). Traditional soyfoods, such as tofu, miso, and tempeh contain from 0.15 to 0.30 % saponins (Kitagawa 1984). Ireland and others (1986) reported that whole soybeans contain about 75% group B saponins and 25% group A saponins. Figures 1 and 2 show group A and group B saponin glycosides, respectively. The group B saponins have a 2,3-dihydro-2, 5-dihydroxy-6-methyl-4*H*-pyran-4-one or DDMP moiety attached to C-22 of the sapogenol. This DDMP saponin bond is labile. Loss of the moiety from soy saponins βg, βa, γg, γa, and αg yields soy saponins I, II, III, IV, and V, respectively. Group A saponins, identified as Aa through Ah, do not contain the DDMP moiety. Hu (2000) contains a more complete review of saponin nomenclature, presence in other plants, and their impact on livestock and other animals.

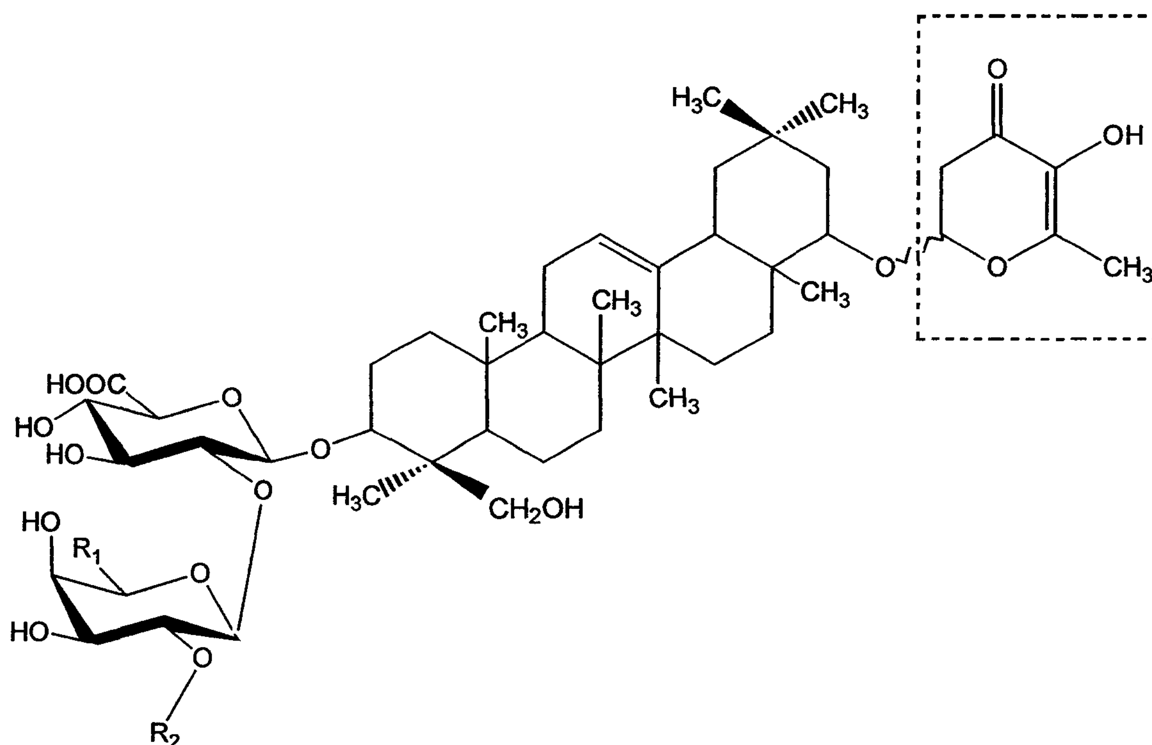
Tables 7 and 8 reveal that saponin content and profile vary among soybean seed components and among soy foods and soy ingredients. Soybean flour primarily contains βg and βa saponins, while soy products and ingredients that have received significant heat treatment contain primarily saponins I and II, demonstrating that heat treatment cleaves the DDMP moiety. Novasoy® is a dried extract resulting from soy ingredient preparation, and due to the processing conditions contains exclusively non-DDMP forms of saponins. As described above, soy protein concentrates may be prepared using an ethanol wash strategy to remove the unwanted oligosaccharides. Table 7 shows that the acid-washed soy concentrates have 9.41 μmol/g group B saponins while ethanol-washed concentrates have only 0.20 μmol/g. This demonstrates that organic solvents are capable of leaching saponins out of soy meal as the oligosaccharides are being removed.

On a g/g basis, the germ, or hypocotyl, contains six to seven times as much group B saponin as the cotyledons. Table 8 shows that Kennong 16 variety soybeans from the 1997 crop year contained 23.89 μmol/g group A saponins in the hypocotyl compared to none



Soyasaponin	R_1	R_2	R_3
Aa = acetyl A_4	CH_2OH	β-D-Glc	H
Ab = acetyl A_1	CH_2OH	β-D-Glc	CH_2OAc
Ac	CH_2OH	α-L-Rha	CH_2OAc
Ad	H	β-D-Glc	CH_2OAc
Ae = acetyl A_5	CH_2OH	H	H
Af = acetyl A_2	CH_2OH	H	CH_2OAc
Ag = acetyl A_6	H	H	H
Ah = acetyl A_3	H	H	CH_2OAc

Figure 1 - Group A acetylated soyasaponin structures.
Glc: glucosyl; Rha: rhamnosyl; Ac: acetyl



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

Figure 2. Structures of group B soyasaponins.
Rha: rhamnosyl; Glc: glucosyl; Y: yes; N: no
DDMP: 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one

Table 7. HPLC analysis of group B saponin content and profile of selected commercial soy products

	μmol/g, as is basis						
Product	V	I	II	αg	βg	βa	Total
Soybean flour[1]	0.00	0.28	0.21	0.17	2.19	0.47	3.31
Tofu[2]	0.00	0.31	0.13	0.01	0.11	0.03	0.59
Tempeh[3]	0.00	0.76	0.39	0.01	0.28	0.09	1.53
Soymilk[4]	0.00	0.22	0.12	0.00	0.09	0.04	0.47
Acid-washed concentrates[5]	0.00	2.41	1.05	0.19	4.90	0.86	9.41
Ethanol-washed concentrates[5]	0.00	0.08	0.12	0.00	0.00	0.00	0.20
Isolated soy protein 500E[6]	0.87	5.73	2.39	0.10	1.20	0.31	10.60
Isolated soy protein Supro 670[6]	0.00	5.59	2.50	0.07	1.01	0.33	9.51
Textured vegetable protein[5]	0.00	1.89	0.87	0.11	1.26	0.38	4.51
Soy hypocotyl[7]	4.41	5.80	0.00	4.71	12.53	0.00	27.46
Novasoy®[5]	0.00	77.55	36.48	0.00	0.00	0.00	114.02

Adapted from Hu and others (2002)
[1]Vinton81, 1994 crop
[2]Mori-nu, firm
[3]Quong Hop & Company
[4]White Wave, Inc.
[5]Archer Daniels Midland Company
[6]Protein Technologies International
[7]Schouten USA Inc., toasted

Table 8. LC-MS analysis of group A saponin content and profile of selected commercial soy products

	μmol/g, as is basis								
Product	Aa	Ab	Ac	Ad	Ae	Af	Ag	Ah	Total
Soy hypocotyl[1]	5.16	4.65	1.08	0.00	1.80	1.14	0.17	0.08	23.89
Soy cotyledon[1]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Defatted soy meal[2]	0.00	0.74	0.25	0.19	0.00	0.30	0.00	0.07	1.55
Molasses[3]	8.38	8.14	1.22	0.00	2.71	1.02	0.24	0.00	21.71

Adapted from Gu and others (2002)
[1]Kennong 16, 1997 crop
[2]Jilin 3, 1997 crop
[3]Shanghai Liantang Food Factory

in the cotyledons. The relative concentration of the saponins, secondary metabolites, in the hypocotyls suggests that they may play a role in defense against oxidative stress, insects, fungi, and bacteria. The hypothesis for this role has been supported by other saponin research. Yoshiki and Okubo (1995) reported that DDMP saponins display oxygen scavenging activity in soybean seeds. Saponin extracts from some plants have been found to be inhibitory against the fungi *Fusarium solani* (Timbekova and others 1996) and the bacteria *Agrobacterium tumefaciens* (Timbekova and others 1996). Tava and Odoardi (1996) demonstrated that a saponin-containing diet led to a rise in larval mortality and a decrease in body weight in grass grubs, locusts, and flour beetles. Since saponins are found in the stems and leaves of plants, as well as seeds, it has been hypothesized that saponins may have been developed by plants as a defense against herbivores, as well as the fungi and insects mentioned above. Cheeke (1996) indicated that swine, poultry, and cattle displayed an aversion to dietary saponins. Some saponins are extremely toxic to fish and some cold-blooded animals (Price and others 1987). Klita and others (1996) showed alfalfa root saponins decreased ruminal protozoal populations and reduced ruminal and total tract apparent digestibilities in sheep. The saponins were introduced into duodenally cannulated wethers, with doses so high that in a preliminary study that one of the wethers did not survive. It is questionable how useful their data is. Malinow and others (1981, 1982) showed that consumption of alfalfa saponins by rats and monkeys (*Macaca fascicularis*) caused no adverse effects. Ishaaya (1969) showed that soyasaponin consumption by chicks, rats, and mice caused no adverse effects. Average daily saponin soy and non-soy saponin intakes for a Western-style diet and Asian vegetarians are about 10 mg/day and 200 mg/day, respectively (Ridout and others 1988). Anti-nutritional effects of soyasaponins in humans are evidently minimal, as no significant health concern has arisen in regard to their consumption.

There does seem to be mounting evidence that soyasaponins may deliver hypocholesterolemic, anticarcinogenic, and immunostimulatory benefits to humans. However, soybean isoflavones, Bowman-Birk inhibitor, and the soy proteins themselves

confound efforts to elucidate the true impact of soyasaponins in these roles. Further confounding these efforts are the extensive use of animal models rather than clinical trials, use of differing animal models, type of diet used in animal trials, and use of different saponins.

The cholesterol-lowering effects of soy protein have reportedly been known for more than 90 years (Anderson and others 1995). There is currently much debate about what component or components of soy are responsible for this effect. More than 20 years ago Potter and others (1979) advanced the hypothesis that the hypercholesterolemic action of soy protein was due to the soyasaponins. They cited the following rationale. First, cholesterol lowering of foods and fibers is due the binding of bile acids or cholesterol, leading to reduced absorption. With reduced absorption and reduced enterohepatic circulation of bile acids there is an increase in bile acid production from cholesterol in the liver. Saponins had been shown to reduce serum cholesterol in animals. An acid hydrolysis of proteins retained hypocholesterolemic activity, an acid hydrolysis of saponins increased their activity, and a mixture of amino acids that matched the profile of the intact proteins did not have activity. Consequently, they stated that the hypocholesterolemic action of soy was due to the saponins rather than the protein.

A review by Oakenfull and Sidu (1990) revealed that saponin-fed animal models, including monkeys, mice, rats, chicks, rabbits, pigs, and hamsters, led to a reduction in hypercholesterolemia in 14 of 20 experiments. They added that of the other six experiments, four did not use a hypocholesterolemic diet and in two the source of saponins was not identified. Thirteen of the 14 experiments employed hypocholesterolemic diets. Proposed mechanisms for the hypocholesterolemic action of saponins were binding of cholesterol and saponins leading to inhibited absorption (Gestetner and others 1972); binding of saponins to enterocrine cells leading to modulation of gastric emptying, intestinal mobility and permeability, nutrient absorption, and appetite (Cooke 1986); binding of bile acids leading to excretion in the feces; and formation of large saponin-bile acid micelles that block reabsorption (Oakenfull 1986).

Oakenfull and Sidhu (1990) also reviewed nine experiments in which saponin-containing materials were consumed by humans, generally at a level of 300-500 mg saponins/d. The sources of the saponins were chickpea, soy, yucca, alfalfa, and ginseng. Plasma cholesterol decreased in six of the nine studies, but of the three studies that used soyasaponins only one study showed a significant decrease.

Some studies to evaluate the effect of saponins on hypercholesterolemia have evaluated incorporation of saponins into the diet material. Oakenfull (1984) added saponins to a casein diet for rats and observed cholesterol-lowering effects coupled with increased fecal bile excretion. Topping and others (1980) spiked saponins into a soy protein-based diet and observed no beneficial effect. Potter and others (1993) built on this work by adding quillaja saponins to soy protein isolate- and casein-based diets. The authors observed a positive effect for the casein diet, a decrease in LDL cholesterol and LDL/HDL cholesterol levels, but not with the soy protein diet. They also observed that the saponins rapidly complexed with the soy protein. They concluded that the effect of saponins on serum lipid profiles was affected by the source of dietary protein.

The Sirtori research group has been aggressive in promoting the 7S soy protein fraction as the component in soy responsible for altering serum cholesterol levels. They point out that soy protein might reduce the risk of coronary heart disease by activating the LDL receptors in liver cells or by modulating synthesis and catabolism of LDL by consuming specific proportions of amino acids corresponding to soy protein. Recent studies by this group have used a human hepatoma cell line (Hep G2) to show that the α'-subunit of β-conglycinin was more effective than whole β-conglycinin, α + α' subunit combination, or the β-subunit for up-regulating LDL receptor activity (Lovati and others 1998, Manzoni and others 1998). Since whole subunits cannot be absorbed and passed into the circulatory system, Lovati and others (2000) evaluated the effectiveness of synthetic sequences, a digestion of an isoflavone-poor soy protein isolate, and undigested isolate. The digest up-regulated the LDL receptor more than the undigested isolate. A synthetic peptide corresponding to the 127-150 positions of the 7S globulin showed a marked up-regulation of LDL activity. The MW of

this unit was 2271 Da, and the authors suggest they have evidence that peptides of >3000 Da can cross the intestinal wall at a percentage of 0.20.

The picture is not clear whether saponins, soy protein, other soy components, or a combination of these is responsible for the hypocholesterolemic effect of soy. Lacaille-Dubois (2000) lists other beneficial saponin activities as cytotoxic, anti-tumoral, and chemopreventive for cancer-related activities and as immunostimulants and immunoadjuvants for immune system activities. Other activities include anti-inflammatory, hepatoprotective, cardiovascular, hypoglycemic, antiviral, antimicrobial, and antifungal. Obviously, a complete review of these properties is beyond the scope of this literature review, but a few a given below. Also, it is important to note that dietary intake of soy may not reap many of these benefits since saponins are generally thought to be poorly absorbed by the digestive system.

Hayashi and others (1997) evaluated the *in vitro* antiviral activities of soyasaponin I and II against herpes simplex virus type 1. Soyasaponin II was found to be more potent than soyasaponin I. Soyasaponin II was also inhibitory toward replication of human cytomegalovirus, influenza virus, and human immunodeficiency virus type 1. The authors claim that the inhibition was due to a virucidal effect rather than inhibition of virus penetration into the cell or inhibition of viral protein synthesis.

Kinjo and others (1998) utilized soyasaponins I, II, II, and IV to evaluate their *in vitro* hepatoprotective actions towards immunologically induced liver injury on primary cultured rat hepatocytes. Effectiveness was soyasaponin III and IV> II and I. The authors hypothesize that the sugar moiety linked at C-3 may have an important role in hepatoprotective actions.

Antimutagenic activity of 2-acetoxyacetylaminofluorene (2AAAF) in Chinese hamster ovary cells was repressed by a concentrated mixture of soyasaponins II, III, IV, V, βg, βa, γg, γa and Be (Berhow and others 2000). Purified soyasapogenol aglycone had a significant activity against 2AAAF. The authors suggest that this is the first time antimutagenic activity of soybean saponins has been demonstrated in mammalian cells.

Oh and Sung (2001) evaluated the effect of soy saponins on HT-29 cell proliferation, differentiation, and apoptosis. The crude soybean saponin extract had only a slight apoptotic effect at the highest concentrations, 240 and 600 ppm. The saponin treatment decreased cell growth in a concentration-dependent manner.

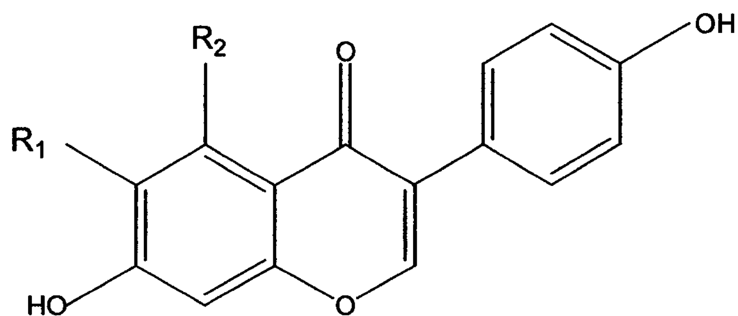
Jun and others (2002) compared the ability of saponins and other antioxidants to inhibit aflatoxin B1 (AFB1)-induced mutagenicity and DNA-adduct formation. Antimutagenicity was found to be butylated hydroxytoluene>saponins>alpha-tocopherol>L-ascorbic acid. The saponins inhibited the mutagenicity of AFB1 by 52, 64, and 81% at concentrations of 600, 900, and 1200 μg/plate, respectively. The authors hypothesized that the saponins blocked initiation of carcinogenesis.

Protection against colon cancer may be the most likely and plausible anticarcinogenic activity of soyasaponins. Koratkar and others (1997) fed soybean saponins to mice at a concentration of 3 g/100 g feed. They observed a decrease in aberrant crypts induced with azoxymethane. It should be noted that this concentration is much higher than the 0.3-0.4% found in soy foods.

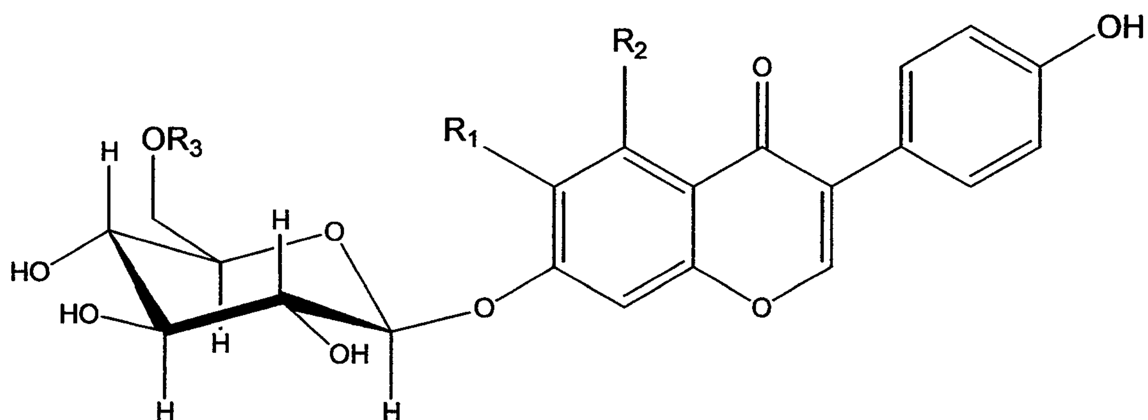
More research is needed to further elucidate the exact role of soyasaponins in decreasing serum cholesterol, as well as their potential role in inhibiting colon cancer. Clearly, further investigation is warranted to understand their chemopreventative, antitumoral, and cytotoxic activities as we search for drugs to combat cancer.

Isoflavones

Isoflavones are a subclass of the isoflavonoids, which are themselves a subclass of the flavanoids. Harborne (1994) indicated that at least 364 known aglucons of isoflavones had been reported. However, few of these isoflavones occur in plants commonly consumed by humans. Genistein, daidzein, and glycitein are the aglucons of soy isoflavones. For each of these aglucons there are three additional major isomeric forms: the β-glucosides (genistin, daidzin, and glycitin), the 6"-*O*-malonylglucosides, and the 6"-*O*-acetylglucosides (Figure 3). Isoflavone data from Wang and Murphy (1994b), using high performance liquid



Isoflavone	**R1**	**R2**
Daidzein	H	H
Genistein	H	OH
Glycitein	OCH3	H



Isoflavone	**R1**	**R2**	**R3**
Genistin	H	OH	H
Glycitin	OCH3	H	H
Daidzin	H	H	H
Malonylgenistin	H	OH	COCH2COOH
Malonylglycitin	OCH3	H	COCH2COOH
Malonyldaidzin	H	H	COCH2COOH
Acetylgenistin	H	OH	COCH3
Acetylglycitin	OCH3	H	COCH3
Acetyldaidzin	H	H	COCH3

Figure 3. Isoflavone structures

chromatography analysis (HPLC), showed that the prevalence of isoflavones in whole soybeans on an aglucon basis was genistein≈daidzein>>glycitein on a mole basis, and relative prevalence of forms within an aglucon family generally was malonylglucoside>β-glucoside>aglycone >acetylglucoside. Wang and Murphy (1994b) also were able to show that genetic differences dramatically affected isoflavone profile. American varieties had malonylglucoside/glucoside ratios of 1-3 compared to 4-6 for Japanese varieties. Crop year was shown to have a greater effect than crop location on overall isoflavone content.

The soy germ isoflavone profile is different from that of whole soybeans, and the concentration of isoflavones in soy germ is about 10 times that in whole soybeans. Tabular data from Hendrich and Murphy (2001) shows that on an aglucon basis the profile for whole soybeans is 42:51:7 and soy germ is 45:15:40 for daidzein, genistein, and glycitein, respectively. If the health benefit differs among the soy isoflavones this may be significant since soy germ is an isoflavone-rich starting material that may be used in the production of isoflavone supplements.

Soy isoflavones have received considerable investigation during the last 20 years, as data suggests that they may have beneficial effects on cardiovascular disease, carcinogenesis, and osteoporosis. The different aglucons and isomeric forms display different biological activities. Consequently, the quantification of these forms in foods and food ingredients is critical to better understand their impact on human health, maintain quality control, and permit accurate labeling and marketing statements.

Much of the literature expresses isoflavone concentrations in μg/g of sample. Since the molecular weights of the β-glucosides, malonylglucosides, and acetylglucosides are substantially different from the aglucons, concentrations should be normalized and expressed in terms of each aglucon or the sum of the aglucons. Murphy and others (1997) indicated that without this correction, summed concentrations may overestimate isoflavone concentrations by almost a factor of two for soymilk samples, but extent of isoflavone overestimations of different samples will vary according to the isomeric profile. In retrospect, it would have been better for authors to express data as μmol/g. Isoflavone

contents of soybeans, soy ingredients, and soy foods are shown in Tables 9, 10, and 11. Table 12 shows isoflavone contents of commercial products on a μmol/g basis, thus enabling a direct comparison to saponins on a molecular basis.

Obviously, the various types of soy foods contain widely varying concentrations of isoflavones. It has been generalized (Messina 1997) that raw soybeans contain 2-4 mg isoflavones/g while soyfoods contain 1-3 mg/g on a dry weight basis. It is difficult to accurately measure isoflavone intake in the U.S. However, the estimated daily median intake of soy isoflavones by postmenopausal women participating in the Framingham Heart Study ranged from 52-177 μg, comprised of genistein and daidzein at 24-57 μg and 28-120 μg (de Kleijn and others 1999), respectively. Kirk and others (1999), using a food frequency questionnaire, estimated that U.S. adults consume 7-10 and 4-6 mg per day of genistein and daidzein, respectively. Estimated dietary intake of isoflavones in Korea was estimated to total 14.88 mg/d, comprised of 7.32 mg/d genistein, 5.81 mg/d daidzein and 1.75 mg/d glycitein (Kim and Kwon 2001). Native Japanese adults have been estimated to consume 30-40 mg (aglucon units) of isoflavones per day (Wakai and others 1999). This demonstrates the disparity of soy isoflavone intake between traditional U.S. diets and Asian diets. Whereas the consumption of traditional soyfoods, such as soymilk and tofu, are rapidly increasing in the U.S., much of the soy isoflavone intake is from soy in the form of ingredients in processed foods.

Bioavailability of isoflavones for humans is affected by their absorption. Hendrich and Murphy (2001) suggested that evidence supports the hypothesis that isoflavone glucosides are not well absorbed in the gut and that human intestinal or gut microflora β-glucosidases must convert the glucosides to aglucons before the isoflavones are absorbed. Brown (1988) reported that the glucosides are poorly hydrolyzed by mammalian intestinal digestive enzymes. Coward and others (1996), employing HPLC analysis, found that isoflavones from soy protein isolate were absorbed more slowly than those in soymilk. It was noted that in isolate the glucoside isoflavone distribution was pre-dominantly malonyl- and acetylglucosides, compared to a predominance of glucosides in the soymilk. It was

hypothesized that the glucosides were more readily hydrolyzed by β-glucosidases than the malonyl- and acetylglucosides. Since individuals may differ in the concentration

Table 9. Isoflavone content (μg/g, as is basis) of soybeans and soy ingredients[1,2]

	β-glucoside			malonyl			acetyl			Aglucon			Totals[6]
Product	Din	Gin	Glin	Din	Gin	Glin	Din	Gin	Glin	Dein	Gein	Glein	
Vinton 81 90H[3]	690^{b}	852^{a}	56^{d}	300^{b}	743^{b}	50^{b}	1^{e}	9^{e}	nd	26^{b}	29^{c}	20^{f}	1636
Vinton 81 91I[3]	180^{e}	394^{de}	53^{def}	241^{c}	738^{b}	61^{a}	tr	2^{e}	35^{de}	7^{f}	17^{f}	20^{ef}	995
Green soybean	451^{d}	430^{d}	48^{f}	515^{a}	851^{b}	57^{ab}	tr	2^{e}	nd	10^{de}	16^{f}	18^{g}	1354
Soy flour	147^{f}	407^{de}	41^{g}	261^{bc}	1023^{a}	57^{a}	tr	1^{e}	32^{e}	4^{g}	22^{e}	19^{fg}	1124
Soy granule	727^{a}	870^{a}	132^{b}	106^{d}	193^{cd}	60^{a}	72^{c}	135^{d}	48^{c}	12^{c}	27^{d}	22^{d}	1464
TVP A[4]	507^{c}	634^{b}	146^{a}	93^{d}	192^{cd}	60^{a}	187^{b}	320^{b}	90^{a}	12^{c}	29^{cd}	25^{c}	1382
TVP B[4]	463^{d}	552^{c}	93^{c}	129^{d}	256^{c}	58^{a}	231^{a}	355^{a}	68^{b}	8^{ef}	22^{e}	26^{b}	1342
Soy Isolate A[4]	tr	137^{g}	34^{h}	20^{e}	100^{d}	39^{c}	6^{e}	nd	33^{e}	63^{a}	136^{a}	53^{a}	466
Soy Isolate B[4]	88^{g}	301^{f}	49^{ef}	18^{e}	88^{d}	36^{c}	74^{c}	215^{c}	46^{c}	11^{cd}	36^{b}	25^{bc}	610
Soy Isolate C[4]	133^{f}	382^{e}	55^{de}	19^{e}	95^{d}	37^{c}	36^{d}	122^{d}	40^{d}	12^{c}	36^{b}	22^{de}	615
Soy concentrate	tr	18^{h}	31^{h}	nd	tr	nd	tr	1^{e}	nd	nd	nd	23^{d}	56
Lsd[5]	31	47	6	41	117	7	14	34	2	2	2	1	

Source: Wang and Murphy (1994a)

[1]Data from three replications. Values in column with different letters were significantly different at α=0.05; dry samples.

[2]Abbreviations: Din, daidzin; Gin, genistin; Glin, glycitin; Dein, daidzein; Gein, genistein; Glein, glycitein; tr, trace; nd, not detected.

[3]90 and 91, 1990 and 1991 crop years; H and I, different locations.

[4]Different commercial sources.

[5]Least significant difference.

[6]Totals are sums of isomers after normalization to aglucon forms.

of β-glucosidases in their gut, possibly due to differences in gut microflora, isoflavone distribution pattern may have dietary significance. Consequently, understanding how soybean storage and processing can affect isoflavone profile and distribution is of interest.

Heat, moisture, and glucosidases are the most important factors in changing isoflavone distribution. Table 9 shows that soybeans and soy flour are high in malonylglucosides, but

heat processed soy products, such as the roasted soybeans and TVP, have distributions shifted substantially from malonylglucosides toward acetylglucosides. The fermented foods, such as tempeh and miso, have their isoflavone distributions shifted toward the aglucons

Table 10. Isoflavone content (µg/g, as is basis) of traditional soy foods[1,2]

	β-glucoside			malonyl			acetyl			Aglucon			Totals[6]
Product	Din	Gin	Glin	Din	Gin	Glin	Din	Gin	Glin	Dein	Gein	Glein	
Roast soybeans	460^{b}	551^{d}	68^{b}	45^{b}	63^{f}	72^{a}	397^{a}	743^{a}	102^{a}	39^{e}	69e	52a	1625
Instant beverage A[3]	444^{b}	775^{a}	76^{a}	39^{b}	144^{d}	40^{c}	5^{b}	24^{b}	33^{b}	18^{g}	44^{g}	20^{d}	1037
Instant beverage B[3]	404^{c}	718^{b}	77^{a}	58^{b}	202^{b}	43^{b}	8^{b}	27^{b}	33^{b}	15^{g}	38^{h}	20^{d}	1014
Instant beverage C[3]	468^{b}	674^{c}	68^{b}	61^{b}	179^{c}	42^{bc}	9^{b}	22^{b}	33^{b}	15^{g}	32^{i}	20^{d}	1001
Instant beverage D[3]	525^{a}	745^{ab}	75^{a}	98^{b}	259^{a}	44^{b}	12^{b}	26^{b}	33^{b}	30^{f}	50^{f}	21^{cd}	1183
Tofu	25^{e}	84^{f}	8^{e}	159^{ab}	108^{e}	nd	8^{b}	1^{b}	29^{b}	46^{d}	52^{f}	12^{f}	337
Tempeh	2^{e}	65^{f}	14^{d}	255^{a}	164^{c}	nd	11^{b}	nd	nd	137^{c}	193^{b}	24^{b}	625
Bean paste	nd	96^{ef}	21^{c}	nd	nd	19^{e}	1^{b}	2^{b}	nd	271^{a}	183^{c}	54^{a}	593
Honsukuri miso[4]	72^{d}	123^{e}	18^{c}	nd	nd	22^{d}	1^{b}	11^{b}	nd	34^{f}	93^{d}	15^{e}	390
Fermented bean curd	nd	tr	nd	nd	nd	nd	nd	nd	nd	143^{b}	223^{a}	23^{bc}	294
Lsd[5]	35	32	3	127	16	3	23	38	8	4	5	2	

Source: Wang and Murphy (1994a)
[1]See Table 9. [2]See Table 9. [3]Different commercial sources, dry powder. [4]Made of rice and soybeans. [5]Least significant difference. [6]Totals are sums of isomers after normalization to aglucon forms.

due to the action of fungal β-glucosidases. Coward and others (1998), using HPLC analysis, evaluated effects of cooking on isoflavones in soyfoods. They found that baking processed soy products led to conversion of malonylglucosides to β-glucosides, but frying tended to promote the conversion of malonylglucosides to acetylglucosides, due to decarboxylation of the malonylglucosides. They indicated that as food received excessive heat treatment, aglucon concentration increased and total isoflavone content decreased. They also suggested that dry heat tends to transform malonylglucosides to acetylglucosides, while moist heat tends to transform malonylglucosides to β-glucosides. In their case, however, the moist

heating was relatively mild, probably less than 100 °C, and under these conditions conversion from malonylglucosides to β-glucosides would be expected.

Wang and Murphy (1996) published the first true mass balance paper for isoflavones, evaluating the effect of tempeh, soymilk, and tofu processing on isoflavone concentration

Table 11. Foods containing significant concentrations (μg/g, as is basis) of isoflavones

Food	Concentration Range	Reference
Soybeans	720-2370	1
Soy flours, defatted	610-2440	1
Soy sprouts	250-530	1
Soy protein isolate	456-1993	1
Soy Protein Concentrate, water washed	20-318	1
Soy Protein Concentrate, ethanol washed	612-1670	1
Texturized soy protein	44-2956	1
Hydrolyzed soy protein (HSP)	127-1621	2
Tuna (packed in water with HSP)	22	2
Doughnuts	85-100	2
Pancake mix	171	2
Soy milk	13-211	1
Low fat soymilk	17-86	2
Tofu	79-635	1
Low fat tofu	194-200	2
Tempeh	69-625	1
Miso	227-892	1
Natto	464-870	1
Soy-based infant formula	202-316	1
Edamame	1354-1860	1
Soybean (vegetable) oil	0	1
Bacon, meatless	121	1
Chicken analog	146	1
Harvestburgers®	82.2	1
Soy cheeses	33-593	1
Soy hotdog and breakfast sausage	34-150	1
Soy sauce	12.7-23.0	1
Commodity hamburgers with soy	5.5-29	1

Adapted from Murphy and Hendrich (2002)
[1]Beecher and others (1999)
[2]Liggans and others (2000)

and profile. During tempeh production, 12% of the isoflavones were leached from the soybeans during the soaking/dehulling step and 49% were lost during cooking. Soaked soybeans had isoflavone distributions that were proportionally higher in daidzein and genistein glucosides and lower concentrations of these malonylglucosides. The β-glucosides and malonylglucosides decreased substantially during cooking, mostly due to leaching with

Table 12. Isoflavone and saponin concentrations in commercial soy products

Product	Isoflavone (μmol/g, as is)	Saponin (μmol/g, as is)
Soymilk	0.5	0.5
Tempeh	5.0	1.6
Textured vegetable protein	8.4	4.8
Soybean flour, 1994	4.2	3.5
Tofu	1.0	0.6
Isolated soy protein Supro 670	7.4	10.1
Isolated soy protein 500E	4.2	11.2
Acid-washed soy concentrate	2.4	10.0
Ethanol-washed soy concentrate	0.6	0.2
Soy hypocotyls	558	30
Novasoy	925	115

Source: Hu (2000)

some conversion to acetylglucosides. Fermentation dramatically shifted the isoflavone profile toward aglucon forms. During soymilk and subsequent tofu manufacture, the most notable changes in isoflavone concentration were losses in the soaking water, okara, and whey fractions. Substantial profile changes occurred during the cooking step, with malonylglucosides being transformed to aglucon and β-glucoside forms.

Franke and others (1999) used HPLC analysis to evaluate 25 multiethnic foods for isoflavone content and distribution. Their results support the leaching and processing trends mentioned in this section. In their summary, they noted that different laboratories measuring the same foods may get different results, due in part to differing analytical techniques, and they emphasized the need for careful quality assurance using external and internal standards as those described by Song and others (1998). Careful choice of extraction solvent is also important to maximize recovery of all soy isoflavone forms.

Murphy and others (2002) evaluated acetonitrile, acetone, ethanol, and methanol as extraction solvents for soy flour, tempeh, tofu, TVP, and soy germ. Acetonitrile, at approximately 53% in water, was determined to be the best choice in most cases. Murphy and others (2002) reported that 53% methanol was significantly less efficient than 53% acetonitrile, acetone, or ethanol in extracting isoflavones and saponins from tofu and textured vegetable protein (TVP). For TVP, 53% methanol was reported to underestimate acetylglucosides by 30-35% and β-glucosides and malonylglucosides by 25%.

The following authors used 80% methanol rather than the 53% acetonitrile solution suggested by Murphy and others (2002). They also did not have standards for all 12 isoflavones, most using just the aglucons and the β-glucosides to prepare HPLC standard curves. Singletary and others (2000) and Mahungu and others (1999) reported the effect of extrusion on corn/soy mixtures was that malonylglucosides decreased while acetylglucosides increased. The glucoside concentrations decreased, but aglucon concentrations did not increase. Total isoflavone concentrations were reduced 24-38% in the extruded material compared to the starting material. Simmone and others (2000) evaluated the impact of processing on isoflavone content and distribution in edamame. They also found that blanching/boiling caused a considerable loss in isoflavones, with the hypothesis that the loss was largely due to leaching of isoflavones. Boiling the immature soybeans caused a substantial increase in genistein and the glucosides daidzin and genistin at the expense of the malonylglucosides. Grün and others (2001) measured changes in isoflavone profile during thermal processing of tofu, Wang and others (1998) measured changes in isoflavone profile during processing of soy protein isolates, and Coward and others (1998) evaluated modification of isoflavones in soyfoods during cooking and processing.

There is interest in isoflavone profile due to reported differences in their bioavailability for humans. Hendrich and Murphy (2001) have reviewed the absorption of soy isoflavones. Glycosidic isoflavones, the malonyl-, acetyl-, and β-glucosides, have not been detected in human blood plasma or urine, suggesting that these forms are transformed by glycosidases in the gut before being passively absorbed. The isoflavones are probably glucuronidated in the

gastrointestinal mucosa and in the liver. About 1/3 of adult humans can metabolize daidzein to equol, another isoflavone, in the gut (Murphy and Hendrich 2002). Setchell and others (1998) determined the plasma half-life of daidzein and genistein to be 7.9 h in adults with peak concentrations occurring in 6-8 hours after consumption. Plasma concentrations of daidzein, genistein, and equol range from 50-800 ng/mL may be achieved when food with about 50 mg/d is consumed. Isoflavones are excreted in urine and feces, but mass balance indicates that further metabolism of isoflavones occurs in the gut due to the actions of microbes (Hendrich and Murphy 2001).

Information concerning the negative health aspects of soy isoflavones is limited. Zhang and others (1999) showed that 10 μM genistein inhibited human natural killer cell activity *in vitro*. Kulling and others (1999) showed that 25 μM genistein caused chromosomal abnormalities in human peripheral blood lymphocytes, but daidzein as high as 100 μM did not yield these abnormalities. It should be noted that these concentrations are much higher than can be obtained through normal dietary means. It does however leave room for concern about abuse of isoflavone supplements. Certainly, isoflavones have been consumed by large numbers of humans for centuries without dramatic detrimental health impacts. However, two population groups, infants and breast cancer survivors, have been suggested to be at risk from soy isoflavones due to their mild estrogenic properties. Isoflavones have been estimated to possess between 10^{-5} and 10^{-2} the activity of 17 β-estradiol on a molar basis (Messina and Lopronzi 2001).

Sheehan (1997) has expressed concerns about the consumption of soybean phytoestrogens by infants and their potential effect on reproductive systems. He calculated that mothers living in Hong Kong and consuming soy in their diet deliver approximately 4 μg isoflavone/kg infant body weight per day. Infants fed soy-based formulas may consume as much as 4000 μg/kg/d. Adults consuming 50 g of soy protein per day might consume 700 μg/kg/d. The author notes that this amount has been shown to cause hormonal effects in premenopausal women. It is mentioned that little is known about the conversion of glucoside forms to aglucon forms in the infant gut. However, Setchell and others (1997) indicated that

β-glucosidase activity is high enough by four months of age to hydrolyze isoflavone glycosides to aglucons. Murphy and others (1997) showed that several major soy-based infant formulas contained isoflavones that were primarily in the glucoside or malonylglucoside forms.

The American Academy of Pediatrics recommends breast feeding or cow's milk-based formulas, but states that soy protein-based formulas are a safe and effective alternative. It has been estimated that approximately 25% of the nearly 4 million newborns in the U.S. consume infant formula (AAPCN 1998). Badger and others (2002) cited several studies that demonstrated normal growth and development of soy formula-fed infants. Strom and others (2001) followed up on a small cohort of 811 male and female subjects, now aged 20-34 years, that had consumed either cow's milk or soy-based formula as infants. No statistically significant differences were found for growth, development, puberty, reproductive function and pregnancy outcomes. Clearly larger cohorts will need to be followed to calm fears about potential estrogenic and reproductive effects on infants.

Part of the basis for this reproductive and developmental concern relates to the observed effects of a clover diet on Australian sheep. The isoflavone formononetin was high in that clover, was converted to equol, and led to reproductive disorders and infertility (Bennetts and others 1946). Although about 1/3 of adult humans can metabolize daidzein to equol in the gut (Murphy and Hendrich 2002). Badger and others (2002) reason in their review of the subject that equol is nearly undetectable in infants fed soy based-infant formula and that the physiological differences between adult sheep and infant humans makes comparison difficult. They also point out that soy products containing isoflavones have been consumed for centuries in countries with high population growth and that sexual development and fertility problems have not been observed in those populations.

Concerns about isoflavone consumption by infants will likely continue to be a topic for considerable debate until definitive studies are done to gauge the impact of the isoflavones on infants. The impact of isoflavones on breast cancer patients will also require further consideration. Conflicting data prevent a confident recommendation for this segment of the

population. Messina and Loprinzi (2001) present a lengthy review of this topic that is far beyond the scope of the current literature review. They cite studies that show isoflavones, particularly genistein, have been shown to both stimulate and inhibit cell or tumor growth depending on the animal model. Since many women who have had breast cancer are now taking tamoxifen, which binds to estrogen receptor sites competitively against estrogen, consideration was given to what interaction or impact isoflavones might have on this drug. Results were conflicting for *in vitro* studies, but *in vivo* studies suggested that there was no harm and potentially a benefit to consuming soy isoflavones. Gotoh and others (1998) evaluated control, 10% miso, tamoxifen, and miso plus tamoxifen diets to rats with N-nitroso-N-methylurea-induced mammary cancer. Tumor incidence was 91, 77, 68, and 10% and tumor multiplicity was 4.5, 2.4, 1.4, and 0.2%, respectively, for the different diets. Constantinou and others (2001) evaluated tamoxifen, soy protein isolate, and the combination on DMBA-induced mammary carcinogenesis. Tamoxifen reduced the number of tumors per rat by 29%, while soy protein isolate and the combination reduced the number of tumors by 37 and 62%, respectively. Messina and Loprinzi concluded that the data does not clearly show whether soy is harmful or beneficial to breast cancer patients, but suggested that if women enjoy consuming soy foods that they should continue to do so in moderation.

The health benefits of isoflavones may include diminished menopausal symptoms, improved cardiovascular health, decreased osteoporosis, and cancer inhibition. It should again be noted that it is difficult to say with certainty that some other component of soy is not responsible for some of the benefits. It is possible that two or more components of soy act synergistically.

Isoflavones, Soy, and Menopausal Symptoms

Hot flushes are one side effect of menopause. Hormone replacement therapy (HRT) has been used to diminish flushes and protect against CHD and osteoporosis. However, recent reviews question the efficacy of HRT for preventing the latter conditions (van der Schouw and Grobbee 2001, Grady and Cummings 2001). HRT is also questionable for women with a

family history of breast cancer. Isoflavones are being promoted as estrogen replacements and many food items and supplements are marketed toward these consumers. Setchell and Cassidy (1999) reviewed recent literature and suggested that phytoestrogens were able to act as weak estrogens, especially in postmenopausal women. Evaluating the effect of soy foods on hot flushes has led to conflicting results and a high placebo effect (Albertazzi and others 1998, Baird and others 1995, Brzezinski and others 1997, Murkies and others 1995).

Isoflavones, Soy, and Cardiovascular Health

In 1999, the US FDA made it clear that they believed there is a relationship between soy consumption and cardiovascular health. A cardiovascular health claim for a soy food can be applied when each serving has 6.25 grams of soy protein, contains less than 3 grams of fat (limit does not apply if all fat is from soy), contains less than 1 gram of saturated fat (total amount of saturated fat must not exceed 15% of total calories), contains no more than 20 milligrams of cholesterol, and contains less than 480 mg of sodium (main dish and meal products must contain <720 or <960 mg sodium, respectively). Studies have shown that soy consumption and/or isoflavone consumption leads to lower serum cholesterol and lower LDL to HDL ratios in humans (Anderson and others 1995), decreased atherosclerosis in monkeys (Anthony and Clarkson 1998), and increased arterial elasticity in women (Nestel and others 1997).

As mentioned when discussing saponins and hypocholesterolemia above, researchers do not agree on the active components in soy for these cardiovascular health benefits. A review by Sirtori and Lovati (2001) presents evidence that suggests that soy proteins, particularly the 7S fraction, are responsible for the hypocholesterolemic effects and point out studies in which ethanol-extracted soy devoid of soy isoflavones had no effect. Cassidy and others (1995) showed that isoflavones reduced total and LDL cholesterol in young women when they received a dose of 45 mg/day. Nestel and others (1997) showed that a 45 mg/d dose of genistein over several weeks did not change lipid profiles in women. Hendrich and Murphy (2001) suggested that soy isoflavones are not the only components of soy responsible for the

hypocholesterolemic effects and point out that the FDA did not include a statement about soy isoflavones in the soy health claim statement.

Murphy and Hendrich (2002) propose mechanisms for the cardiovascular protecting effects of soy and soy components as: plasma lipid and profile improvement, mediated effects on blood pressure, effects on vascular and endothelial cell functions, platelet aggregation, activation and serotonin storage, LDL oxidation state, smooth muscle proliferation, estrogen receptor mediated-effects, and LDL receptor interactions. Research will continue in the attempt to elucidate the soy components and their roles in minimizing cardiovascular disease.

Isoflavones, Soy, and Osteoporosis

Soy seems to play a role in diminishing or preventing osteoporosis. Again, whether the active component is isoflavones or some other component of soy is unclear. Some evidence suggests that isoflavones likely play a role. A synthetic isoflavone, ipriflavone, lacks estrogenic activity but is structurally similar to daidzein and genistein, and one of its metabolites is daidzein. Ipriflavone is approved as an alternative to hormone replacement therapy for bone loss prevention. Arjmandi and others (1996) fed ovarectomized rats isoflavone-containing soy or casein. Bone retention was observed for the rats containing the soy diet. Messina and others (2002) pointed out in their review that three human studies with women indicated that isoflavone-containing or isoflavone-spiked soy diets favored bone density retention while another study did not.

Murphy and Hendrich (2002) concluded that there is not enough evidence for a osteoporosis health claim for soy or isoflavones, but suggested that 60-90 mg of isoflavones per day, approximately 2-3 servings of traditional soy foods, may be useful for women concerned about osteoporosis to consume if they choose not to undergo hormone replacement therapy.

Isoflavones, Soy, and Cancer

Barnes and others (1990) proposed that isoflavones in soybeans were responsible for tumor inhibition in chemically induced mammary cancer in rats. They fed both raw and autoclaved soy. Theoretically, 90% of the protease inhibitors should have been destroyed during autoclaving, with some Bowman-Birk inhibitor remaining. Yamashita and others (1990) showed that genistein inhibited tyrosine protein kinases, DNA topo-isomerases, and S6 kinases, at least in during *in vitro* experiments. Lamartimiere and others (1998) presented data that suggested genistein, when given in pharmacological doses to pregnant rats, caused mammary gland differentiation or maturation in their pups that yielded tissue less susceptible to carcinogenic insult. Messina and others (2002) described a study in which 100 mg/d isoflavone supplement was consumed by premenopausal women. No change in tissue density was observed. Decreased breast tissue density, such as that usually seen with tamoxifen use, generally is related to reduced cancer risk. They related that another study in women consuming 40 mg/d isoflavones from red clover did experience significantly reduced tissue density. These are good signs that isoflavones do not promote breast cancer and may reduce cancer risks. Murphy and Hendrich (2002) indicate that many in vitro studies have shown that genistein or daidzein alter cancer cell proliferation, but these occurred when using concentrations greater than that would be experienced from dietary soy and isoflavone consumption, considered to be in the range of 0.01 to 5 μM.

Asian men have lower incidences of prostate cancer than Europeans (Morton and others 1997) and exhibit a higher concentration of equol and daidzein in their plasma and prostatic fluid. Since prostatic cancer responds to estrogen therapy, the authors suggested that phytoestrogens may play a role in reduced risk in prostate cancer. Pollard and Luckert (1997) showed that a rat model consuming an isoflavone-rich diet had a reduced rate of prostate cancer and a prolonged disease-free period when fed a soy-rich diet. Peterson and Barnes (1991 and 1993) were able to show that genistein and biochanin A inhibit growth of prostatic cancer cells in vitro. These results are promising, but more and long-term studies

need to be done in humans to determine the true effect of soy and soy phytochemicals on prostate cancer.

E. Quantification of Isoflavones and Saponins and Preparation of Enriched Fractions

Isoflavone Quantification

Isoflavones are extracted by aqueous organic solvent solutions. A comparison of effective solvents has been reported by Murphy and others (2002). High performance liquid chromatography (HPLC) analysis has been the workhorse for quantification of isoflavones in food samples, as detailed in the "Isoflavone" section of this literature review. To accurately quantify soybean isoflavones, all 12 of the major isoflavone forms (aglucons, β-glucosides, 6"-O-acetylglucosides, and 6"-O-malonylglucosides) must be purified and standard curves must be prepared for each. Purified standards of the aglucons and β-glucosides are commercially available. LC Laboratories (Woburn, MA) and Indofine Chemical Company, Inc. (Somerville, NJ) claim to have all 12 soy isoflavones commercially available. However, difficulties have been encountered purchasing the malonylglucosides. These isoflavones are labile, making them difficult to purify and retain in purified form without conversion. All 12 of the major soy isoflavone forms (aglucons, β-glucosides, 6"-O-acetylglucosides, and 6"-O-malonylglucosides) have been purified and quantified in our laboratory and used to develop the necessary standard curves. Klump and others (2001) have developed a process for alkaline heat extraction of isoflavones in which malonyl- and acetylglucosides are converted to β-glucosides for analysis. This is now an AOAC method and presents the advantage that laboratories only need to prepare standard curves for the aglucon and β-glucosides. The disadvantage of this method is the inability to evaluate the true isoflavone profile of the sample material.

Murphy and others (1999) have detailed a quantification procedure employing HPLC analysis and have used it to build a USDA database for isoflavones in food products, as well

as measure isoflavones in urine, fecal, and plasma samples. HPLC analysis is an excellent method for quantifying isoflavones, but some have felt that the approximately one hour per sample run time was too slow and that other methods were more sensitive.

Wang and Sporns (2000) have described a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method for isoflavone quantification. The authors report that a MALDI-TOF MS analysis can provide an isoflavone profile in 2 min. In their study, only daidzin and genistin were evaluated as standards. HPLC coupled with MALDI-TOF allowed them to evaluate peaks that they assumed were other isoflavones, basing this assumption on their HPLC retention times compared to those reported by other researchers.

Fang and others (2002) characterized isoflavones in rat urine using liquid chromatography/mass spectrometry/mass spectrometry. This method was not faster than regular HPLC analysis, actually taking 80 min per sample, but did offer the advantage of assisting in identifying isoflavones and their metabolites. Daidzein, genistein, biochanin A, glycitein, dihydrodaidzein, dihydrogenistein, and *O*-desmethylangolenstein were used as standards. The authors reported the method to be simple and sensitive.

Holder and others (1999) reported the use of liquid chromatography/electrospray-mass spectrometric quantification of genistein and daidzein and conjugated isoflavones in rat blood. Their extraction efficiency, using acetonitrile followed by a lengthy sample preparation process, was approximately 85% for genistein and the detection limit was reported to be 15 nM. The authors reported that an advantage of the LC/ES-MS method over LC/MS was the ability to measure isotopically-labeled internal standards.

Some reported isoflavone quantification methods do not use HPLC. Creeke and others (1998) and Bennetau-Pelissero and others (2000) have described the use of ELIZA to measure pg and pmol quantities of soy isoflavones extracted from plasma or soy samples, respectively. Gas chromatography has been used to measure isoflavones in foods and non-food seeds (Mazur and others 1998, Liggins and others 1998).

A problem with most of these methods is the cost to purchase, maintain, and effectively operate the required equipment. HPLC analysis will likely continue to be the preferred method for isoflavone analysis.

Saponin Quantification

Hu and others (2002) describe a method for quantification of Group B saponins. Previously, there was not a method to quantify saponins V, I, and II and their DDMP-conjugated forms (αg, βg, and βa) in the same analysis. Formononetin was used as an internal standard. Ireland and others (1985), Oleszek and others (1990), and Tsukamoto and others (1995) have reported methods for HPLC-mass detection or ultraviolet absorbance detection to measure different saponin forms, either modified or unmodified.

Dalluge and others (2003) have developed a liquid chromatography/electrospray ionization-mass spectrometry method to simultaneously identify all 12 soy isoflavones and 13 soyasaponins, as well as quantify soyasaponin B_b. Glycyrrhizin was used as an internal standard.

Gu and others (2002) used liquid chromatography-mass spectrometry to simultaneously quantify all group A and group B soyasaponins. The structural similarity of these compounds has made them difficult to resolve by other methods. Samples were treated under mild conditions with sodium hydroxide to deacetylate group A saponins and cleave the DDMP moiety from the group B saponins. According to the authors, "Soyasaponins originating from the same initial structures were unified into well-defined structures and then quantified individually using the selective ion recording of their $[M-H]^-$ ions." Quantification limits of group A and group B saponins were 1.74 and 1.89 ng, respectively. Recovery rates were 94.1 and 96.9%, respectively.

Hemolysis (Birk and others 1969), colorimetric (Hostettmann and Marston 1995), gas chromatography-flame ionization detector (Meyer and others 1990), and gas chromatography-mass spectrometry (Price and others 1984) have been used for saponin quantification. Thin layer chromatography, a relatively simple, inexpensive method for

qualitative and quantitative analysis, has been an important tool for evaluating samples. Various capacities of this technique have been described by Shimoyamada and others (1990), Shiraiwa and others (1991), Price and others (1986), Curl and others (1988), Karikura and others (1991), and Hasegawa and others (1996).

Most laboratories may find that equipment costs and technical expertise may limit them to using HPLC for the qualitative and quantitative analysis of soyasaponins.

Preparation of Enriched Fractions

Soy isoflavones are currently a desired ingredient for the supplement market and soyasaponins may be the next ingredient of interest. This is a brief review concerning methods to prepare enriched fractions.

Kelly and others (2000) have described a pilot-scale method to extract aglucon isoflavones from defatted soy flour. A 7,000-kg quantity of soy flour was placed in a 10,000-L vessel containing 5,000 L of water and 10 kg of β-glucanase. A 1,000-L volume of ethyl acetate was layered on top. Glucoside isoflavones leached from the soy flour during agitation were hydrolyzed to the aglucon form and removed into the ethyl acetate phase. After about 18 h the agitation was stopped, separation of the phases occurred, and the ethyl acetate layer was removed. The ethyl acetate was evaporated until about 20 L remained. To this slurry 200 L of hexane were added and mixed for five min and then allowed to stand overnight. This produced a sludge, which was collected and dried at 85 °C. This material contained about 60% isoflavones. A more enriched fraction can be produced if the starting material is soy hypocotyls. A genistein-rich fraction can be recovered from the isoflavone-rich dried sludge produced from soy flour or soy hypocotyl material. Approximately 3 kg of material was mixed with 1,000 L of acetone for about 2 h. After settling, the solvent was removed and evaporated leaving a genistein fraction that was about 92% pure, with the residual being daidzein and glycitein. Genistein purity was further increased to about 98.5% by employing an additional extraction step using ethanol at 80 °C, an ethanol volume reduction of approximately 70%, followed by crystallization of genistein upon cooling.

Gugger and others (2001) disclose a process by which soy solubles were heated to 80 °C, then passed through an ultrafiltration system with a 10,000 MW cutoff. From 15.26 kg of starting materials 9.4 kg of permeate were recovered. The permeate contained 6.6 g of isoflavones. Diafiltration of the retentate yielded another 2.14 g of isoflavones. Also described is the further purification of the permeate using a resin-packed glass liquid-chromatography column.

Waggle and others (2002) have described a method to recover isoflavones from soy molasses, the liquid material produced during aqueous acid or aqueous ethanol leaching of defatted soy flakes to produce soy protein concentrate. Enzymes were added to the molasses to convert almost all glucoside isoflavones to aglucons. Aglucons were precipitated by lowering the pH to about 4.5 and chilling to about 4 °C. The precipitate was formed into a cake by centrifuging, and the cake contained about 2-5% isoflavones by weight.

Because isoflavones and soyasaponins are amphiphilic compounds that are extractable with weak organic solvent in water mixtures, soyasaponins are a likely contaminant of the enriched isoflavone fractions described above. Indeed, Dobbins (2002) described a method to recover an enriched saponin fraction starting from commercial soy isoflavone concentrates. Isoflavone concentrate was added to a 4:1 acetone and water solution, and then refluxed at 56 °C for 90 minutes. This material was filtered while maintaining temperatures above 50 °C. The filtrate was cooled to 5 °C and the precipitate that was formed was recovered by filtration. The recovered precipitate was 86% saponins and contained 0.2% isoflavones by weight. Isoflavones could be recovered from the stripped filtrate by extracting with hexane at 60 °C, mixing, settling, and dividing the two layers. Lecithin and fats remained in the hexane layer, while isoflavones were precipitated out of the aqueous layer at $<$10 °C. This filtered precipitate was found to be 72% isoflavones.

Dobbins' process demonstrates the difficulty in separating isoflavones from saponins. Preparation of very pure fractions can only be accomplished with extensive chromatography. This separation problem will plague or add expense to projects designed to elucidate the biological properties of these two phytochemicals.

F. Soy Protein Structure

On a dry weight basis, average soybeans contain 40% protein, and the protein is stored in protein bodies, which usually have a diameter of 5-8 μm but can range in size from 2-20 μm. There has been some disagreement whether protein bodies are bound by membranes. Pernollet (1978) indicated that crystalloid protein bodies did not have membranes. Recent work, however, suggests that they are bound by Golgi- or endoplasmic reticulum-derived membranes (Herman and Larkins 1999, Kinney and others 2001). Boatright and Kim (2000) were able to show that membrane-bound crystalloid structures were visible with transmission electron micrography when samples were fixed at pH 6.4 and 5.6, but were not observed well when fixed at pH 7.2, a pH in the range of 7.0-7.4 commonly used for biological tissues.

Ultracentrifugation of water-soluble protein extracts shows four major fractions with sedimentation values of 2S, 7S, 11S, and 15S. Wolf (1969) provided a sedimentation profile of these fractions. The 2S fraction contains, among other components, Kunitz and Bowman-Birk protease inhibitors and cytochrome *c*. The 7S fraction contains the α-, β-, and γ-conglycinin proteins, with β-conglycinin being the predominant form at about 50% of the three proteins. The 7S fraction may also include lectin, lipoxygenase, and β-amylase. The 11S fraction contains the glycinin protein fraction. The 15S fraction is thought to be an aggregation of glycinin. At low ionic strength (e.g. 0.1 vs. 0.5 M) a 9S fraction exists. The 9S fraction is thought to be a dimer of the 7S fraction. Nielsen (1985a) suggested that the proportions of these fractions are about 20, 33, 33, and 10% for 2S, 7S, 11S, and 15S, respectively. Lehnhardt and others (1983) reported that a commercial extraction of soy will contain these protein fractions at 22, 37, 31, and 11%, respectively.

Because the 7S and 11S globulin fractions comprise the majority of the extractable proteins, they have received the most attention. β-Conglycinin, the predominant 7S protein, is a trimer with a molecular mass of 150-200 kDa. It is a glycoprotein with a carbohydrate content of about 5%. Three major and one minor subunits have been identified: α, α', β, and γ (minor). Thanh and Shibasaki (1976a) determined that the molecular mass in kDa of α, α',

β to be 57, 57, and 42 by electrophoretic mobility in SDS-polyacrylamide gels, respectively, while Medeiros (1982) reported these values to be 72, 68, and 52 kDa. These subunits have been found to combine in several different combinations within β-conglycinin trimers: $\alpha'\beta_2$, $\alpha\beta_2$, $\alpha\alpha'\beta$, $\alpha_2\beta$, $\alpha_2\alpha'$, α_3, and β_3 (Thanh and Shibasaki 1978, Yamauchi and others 1981).

The 11S glycinin is a hexamer with a molecular mass of 300-380 kDa. Each of the subunits comprising the hexamer has an acidic polypeptide (acidic pI, ~35 kDa) and a basic polypeptide (basic pI, ~20 kDa), and these polypetides are linked by a disulfide bond. Six acidic and five basic subunit types have been identified and have been observed to naturally assemble as: $A_{1a}B_{1b}$, A_2B_{1a}, $A_{1b}B_2$, $A_5A_4B_3$, and A_3B_4 (Nielsen 1985b).

Utsumi and others (1997) and Liu (1997) provide more complete reviews about 11S and 7S structure and amino acid sequence. The 11S and 7S, or glycinin and β-conglycinin fractions, are primarily responsible for the functional properties of soy proteins. Fractionation of these proteins is of interest to better understand their separate contribution to functionality, to evaluate their impact on nutrition and potential health benefits, and to identify opportunities for new product development.

G. Fractionation of Soy Proteins

Differing physicochemical characteristics of the 11S and 7S protein fractions, such as isoelectric point, thermally-induced precipitation behavior, chemical interaction with reducing agents, and sensitivity to ionic strength, are among those used to aid in the fractionation of these proteins. Early research evaluated these parameters at the bench scale, but more recently these have been explored at pilot and industrial scales.

Osborne and Campbell (1898) used a 10% sodium chloride solution to extract soy protein. The protein remaining after dialysis against water was termed glycinin. They also reported preparing three other fractions. Other researchers reported preparing soy extract fractions that had different isoelectric points and displayed different nitrogen concentrations. Consequently this "glycinin" fraction was recognized as being very heterogeneous. Nielsen

(1985a) credits Roberts and Briggs (1965) as one of the early researchers isolating and characterizing conglycinins and Catsimpoolas and Ekenstam (1969) for coining the three immunologically distinct fractions of conglycinin as α, β, and γ.

Wolf and Sly (1967) used ultracentrifugation to explore whether meal to extraction ratio, extraction temperature, rate of cryoprecipitation, pH, and addition of reducing agents, sucrose, or salts improved cryoprecipitation of 11S proteins from 15S (soy protein polymers), 7S, and 2S (containing trypsin inhibitors) proteins. They observed a direct relationship between an increase in protein extraction temperature and contamination of the 11S fraction by 15S, 7S, and 2S proteins, but 11S concentration from extract produced at 40 °C was twice that produced at 25 °C. Cryoprecipitation of 11S in 10 mL of extract at 0-2 °C was generally complete in less than one hour. Concentrations of sodium chloride and sucrose greater than 0.3 and 0.6 M, respectively, inhibited cryoprecipitation, but calcium chloride enhanced quantitative isolation of the 11S fraction. Reducing agents did not affect cryoprecipitation. These data were generated from bench-scale experimentation. Eldridge and Wolf (1967) and Wolf and Briggs (1959) also describe bench-scale purification and characterization of 11S protein.

Roberts and Briggs (1965) described a bench-scale isolation and characterization of 7S protein accomplished by sequential salting out with ammonium sulfate coupled with cold treatments. A 2% globulin extraction in 0.5 ionic strength buffer at pH 7.6 was treated with ammonium sulfate or cold treatment to produce precipitate fractions. Precipitates were removed at: 1) 0.40 saturation, 2) 0.50-0.55 saturation, 3) cold treatment, 4) 0.60 saturation, 5) cold treatment, and 6) 0.80-0.90 saturation. Fraction 1 contained primarily 2S proteins. Fraction 2 contained most of the 15S proteins and primarily 11S proteins with contaminating amounts of 2S and 7S. Fractions 4 and 5 were both comprised of 70% 7S and 30% 11S proteins. Fraction 6 contained 85% 7S, 5% 2S, and 10% 11S. This may have been suitable for producing small quantities of enriched 7S, or β-conglycinin fraction, but it is not practical for large-scale production of that fraction.

Thanh and Shibasaki (1976b) described a straightforward means to sequentially fractionate the 11S and 7S fractions. They used a meal to 0.03 M Tris-HCl buffer ratio of 1:20 at pH 8.0 containing 0.01 M 2-mercaptoethanol to extract the soluble soy protein. The insolubles were removed by centrifugation, then the supernatant was acidified to pH 6.4 with 2N HCl. The 11S (glycinin) precipitate was removed by centrifugation. The supernatant was acidified to pH 4.8. The resulting 7S (β-conglycinin) precipitate was suspended in 0.03 M Tris-HCl and dissolved by increasing the pH to 7.6 with 2 N NaOH. Then the pH was adjusted to 6.2. After centrifugation the supernatant fraction contained the 7S globulin fraction and the precipitate fraction contained the 9S, or dimerized 7S fraction. Thanh and Shibasaki claimed that there was very little cross contamination between the two fractions. However, Iwabuchi and Yamauchi (1987), using immunological methods, determined that the method of Thanh and Shibasaki produced a glycinin fraction that was 79% glycinin, 6% β-conglycinin, and 15% other, whereas the β-conglycinin fraction contained 52% β-conglycinin, 3% glycinin, and 45% other.

O'Keefe and others (1991) modified Thanh and Shibasaki's method. Instead of using precipitation at 6.4 and 4.8 to recover the glycinin and β-conglycinin fractions, they incorporated an additional precipitation step at pH 5.3. This method produced a crude β-conglycinin fraction that had less contamination by glycinin, verified by SDS-PAGE, but the authors reported a decrease in yield of β-conglycinin fraction. Some of the loss undoubtedly occurred in the intermediate fraction precipitated at pH 5.3. Immunoelectrophoresis indicated no immunoreactive glycinin in the β-conglycinin fraction and only 0.06% β-conglycinin in the glycinin fraction. It should be noted, however, that denatured forms of contaminating glycinin and β-conglycinin would not have been quantified by the immunoelectrophoresis, possibly suggesting less contamination than was actually present.

Nagano and others (1992) also developed their own modified version of Thanh and Shibasaki's method. They used a meal to solvent ratio of 1:15 rather than 1:20, an extraction pH of 7.5 rather than 8.0, and water as the extracting solution rather than 0.03 M Tris buffer. After insolubles were separated from the solubilized protein, sodium bisulfite at (0.98 g/L)

was added, rather than 2-mercaptoethanol. The supernatant was acidified to pH 6.4 and the mixture was chilled overnight on ice. The glycinin precipitate was removed by centrifugation, and then the ionic strength of the supernatant was increased by adding sodium chloride until the concentration was 0.25 M. This solution was then acidified to pH 5.0. This produced an intermediate fraction than Thanh and Shibasaki's method did not produce, but similar to the intermediate fraction prepared by O'Keefe and others. After the intermediate fraction was removed by centrifugation, a two-fold dilution of distilled water followed by acidification to pH 4.8 was used to precipitate the β-conglycinin fraction. Nagano and others indicated that their glycinin and β-conglycinin fraction purities were both greater than 90% according to SDS-PAGE densitometry results.

Several U.S. patents have been awarded for protein fractionation processes. Davidson and others (1979) disclosed a pilot-scale process by which four different protein fractions with different functional properties could be produced from a soy protein extract. Defatted soy flakes with a minimum nitrogen solubility index of 60 were agitated with water at a 1:5 ratio. Extraction was carried out at 55 to 70 °C for 15 to 50 minutes at pH 6.2 to 6.8, the pH of an aqueous extraction of the flakes without additional pH adjustment. Insolubles were removed by centrifuge or by screening. The authors stated that to improve protein recovery from the insoluble fraction two extraction passes could be employed using a 8:1 solvent-to-flake ratio. A counter-current process was described by which the extract from a previous extraction was used to extract proteins from unextracted flakes. Flakes that have been extracted once were exposed to fresh water for the second extraction. The extraction was carried out at about 65 °C with 15 minutes of extraction time for each step. The resulting extract was about 10 to 11% solids and 6 to 7% protein. The recovered extract was cooled to 5 to 10 °C over one to three hours, then held at that temperature for about one hour. This first precipitate fraction (Fraction 1) was then removed by centrifugation, yielding a yellow, stringy curd. The precipitate, or curd, was then either freeze-dried, spray-dried, or received additional washing. Before washing the curd, the pH was lowered to 5.3, then washed at

27 °C, and then the pH was adjusted to 6.3 to 6.8 before drying. The supernatant was further processed in one of two ways.

The first process method included decreasing the pH of the supernatant to 4.5, near the isoelectric point. The resulting curd (Fraction 2) was removed by centrifugation and dried. The second process began by heating the extract back to 32 to 38 °C, then acidifying to 5.3. The resulting precipitate (Fraction 3) was removed by centrifugation, washed, neutralized to pH 6.5, and then dried. The whey from the previous step was brought to 10 to 16 °C and acidified to pH 4.5. The resulting curd (Fraction 4) was rubbery and was freeze-dried rather than spray-dried due to the difficulty encountered in preparing a suspension. It could be re-dissolved by adding water and adjusting the pH to 6.8, after which it could be spray dried.

The patented process of Davidson and others was essentially a two-stage process yielding glycinin and β-conglycinin fractions or a three stage process yielding a glycinin, intermediate, and β-conglycinin fraction. The low extraction pH likely led to a poor recovery of soluble proteins as it was at or near the pH of 6.4 used to precipitate glycinin in other fractionation processes. Additionally, freeze drying would not be practical for pilot- or industrial-scale processes.

A patent by Howard and others (1983) disclosed a method to make 7S and 11S fractions. They referenced early patents concerning soy protein isolate production and isolation of glycinin. Their invention suggested the use of defatted soybean meal, soy flour, soy grits, soy protein concentrates, and/or soy isolates as starting materials. Solvent-to-flake ratios of 5:1 to 20:1 were specified, with a ratio of 10:1 reported as optimal for 7S and 11S extraction. While extractions could be carried out at 6.0 to 10.0, they indicted that 7.0 to 8.5 was best to preserve the native characteristics of the 11S and 7S proteins. A 20 to 40 °C temperature range was cited as best for extraction, but it was indicated that temperatures of up to 50 °C could be used for short periods, i.e. two minutes or less.

The first example of the process of Howard and others (1983) produced three protein fractions. Fraction 1 was 95% 11S as determined by SDS-PAGE, while the second fraction contained 30% 7S and 70% 11S. The third fraction reportedly was 95% 7S. These products

were produced from defatted soy grit. The grits were extracted with a 10:1 solvent-to-flake ratio at pH 8.0 for 30 minutes at 22 °C. The extracting solution also contained 0.03 M sodium chloride and 0.77 mM sodium bisulfite. The extract was acidified to pH 6.0 to recover the 11S fraction. The resulting supernatant was acidified to pH 5.5 to recover the mixed 11S and 7S fractions. Acidification of the resulting supernatant to pH 4.5 precipitated the 7S fraction.

Also disclosed in the patent by Howard and others (1983) was the effect of varying the ionic strength and reducing strength of the extraction solution. At pH 6.0 the percent curd yield increased dramatically from 3.2 to 19.7% as sodium chloride concentration was increased from 0 to 0.02 M. Contamination of 11S curd by 7S generally decreased as sodium chloride concentration was increased. It was shown that when sodium bisulfite was omitted or was present at concentrations greater than 2.5 mM a significant portion of the 7S fraction was insoluble and contaminated the 11S curd. A range of 0.48 to 1.92 mM was determined to be acceptable to keep the 7S fraction soluble during acid precipitation of the 11S and mixed 11S/7S fractions. Solubility of the 7S fraction was less than 80% in this bisulfite range at pH 5.5.

Lehnhardt and others (1983) disclosed a dramatically different approach in the patent for pilot-scale 11S and 7S protein fractionation. In their first example they used a 14:1 solvent-to-defatted soy grit ratio stirred slowly for one hour at 40 °C and pH 8.0. The extract was acidified to pH 4.3. The isoelectrically precipitated curd was isolated by centrifugation. The curd was dispersed in water at a ratio of 15 (dry solids basis) to 85. Sodium bisulfite and sodium chloride were added to prepare concentrations of 7.5 mM and 0.1 M, respectively, and this solution was stirred at 21.5 °C for 30 minutes at pH 4.5. The pH was then adjusted to pH 5.3 and stirring was continued for one hour. The resulting suspension was centrifuged. The resulting 11S curd reportedly was comprised of 70.5% 11S and 29.6% 7S as determined by SDS-PAGE. The 7S-containing extract was again acidified to precipitate the 7S fraction, which was removed by centrifugation. The 7S fraction reportedly had only 11% contamination.

Lehnhardt and others (1983) also evaluated the effect of the 7S extracting medium pH on the composition of the 7S soluble and 11S insoluble fractions. As the pH was increased from 5.1 to 5.6, the percentage of 7S in the 7S fraction decreased from 94.9 to 46.6%. The 7S composition of the insoluble 11S fraction decreased from 40.0 to 23.1% over the same pH range. The composition of the pH 4.3 curd was 37.4 % 7S and 51.0% 11S. The authors indicated that it was desirable to allow some contamination of the 7S fraction by 11S to provide ease of manipulation of the 7S fraction, which otherwise would be a highly visco-elastic gum.

Wu and others (1999) described a pilot-scale fractionation process yielding kilogram quantities of fractionated soy storage proteins. The process parameters were quite similar to that of Nagano and others (1992). Fifteen to twenty kg of defatted soy flakes were suspended in water at a solvent-to-flake ratio of 10:1 to 15:1. The pH was adjusted to 8.5 and the suspension was stirred for one hour at 20 °C. The insolubles were removed by centrifugation, and then mixed with water at a 1:10 or 1:5 ratio. The second extraction, also at 20 °C, lasted for 30 minutes. Insolubles were again removed by centrifugation. The extracts from the first and second extraction steps were combined, sodium bisulfite added at 0.98 g/L, the solution was acidified to pH 6.4, and the suspension was chilled overnight at 4 °C. The glycinin, or 11S, precipitate was removed by centrifugation. Sodium chloride was added to the supernatant to prepare a 0.25 M concentration and the solution was acidified to pH 5.0. After stirring for one hour, the suspension was centrifuged to remove an intermediate fraction comprised primarily of glycinin and β-conglycinin proteins. Water was added 2:1 to the supernatant, the pH was lowered to 4.8, and the suspension was chilled at 4 °C overnight. The β-conglycinin fraction was recovered by centrifugation.

Wu's process was sequential in nature and utilized continuous centrifuges. Continuous centrifuges may not return yields as high as those produced at the bench scale using high-speed, batch centrifuges, because the de-watering capabilities are not as good. However, the process clearly demonstrated scale-up potential. The percent yield on a dry basis for Wu's bench-scale glycinin, β-conglycinin, and intermediate fractions were 12.9, 9.8, and 10.1,

respectively, while those for the pilot-scale process were 11.2, 10.9, and 9.1. SDS-PAGE densitometry indicated that purities of the bench-scale and pilot scale glycinin fractions were 95.7 and 84.2%, respectively, while those for β-conglycinin were 77.6 and 71.8%.

Other fractionation methods have been published. Wu and others (2000) used pH adjustment and ultrafiltration as a simplified method to fractionate glycinin and β-conglycinin. Production of the solubilized protein extract was the same as that for Wu and others (1999). Sodium bisulfite was added to the extract to bring the concentration to 0.03 M, the pH was lowered to 6.0, and the suspension was chilled overnight at 7 °C. Centrifugation recovered the glycinin fraction. The supernatant was adjusted to pH 7.0 and concentrated with an RC-100 membrane feed and bleed system. The retentate was the β-conglycinin fraction. An intermediate fraction was not produced.

Wu's simplified method had dry basis yields of glycinin and β-conglycinin at 9.7 and 19.6%, whereas the sequential fractionation method had yields of 9.4, 10.3, and 4.8% for glycinin, β-conglycinin, and intermediate, respectively. Protein content and purity of the glycinin fractions was reportedly similar between the two methods. The β-conglycinin purity of the simplified method was 9% lower than that of the sequential fractionation method.

Saito and others (2001) developed a novel bench-scale method for fractionating glycinin and β-conglycinin employing phytase. Soymilk was produced from defatted soy flakes, and the pH of the soymilk was adjusted to 6.0. Phytase was added to this soymilk, and the mixture was held 1 hour at 40 °C. The precipitated protein fraction was removed by centrifugation, yielding the glycinin fraction. The supernatant was acidified to pH 5.0, and the resulting precipitate, the β-conglycinin fraction, was removed by centrifugation. The authors reported that the purity of both fractions was greater than 80%. The potential advantage of this method is that the treated extract does not need to be chilled to precipitate glycinin. It also has the added benefit of reducing the phytate content of soy.

Currently, glycinin (11S) and β-conglycinin (7S) fractions are not commercially available. The additional processing steps to prepare the fractions are time-consuming and involve much more extensive use of processing equipment compared to production of soy

concentrates and soy protein isolates. The functional properties or nutritional aspects of the glycinin and β-conglycinin fractions may not offer cost-effective advantages compared to existing soy ingredient products. Since only very small amounts of these products have been available for applications evaluation, little information concerning this experimentation exists in the literature. Bringe's (2001) patent disclosed a soybean line that had a high β-conglycinin content and described application of the high β-conglycinin isolate in regard to very successful beverage and cheese production, as well as its functionality as an emulsifier and gelling agent. If plant breeding efforts to shift glycinin and β-conglycinin ratios are successful, the need to fractionate soy proteins through processing techniques may become moot.

H. Soy Protein Functionality

Kinsella (1979) defined protein functionality as the physicochemical properties of the proteins and their interactions with other food components. Examples of functional properties are solubility, emulsification, foaming capacity and stability, gelation, water- and fat-binding capacity, hydrophobicity, viscosity, and flavor-binding. Many frustrating factors confound comparisons of results among published data. First, preparation of the protein is critical. Processing steps that differ in heat treatment and duration, pH, contaminating ions and ionic strength, and type of isolation and purification buffers and conditions are a few of the considerations that must be taken into account. Each condition will play a role in the configuration of the protein relative to its native state and how that configuration reacts in a particular environment. Even the ratio of the types of proteins comprising the protein sample, such as the 7S and 11S fractions in soy, and the type and prevalence of subunits comprising each particular protein, such as the α, α' and β subunits of β-conglycinin, affect protein functionality. These subunit differences could occur between the same plant genetic source grown in different environmental conditions or be due to varietal differences. Finally, the functionality tests themselves can be maddening. There are many published methods to

measure a particular functionality, most being empirical rather than intrinsic, but ensuring uniform testing between laboratories can be difficult. Regenstein and Regenstein (1984) pointed out several considerations for performing solubility testing including dissolving time, concentration of material, dissolving conditions, stirring, appropriateness of the solvent, and relationship of the measurement to the final use in the product.

Functionality of proteins can be purposely altered by physical methods, such as heating, acidification, or alkali treatments that change the secondary and tertiary structures of a protein; by chemical methods, such as acylation, phosphorylation, and deamidation; and by enzymatic methods leading to proteolysis with enzymes, such as trypsin, pepsin, and bromelin. Liu (1997) presents a short review of each of these topics. Boye and others (1997) and Schwenke (1997) present more extensive reviews.

Volumes could easily be written reviewing functionality testing for soy products alone. This review will cover the functionality tests most relevant to my research.

Solubility

Different protein solubilities are necessary to produce a wide variety of food systems. To produce a protein drink in which the protein stays in solution, a highly soluble protein is desired. Processed meats, for which water and fat binding is desired, utilize proteins with low solubility so that the proteins are not lost during heat processing.

Protein solubility is influenced by, among a host of other factors, the relative proportion of hydrophilic and hydrophobic areas on the exterior of a protein, the charge density of the protein, pH and ionic strength of the solution, and temperature (Vojdani 1997). Terms to express protein solubility are protein dispersibility index, water dispersibility protein, nitrogen solubility index, solubility index, water soluble protein, percent protein or nitrogen solubility, degree of turbidity, percent insolubility, dispersibility and maximum protein solubility. Protein solubility is usually determined in salt-free water or buffered water at a specified ionic strength, although the buffer can influence solubility. Methods used to measure liquid or solid protein samples are Kjeldahl, Dumas, spectrophotometric at 280 nm

or 191-194 nm, Lowry, bicinchoninic acid, Biuret, and dye-binding. Each of these methods has advantages and disadvantages.

Solubility is measured by adding a known amount of sample or protein to water or buffer. If water is used, acid or base can be used to adjust pH as desired. After a suitable solubilization and stabilization period (e.g. 1 h with stirring that does not produce foaming), the solution is clarified, usually by centrifugation. Protein content of the extract, and sometimes the precipitate, is measured.

Shen (1976, 1981) showed the effects of pH and ionic strength on soy protein isolate and described the effects of various salts on soy protein solubility. The data show evaluation of protein solubility at one pH is not sufficient to compare different proteins, although a one point evaluation is not uncommon. For instance, Khatib and others (2002) evaluated the solubility of glycinin and β-conglycinin fractions from four different soybean varieties. The solubility of β-conglycinin fractions at pH 7 ranged from 55 to 67%, while glycinin solubility ranged from 65 to 84%. This gives a limited picture of two very different soy proteins. Others have attempted to give a more complete description of solubility. Lakemond and others (2000) reported that at an ionic strength of 0.2 their purified glycinin exhibited nearly 100% solubility at pH<4 and >6.5 and about 10% at pH 4.6 to 5.8. Wu and others (1998) evaluated the effects of papain modification of soy protein isolate. The solubility of the isolate was approximately 38, 4, 55, 85, and 92% at pH 3, 5, 7, 9, 11, respectively. Enzymatic treatment increased solubility. Exhaustive analysis at over a range of pH values and ionic strengths generated by different cationic and anionic salts provides the best understanding of solubility for a particular protein, but what is practical must also be considered.

Gelation

A gel network is necessary in many food systems to hold moisture, fat, flavors, and water-soluble components. Gels also provide the characteristic and customary textures of many foods.

According to Matsumura and Mori (1996), gelation is a two-step process involving conformational change or partial denaturation of protein followed by gradual association or aggregation of the protein molecules. Tombs (1974) describes gelation of globular proteins as a highly oriented 'string of beads', generally yielding a transparent or translucent appearance, or a random aggregation, generally yielding a turbid appearance. Chemical forces involved in gel formation are hydrophobic interactions, hydrogen bonding, electrostatic interaction, and disulfide bonding. Some of the research reviewed below evaluates how these types of interactions can be enhanced or disrupted with regard to gel formation.

Yamauchi and others (1991) published an extensive review covering the heat-induced gelation of the soy proteins, glycinin, and β-conglycinin, as related to structure of the proteins. Effects of protein concentration, reducing agents, ionic strength, protein subunit contribution, and temperature of denaturation were also considered.

Choice of gelation temperature is aided by differential scanning calorimetry (DSC) data. Onset (T_0), peak (T_d), and final (T_f) protein denaturation temperatures can be obtained from DSC data. When a protein solution is heated in the range of T_0 to T_d, a weak gel forms. Gel strength increases from T_d to T_f, due to the increased number of denatured protein molecules available to aggregate and form a network. Above T_f the gel hardness decreases due to excessive heat damage. Puppo and Anon (1999) showed that 20% (w/w) solutions of 7S and 11S fractions at pH 8.0 had peak denaturation temperatures of 79 and 90 °C, respectively. Yamauchi and others (1991) cited peak denaturation temperatures of 75 and 90 °C, respectively. Sorgentini and others (1995) reported the temperatures as 74 and 83 °C, respectively. Denaturation temperatures are affected by pH and salt concentration (Kinsella and others 1985), so heating conditions must be chosen carefully to ensure that the proteins denature during the heating process.

Gel properties can be measured by two different methods (Matsumura and Mori 1996). The first involves small deformation using oscillatory rheology, which provides fundamental information. A complex shear modulus G* is defined by:

$$G^* = G' + iG''$$

where G' is the storage (elastic) modulus, G" is the loss (viscosity) modulus, and i is a complex number. The second method uses large deformation force and provides empirical information, such as firmness or hardness measured in units such as Newtons. Usually the sample is pierced or deformed with a plunger causing rupture of the gel structure.

Hatta and others (1986) explored the effects of pH (2-9) and sodium chloride concentration (10-80 mM) on gel strength of a 5% ovalbumin system heated at 80 °C. Peak gel strengths occurred at pH 3 and 7 for all NaCl concentrations, with these strengths being highest at 30 and 40 mM sodium chloride concentration. For all ionic strengths, gels were generally weak between pH 4-5 and were very weak or nonexistent at pH 2 and 8.

Nakamura and others (1986) indicated that minimum glycinin and β-conglycinin concentrations for gel formation at 100 °C and 0.5 μ were 2.5 and 7.5%, respectively. Khatib and others (2002) reported that the storage modulus (G') of glycinin and β-conglycinin gels from four soybean varieties ranged from 4.43 to 5.39 and 4.11 to 4.64, respectively, indicating that the elastic characteristics were not very different for the two major storage proteins.

Nagano and others (1996) evaluated gelation of preheated soy protein isolates prepared from a control, a glycinin-rich, and a β-conglycinin-rich soybean variety. A 12% (w/w) solution of each was heated from 30 to 80 °C, and then cooled to 20 °C at 2 °C/min. The G'/Pa, an expression elasticity or strength, for the glycinin-rich and control samples were still zero at 80 °C, but the β-conglycinin-rich line was about 60. Final G'/Pa were about 250, 60, and 2 for the β-conglycinin-rich, control, and glycinin-rich varieties, respectively. The data may have been more useful had the temperatures been taken to 90 or 100 °C for a longer time, permitting denaturation of the glycinin in samples. These data do indicate, however, that a β-conglycinin isolate might be more useful than soy protein isolate for gelling in meat systems that are cooked to 160 °F (71.1 °C).

Wang and Damodaran (1991) used a puncture method to evaluate gel structure or hardness of soy isolate, 11S (glycinin), and 7S (β-conglycinin) gels of varying protein

concentrations and ionic strengths, contributed by 0.5 M NaCl and $NaClO_4$ (a structure destabilizer). Concentrations of soy protein ranging from 8-12% in 20 mM sodium phosphate buffer were heated at 90 °C for 30 min then cooled to 4 °C for 20 h. Gel hardness increased with increasing protein concentration for samples in buffer, with hardness (gm x 10^{-2}) at 12% protein being about 10, 5, and 10 for 11S, 7S, and soy isolate gels, respectively. At 0.5 M NaCl, the 11S sample did not form gels at any protein concentration. The 0.5 M $NaClO_4$ diminished gel strength somewhat. In contrast, the salt treatments did not dramatically affect 7S gel strength.

Whereas each laboratory is able to make comparisons between controls and treatments for their experiments, comparison of data among research projects is extremely difficult due to the difference in assay technique and sample preparation.

Emulsification Capacity

Many foods are protein-stabilized emulsions or foams. Examples are milk, butter, margarine, salad dressings, hot dogs, and ice cream. It is important to understand why different food proteins act differently and to predict how they will perform in food applications relative to other potential protein ingredients.

An emulsion can be defined as a dispersion of two immiscible liquids. There are two types of emulsions: water in oil, such as butter, or oil in water, such as milk. Over time, the dispersed phase of an emulsion will generally move within the continuous phase, causing creaming, coalescence, or flocculation. Creaming occurs if the dispersed phase is of lower density than the continuous phase, resulting in a change in distribution of the disperse phase. Coalescence occurs when dispersed droplets approach and merge into larger droplets. Flocculation occurs when dispersed droplets become associated with other droplets, but do not merge.

The stability of an emulsion depends on a balance of the forces associated at the interface of the oil and water. The van der Waals forces are attractive forces, but these tend to be balanced by electrostatic repulsive forces. Proteins, containing hydrophobic and hydrophilic

regions, may align themselves between the aqueous and disperse phases and provide steric forces that usually inhibit coalescence. Polymers in a system, such as starches, can prevent movement and association of the dispersed phase, but may enhance attraction in voids or regions where the polymer is lacking.

Proteins function as emulsifiers by decreasing the interfacial tension between the oil-water interface and by forming a barrier, comprised of electrostatic, structural, and mechanical components, that diminishes destabilization of the interface. Effectiveness of a protein in performing as a surfactant or emulsifier depends on its size, conformation (e.g. available hydrophobic and hydrophilic regions), viscosity relative to the system, the pH and ionic strength of the system, oil type, temperature, phase volumes, protein concentration, and the amount of mechanical energy applied to provide a homogeneous distribution of the protein. Kinsella (1979) describes soy globulins as slow to diffuse, but reduce interfacial tension between phases as protein concentration is increased. Salt addition may enhance diffusion rate, reduce charge repulsion between proteins, and improve interface hydrophilic associations.

Emulsification capacity is commonly defined as the mL of oil per g of protein added to a solution to cause an inversion from oil-in-water to water-in-oil emulsion. Oil is added at a uniform rate to a low-concentration protein solution that is being rapidly stirred in a mixing or blending action. Inversion from oil in water to water in oil is usually determined visually (change in appearance or sudden drop in viscosity) or by conductimetry (sudden increase in electrical resistance at inversion point). Mixing rate, container shape, mixing blade shape and size, and protein sample volume and concentration are some of the physical factors that will influence the result.

Liu's review (1997) summarizes that soy protein solubility is positively correlated with emulsification capacity. Emulsification capacity is decreased above 50 °C, but increases when ionic strength is increased from 0.03 to 0.05 at pH 7. Emulsion properties tend to be better at alkaline pH and poor at pH 5 to 6.

Ochiai and others (1981) showed that solubility of tryptic digested crude glycinin did not necessarily correlate with emulsification capacity. Heat-denatured and native state glycinin were enzymatically digested, and then the extract of the heat-denatured, enzymatically treated glycinin was separated into two fractions of differing molecular weight. The digested native-state glycinin, the high-molecular weight fraction, and the unhydrolyzed glycinin had solubility profiles that were near zero at about pH 5 and nearly 100 % within +/- one pH unit from pH 5. The emulsification capacity profiles followed that trend as well, but emulsification capacities at pH 5 were only half that of capacities below pH 4 and above pH 7. The low molecular weight fraction no longer displayed the typical solubility curve and displayed solubility near 80% for the entire pH profile. The emulsification capacity profile for the low molecular weight fraction across different pH values was fairly flat as well, but was generally lower than that for other treatments. Consequently, it is difficult to say that solubility is always positively correlated with emulsification capacity.

Bernardi Don and others (1991) also demonstrated that solubility of enzymatically-treated soy does not translate into improved emulsification capacity. A bacterial and a fungal protease were used to digest the soy protein. Solubility was improved by proteolysis, but emulsification capacity was not.

De Kanterewicz and others (1987) developed an index that described the relative hydrophobic-lipophilic nature of several proteins using water and oil uptake to characterize these attributes. The water-oil absorption index for a given protein was the mL water/g sample divided by the mL oil/g sample absorbed. They found that emulsification capacity was highest when the index value was near two and poor at extremes of the index value. They concluded that proteins need to have a suitable balance between lipophilic and hydrophilic characteristics. It was shown that this prediction method was less useful when applied to several different soy proteins at different concentrations. As concentration was increased from 0.5 to 4.0% there was a lower dependence of emulsification capacity on the index value.

Dagorn-Scaviner and others (1987) showed that emulsification capacities of pea legumin and vicilin, bovine serum albumin (BSA), and casein were 58, 61, 112, and 90 g of oil/100 mg protein, respectively. Bovine serum albumin has a molecular weight of about 66 kDa, is nearly 100% soluble from at least pH 3-9, and exhibits high surface hydrophobicity as measured by probe spectrofluorometry using 1-anilinonaphthalene-8-sulfonate (ANS). Still, Dagorn-Scaviner and others have shown, as we have in our laboratory, that BSA has a very high emulsification capacity.

Clearly, there are many confounding factors to consider when trying to find a predictor of emulsification capacity. Comparison of published results among laboratories is also extremely difficult given the assay variability mentioned above.

The emulsifying ability of soy proteins has been studied at the subunit level. Maruyama and others (1999) presented data concerning the structure-physicochemical function relationships of soybean β-conglycinin subunits. The α and α' subunits contain extension regions that are glycosylated. They suggested that solubility (at $\mu = 0.08$), heat-induced association, and emulsifying activity were affected substantially by the carbohydrate moieties, as well as the core regions of the subunits. This was determined using recombinant nonglycosylated subunits and deletion mutants. Maruyama (2002) evaluated the emulsifying ability of all possible β-conglycinin heterotrimers. Emulsion particle size distribution revealed that the jump from one β unit per molecule to two led to particle-size distributions skewed toward larger emulsion particles as determined by using a laser light scattering instrument. Additional studies such as these may help direct plant breeding to produce soybeans with preferred functionality traits.

Emulsification Activity and Stability

The ability of a protein to aid in formation and to stabilize a freshly prepared emulsion is termed its emulsifying activity (EA) or emulsifying activity index (EAI). Several methods to understand this functional property have been invented, unfortunately comparison of results among the methods is difficult if not impossible. Generally, a pre-determined volume of oil

and protein solution are combined and then blended aggressively to form an emulsion. Hill (1996) briefly reviews several of the methods. Determination of particle-size distribution of the dispersed phase, using microscopy, Coulter counting, spectroturbidity, and laser scattering techniques, is one way to determine EA and EAI (one definition being area of interface stabilized per unit weight of protein). Emulsifying activity has also been defined in terms of conductivity differences between a protein solution and a fresh emulsion of that solution (Kato and others 1985).

Emulsification stability is generally a temporal extension of the emulsification activity assay. It is a measure of the separation of the dispersed oil phase from the aqueous phase at prescribed temperature, gravitational field, and duration. Stability is measured in regard to creaming, flocculation, and coalescence as measured by visual, light-scattering techniques, ultrasound, and microscopy (Hill 1996). Destructive sampling of layers has also been utilized.

Dagorn-Scavinger and others (1987) evaluated the EAI of pea legumin and vicilin, BSA, and casein. Ten mL of oil were dispersed into 30 mL of 1.3 mg/mL protein solution at 20,000 rpm for 30 sec. One-mL aliquots of emulsion were diluted 250 fold with 0.1 M sodium chloride solution plus 0.1% sodium dodecyl sulfate at pH 7. Absorbance was immediately measured at 500 nm. Their reported EAI was in units of m^2/g, relating interfacial area stabilized to weight of protein. The EAI for pea legumin and vicilin, BSA, and casein were 60, 111, 133, and 110, respectively, differing somewhat in relation to their rankings for their emulsification capacities of 58, 61, 112, and 90 g oil/100 mg protein, respectively. The authors noted that their results were somewhat different from those of Pearce and Kinsella (1978) for BSA and casein, reporting values of 197 and 166, respectively. Experimental conditions were reportedly similar except for the type of blender. Although the EAI values for the two proteins were different between research groups, the BSA/casein ratios were the same.

Wu and others (1998) evaluated the emulsifying properties of soy protein isolate (SPI) with and without proteolytic enzyme modification by papain. Hydrolysates of 10, 30, and 60

min were produced, with a portion of the 60 min hydrolysate being separated by ultrafiltration into 20, 50 and 100 kDa fractions. Corn oil (2 mL) and 0.1% protein solution (6 mL) at pH 7 were combined and mechanically homogenized at high speed for 1 min. At 0 and 10 min 50 μL were pipetted from the bottom of the emulsion and diluted with 5 mL of 1% sodium dodecyl sulfate solution. Absorbance was measured at 500 nm. EAI was calculated as:

$$\text{EAI (m}^2\text{/g)} = 2T(A_0 \text{ x dilution factor/C x } \Phi \text{ x } 10{,}000)$$

where T = 2.303; A_0 = absorbance measured immediately after emulsion formation; dilution factor = 100, C = weight of protein/unit volume (g/mL) of aqueous phase before emulsion formation; and Φ = oil volume fraction of the emulsion. ESI was calculated as :

$$\text{ESI (min)} = A_0 \text{ x } \Delta t/\Delta A$$

where Δt = 10 min and $\Delta A = A_0 - A_{10}$.

Wu and others (1998) found that the SPI had an EAI of 100, while the EAI for the digests and ultra-filtered fractions were 200-230 and 280-315, respectively. The ESI for SPI was about 37, whereas the ESI for digests and most ultra-filtered fractions were 42-48. The ESI for the lowest molecular weight fraction was about 22. The data suggest that proteolysis and fractions with diminished molecular weights increased EAI, but did little to improve ESI. ESI was greatly reduced when molecular weights were as low as 20 kDa. Surface hydrophobicity increased from about 12 to 27 with digestion, but the ultra-filtered fractions had surface hydrophobicities of only about 2. The cleaved fractions were not terribly hydrophilic, but enzyme modification probably had a molecular unfolding effect, opening the molecule to expose previously-hidden hydrophobic areas. From this data it seems difficult to generalize or predict ESI and EAI based on hydrophobicity alone.

Chove and others (2001), using the methodology of Wu and others (1998), evaluated ESI and EAI of soy protein isolate fractions produced by sequential isoelectric precipitation at pH 5.6, 5.1, and 4.5 and non-sequential precipitation at these values. The authors concluded that

fractions high in 7S globulin protein fractions had better functionality in terms of ESI and EAI.

Khatib and others (2002) compared four soybean varieties for EAI and ESI of their respective glycinin and β-conglycinin fractions. EAI of the glycinin and β-conglycinin fractions ranged from 300 to 381 and 400 to 434 m^2/g, respectively, while ESI ranged from 160 to 191 h and 161 to 200 h, respectively. Emulsion was prepared by blending 10 mL of peanut oil with 30 mL of protein solution. Absorbance was read at 540 nm and initial and final ESI turbidity readings were taken at 0 and 24 h.

Liu and others (1999) evaluated the emulsifying properties of 11S and acidic 11S proteins at various protein concentrations (0.1 to 1.0%) and ionic strengths (0.05 to 0.50). Emulsifying properties of acidic subunits of 11S (AS11S) were found to be better than those of 11S and heat-denatured 11S. The EAI of the AS11S decreased from 1988 to 349 cm^2/mg, as protein concentration was increased. At pH 7 and 0.5% (w/v) emulsion the droplet size of the AS11S increased only slightly from 0 to 140 h at 0.05-0.15 ionic strength, but increased dramatically at ionic strengths of 0.2 to 0.5.

Additional research in this area is needed to better understand what factors influence emulsion stability and how to apply this information when developing food systems, since food systems are vastly different from well-defined laboratory conditions.

Foaming

Meringues, mousses, and beer are examples of air-protein food foams. Two thin air/water interfaces need to be created and maintained to prepare foams. Proteins adsorb at this interface, reduce surface tension, and interact via electrostatic, hydrophobic, hydrogen and covalent bonds that permit some stretching and deformation of the films. Mechanically-induced foams are prepared in at least four ways: sparging of gas through a column of protein solution, whipping, shaking, and pouring. Evaluations of protein foams usually include one or more of the following measurements: foam volume, drained liquid volume, time to foam to a given volume, stability of foam based on time required for a specific volume of the foam

to collapse. As with the other functionality tests described above, experimental conditions such as protein solution concentration, volume of solution, sparger pressure and frit size, blending speed, and container characteristics are among the variables that can greatly influence the foaming results.

Wilde and Clark (1996) reviewed foam formation and stability. They suggested that the amphipathic character of the amino acid side chains was responsible for the adsorption of proteins at interfaces. To effectively form interface films, proteins must move from the bulk solution to the interface, penetrate and incorporate into the surface layer, and undergo some degree of surface denaturation in the adsorbed layer. Protein size, surface hydrophobicity, and structural flexibility impact efficient protein film formation. Foams tend to be more stable around their isoelectric point since the charges are balanced and repulsion is minimized. Foams that maintain more liquid between the opposing interfacial films are more stable, preventing the films from contacting and rupturing. Liquid drainage from films is affected by bulk and surface viscosity and electrostatic repulsion of the two interfaces. Glycoproteins that contain large oligosaccharides that protrude into the lamellar liquid between the two interfaces have a stabilizing effect by reducing drainage.

Attempts have been made to improve foaming properties by modifying the proteins, e.g. enzymatic digestion, enzymatic modification, and chemical derivatization, or by modifying the solution conditions, e.g. pH, ionic strength, or viscosity. Reducing molecular weight will improve rate of diffusion to the interface. Some modifications have increased hydrophobicity, changed surface charge, and improved flexibility, thus improving foaming functionality.

Bernardi Don and others (1991) evaluated enzymatic modification of soy protein concentrates to improve functionality. Foaming capacity, the relative ability to produce foam, was evaluated by placing 30 mL of 3% (w/w) dispersion in a graduated cylinder and stirring it at 6000 rpm for 3 min. The percent volume increase of the system was calculated as a measure of foaming capacity. Foaming stability was determined by measuring the liquid drained from 10 mL of foam as a function of time. As degree of protein hydrolysis increased

from 0 to 10% the percent volume increased from 100 to 400%. There was little change in foaming capacity from 10 to 30% hydrolysis. However, foam stability was maximal at a 10% degree of hydrolysis, being about twice as stable as at 0 and 20% hydrolysis.

Wagner and Gueguen (1999) explored the effects of acid treatment and deamidation, as well as reducing treatment, on the foaming properties of glycinin. Foaming properties were measured by conductimetry. Foam was produced by sparging air into a column of protein solution at a rate of 10 mL/min until foam volume reached 75 mL. Maximum volume of liquid incorporated into the foam, rate of liquid incorporation, foam conductivity, and rate of liquid drainage, a measure of foam stability, were measured. The authors suggested that treatments, such as dissociation, deamidation, and reduction, that increased surface hydrophobicity, increased net charge, and decreased molecular size enhanced foam forming and stabilizing capacities.

Lipids can destabilize foams from the soy proteins in flour and concentrates. Table 13 displays foaming characteristics of some soy protein products. Isolate is at least 90% protein, whereas concentrate is only 60-70% protein with protein dilute by ash and fiber. Flour has the ash and fiber plus soluble carbohydrates. As components other than protein are removed, foaming capacity and foam stability are improved.

Detail of the methods used by the authors in this section was included to reveal the variety of assays and conditions used to evaluate foaming. There is need for standardization of methods and publication of additional research to promote the understanding of the factors at work in foam production and stability and how this information relates to actual food systems.

Surface Hydrophobicity in Relation to Protein Functionality

Surface hydrophobicity has been used to try to better understand and predict functionality properties. Hydrophobic characteristics are important in the amphipathic nature of proteins that stabilize interfaces, as well as how they affect solubility.

The surface characteristics of proteins, and especially those modified by heat, enzymatic, or chemical treatment, is not always known. Thus, we cannot always know or predict the

Table 13. Foaming capacity and stability of soy protein products

		Foam Stability – Volume after time (min)			
Product	Foaming Capacity (% volume increase)	1	10	30	60
Flour	70	160	131	108	61
Concentrate A	170	400	28	13	8
Concentrate B	135	370	265	142	30
Isolate A	235	670	620	572	545
Isolate B	230	660	603	564	535

Source: Lin and others (1974)

surface hydrophobicity. There are empirical methods to assess this characteristic. Among those are partitioning of proteins into aqueous two-phase systems (e.g. dextran and polyethylene glycol); HPLC; binding and measurement of associated non-polar or polar ligands; contact angle measurement; intrinsic fluorescence of tryptophan, tyrosine, and phenylalanine; and probe spectrofluorometry, which includes using amphiphilic 1-anilinonaphthalene-8-sulfonate (ANS) or cis-parinaric acid (Nakai and others 1996). Each of these methods has advantages and disadvantages, but detailing these is beyond the scope of this review.

The ANS method is quite popular because it is fast and, beyond the possession of a fluorometer, is relatively inexpensive. ANS binds to membranes or hydrophobic cavities, and bound ANS fluoresces in organic solvents. Nakai and others (1996) warn that some solvents that are non-polar may still favor the rigid, planar configuration of the ANS

molecule responsible for fluorescence. Also, electrostatic and hydrophobic interactions may alter interaction of the protein and probes.

Nakai (1983) reviewed the impact of protein hydrophobicity on structure-function relationships. It was suggested that hydrophobic and solubility information, when used in tandem, were better predictors of protein functionality than solubility alone. Kato and Nakai (1980) used fluorometry to evaluate several types of protein and determined there was a significant correlation ($P<0.01$) between hydrophobicity and emulsifying capacity. Emulsifying activity increased with respect to hydrophobicity, while interfacial tension declined.

Ovalbumin and lysozyme were heat treated without coagulation by Kato and others (1981). This caused an increase in surface hydrophobicity, a decrease in surface tension, and an increase in emulsifying and foaming capacities and stabilities.

Commercial and laboratory-prepared soy protein isolates were evaluated for solubility and surface hydrophobicity by the ANS method by Wagner and others (2000). After creating a scatter plot of these data, the authors grouped the results into three zones: A, B, and C. Group A isolates were shown by DSC to be high in native state glycinin and β-conglycinin and were prepared in the laboratory under mild conditions. Group A varied little by surface hydrophobicity, 170-200, but solubility ranged between 80 to nearly 100%. The isolates that had the highest solubility had been washed extensively with water, resulting in a decrease in salts, whey proteins and sugars, then re-dissolved to pH 8 and freeze dried with a low protein concentration. It was noted that all of these treatments tended to have a positive effect on solubility. Group B isolates had hydrophobicity values of 210 to 640 while having solubilities of about 65 to 98%. No obvious trend could be observed for solubility with regard to hydrophobicity. These isolates were prepared under conditions differing in time, temperature, and treatment with denaturing chemical agents. The authors tried to rationalize differences in solubility and hydrophobicity based on these differences, as well as differences in protein concentration during freeze drying. Group C isolates were dispersions of 8-13% protein that received substantial thermal treatment or 3% dispersions thermally treated in the

presence of calcium. For this group as surface hydrophobicity decreased solubility decreased. The authors hypothesized that "(a) the protein species undergoing aggregation are the more hydrophobic, so that only the hydrophilic ones remain soluble; and (b) as the proteins aggregate, they hide or occlude the hydrophobic zones, leaving part of the proteins as soluble aggregates of low surface hydrophobicity."

Maruyama and others (1999) evaluated the surface hydrophobicity of normal β-conglycinin subunits (α, α' and β) and deletion mutants of α and α' lacking the N-glycosylated extension regions. The hydrophobicity under all conditions, varied by ionic strength and pH, was α >α'>β, with the deletion mutants having nearly identical hydrophobicity compared to their α and α' counterparts. This indicates that the extension regions do not significantly affect hydrophobicity. The authors provided data suggesting that interactions between the subunits do not affect native state β-conglycinin hydrophobicity.

Wu and others (1999) used the ANS method to evaluate surface hydrophobicity of glycinin, β-conglycinin, and an intermediate fraction, containing both glycinin and β-conglycinin, prepared in during pilot-plant fractionation of the proteins. These fractions were evaluated against soy protein isolate, also prepared at pilot scale, and casein. Isolate, β-conglycinin, glycinin, intermediate, and casein had surface hydrophobicity values of approximately 600, 350, 275, 260, and 320. Wu hypothesized that the high value of the isolate was due to high salt content, which can increase protein-ANS interactions. The value for β-conglycinin with respect to glycinin was thought to be due to the amino acid composition and partial denaturation of the β-conglycinin. A significant portion of the glycinin and β-conglycinin fractions in the intermediate fraction was denatured, which one might expect to expose hydrophobic portions from the interior of the proteins. The somewhat lower hydrophobicity value of the intermediate fraction was thought to be due to lower protein content of the fraction.

Surface hydrophobicity may be useful for understanding protein functionality for certain protein products or under certain well-defined conditions. It should not be used to make generalizations about solubility or emulsifying ability.

Viscosity

Viscosity is a measure of the resistance of a medium to flow. It is important in food systems as it affects the ability of a protein to disperse and stabilize foams and emulsions, and it is also responsible for texture and mouth feel attributes. Protein concentration, dispersion conditions, pH, and ionic strength can all affect the viscosity of a solution or dispersion. Viscosity (η) is expressed in terms of shear stress (σ, force/unit area) and shear rate (γ, velocity/sample thickness). Newtonian fluids (e.g. water) have constant shear stress as shear rate is increased. Dispersions, emulsions, polymer solutions and colloids tend to display shear-thinning behavior. As shear rate is increased, shear stress increases somewhat rapidly, but as shear rate increases further shear stress increases more slowly. This is generally due to breakdown of structure within the liquid. A model often used to describe a simple shear-thinning liquid is the power law model:

$$\eta = K\dot{\gamma}^{n-1}$$

where η = apparent viscosity, K is a viscosity constant, $\dot{\gamma}$ = shear rate, and n = the power law index, which is less than 1 for shear-thinning liquids.

Measuring shear viscosity is often done with rotational viscometers, rate-controlled viscometers, or stress-controlled viscometers. Each of these techniques has advantages and disadvantages. These are outlined by Schenz and Morr (1996). Capillary viscometry is very accurate and provides intrinsic viscosity, but it is very time consuming. Surface viscometry is important in evaluating proteins used in emulsions and foams and measures the deformation and flow of film at a fluid interface with respect to adsorption or spreading.

Circle and others (1964) published a detailed evaluation of rheology of heated and unheated protein dispersions. Viscosity measurements were made on a Brookfield LVT viscometer using a Brookfield Helipath stand and appropriate spindles. The apparent viscosity of unheated proteinate increased from about 0.06 to 4 poises as proteinate concentration was increased from 2 to 10% by weight at 60 rpm . Proteinate dispersions of 8 to 12% were evaluated for unheated and heated (100 °C, 30 min) samples. Unheated samples increased from about 8 to 500 poises as concentration was increased, and heated

samples increased from about 75 to 1600 poises. As shear rate was increased from 3 to 60 spindle revolutions per minute, a 6% (w/w) dispersion experienced a drop in apparent viscosity from about 2 to 0.75 poises. When dispersions of 8 and 10% were measured at 0.2 to 60 rpm, apparent viscosity decreased from 50 to 4 poises and 400 to 9 poises, respectively. In another experiment, as pH of a 10% unheated dispersion was increased from 6 to 9 apparent viscosity increased from 9 to about 125 poises, with 99 poises at pH 7. Effects of various additives at different concentrations at pH 7 are shown below in Table 14.

The data in the table generally show that as salt concentration was increased, viscosity decreased. For food additives that are emulsifiers or thickeners, as concentration was increased viscosity increased. This is a useful table when trying to understand how additives might affect a food system using protein to provide viscosity.

Anon and others (2001) measured the apparent viscosity of commercial and laboratory soybean isolates at different water imbibing capacities (WIC). A Haake Rotavisco RV2 viscometer was used at 20 °C and rotor speed was varied from 0 to 128 rpm in 2 min and then held for 1 min at 128 rpm. Dispersions of 1.6 to 16% w/w were used. As WIC increased, the apparent viscosity increased, although laboratory isolates had values 10 times less than those of commercial isolates. Differences in particle density and shape of commercial isolates (spray-dried, small, spherical particles of ~1 g cm^3) and laboratory isolates (lyophilized, irregular, flat, and compact plaques of ≥1.2 g/cm^3) may have impacted apparent viscosity differences. Particles that align easily and do not resist flow exhibit a lower viscosity than particles that do not align well and physically interact more easily. The body of literature for viscosity of unheated soy protein isolates and other products is relatively small compared that for solubility and emulsification. Available rheology information tends to be from gelled proteins. Building literature regarding viscosity, especially for modified or novel preparations, should be useful since viscosity is such an important aspect in emulsion and film formation. This information would be useful for both food and industrial applications.

Table 14. Effect of various additives on apparent viscosity of a 10% soy proteinate dispersion

Additive	Additive concentration (%)	Poises
Control		43
NaCl	0.1	32
NaCl	1	18
$NaNO_3$	0.05	53
$NaNO_3$	0.5	37
$NaH_2PO_4 \bullet H_2O$	0.05	45
$NaH_2PO_4 \bullet H_2O$	0.5	28
$(NaPO_3)_n$	0.05	47
$(NaPO_3)_n$	0.5	25
Degummed soy oil	5	800
Degummed soy oil	20	3270
Soy lecithin	5	1330
Soy lecithin	10	3130
Wheat starch	5	48
Wheat starch	10	117
Carboxymethylcellulose	0.1	300
Carboxymethylcellulose	1	2600
Carrageenan	0.1	467
Carrageenan	1	2130
Na_2SO_3	0.01	14.7
Na_2SO_3	0.1	0.4
Na_2SO_3	0.5	0.2
$NaNO_2$	0.01	18.8
$NaNO_2$	0.1	28.7
$NaNO_2$	0.5	20

Table 14. (cont'd)		
$NaH_2PO_2 \cdot H_2O$	0.05	52
$NaH_2PO_2 \cdot H_2O$	0.5	33
Cysteine•HCl	0.05	0.4
Cysteine•HCl	0.5	27
Ascorbic Acid	0.05	49
Ascorbic Acid	0.5	467

Source: Circle and others (1964)

Conclusions

It is evident that there are difficulties in trying to predict performance relative to other proteins or preparations based on a single functionality test, such as solubility or hydrophobicity. Functionality testing on purified proteins, such as glycinin or β-conglycinin, or soy protein products, such as flours, concentrates, or isolates, provides basic research data that may assist in understanding what molecular aspects drive certain functionality characteristics. However, foods are complex systems. It may be useful to develop an understanding concerning how a protein's functionality in a well-defined, controlled system compares with the functionality of the protein in a real food system. This information may be extremely useful to small companies that don't have R&D resources to do extensive product development or functionality testing.

I. Conclusions

The soybean has probably received more scrutiny and attention than any other oil seed or cereal grain other than wheat. Given the rising world population and the soybean's position as a source of relatively low-cost and high-quality protein, worldwide soy consumption will continue to rise. The health benefits of its components will continue to be explored for years

to come. Developing the functional aspects of soy protein will continue to fuel its use in new and traditional food applications.

References

Albertazzi P, Pansini F, Bonaccorsi G, Zanotti L, Forinin E, DeAloysio D. 1998. The effects of dietary soy supplementation on hot flushes. Obstet Gynecol 91:6-11.

American Academy of Pediatrics Committee on Nutrition. 1998. Soy protein-based formulas: recommendations for use in infant feeding. Pediatrics 101:148-153.

American Soybean Association. 2003a.Defatted soy flour reference guide. http://soyasa.org/documents/0/DSF_RefGuide.htm.

American Soybean Association. 2003b. Soy protein concentration reference guide. http://soyasa.org/documents/0/SPC_RefGuide.htm.

American Soybean Association. 2003c. Soy protein isolate reference guide. http://soyasa.org/documents/0/SPI_RefGuide.htm.

American Soybean Association. 2003d. Textured soy protein reference guide. http://soyasa.org/documents/0/TSP_RefGuide.htm.

Anderson JW, Johnsstone BM, Cook-Newell ME. 1995. Meta-analysis of the effects of soy protein intake on serum lipids. N Engl J Med 333:276-282.

Anderson RL, Wolf WJ. 1995. Compositional changes in trypsin inhibitors, phytic acid, saponins, and isoflavones related to soybean processing. J Nutr 125: 581S-588S.

Anon CM, Sorgentini DA, Wagner JR. 2001. Relationships between different hydration properties of commercial and laboratory soybean isolates. J Agric Food Chem. 49:4852-4858.

Anthony MS, Clarkson TB. 1998. Comparison of soy phytoestrogens and conjugated equine estrogens on atherosclerosis progression in post-menopausal monkeys. Circulation 97:829.

Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M, Guo P, Kukreja SC. 1996. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 126:161-167.

Badger TM, Ronis MJJ, Hakkak R, Rowlands JC, Korourian S. 2002. The health consequences of early soy consumption. J Nutr 132:559S-565S.

Baird DD, Umbach DM, Lansell L, Hughes CL, Setchell KDR, Weinberg CR, Haney AF, Wilcox AJ, McLachlan JA. 1995. Dietary intervention study to assess estrogenicity of dietary soy among post-menopausal women. J Clin Endocrinol Metab 80:1685-1690.

Barnes S, Grubbs C Setchell S, Carlson J. 1990. Soybeans inhibit mammary tumors in models of breast cancer. In: Pariza MW, Aeschbacher HU, Eton JS, Sato S, editors. Mutagens and carcinogens in the diet. New York, Wiley.

Beecher GR, Holden J, Bhagwat S, Haytowitz D, Murphy PA. 1999. http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav/html

Begbie R, Pusztai A. 1989. The resistance to proteolytic breakdown of some plant (seed) proteins and their effects on nutrient utilization and gut metabolism. In: Friedman M, editor. Absorption and utilization of amino acids, CRC: Boca Raton, FL p 231-242.

Bennetau-Pelissero C, Le Houerou C, Lamothe V, Le Menn F, Babin R, Bennetau B. 2000. Synthesis of haptans and conjugates for ELISAs of phytoestrogens. Development of the immunological tests. J Agric Food Chem 48:305-311.
Bennetts HW, Underwood EJ, Shier FL. 1946. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Aus Vet J 22:2-12.
Berhow MA, Wagner ED, Vaughn SF, Plewa MJ. 2000. Characterization and antimutagenic activity of soybean saponin. Mutat Res 448:11-22.
Berk Z. 1992. Isolated soybean protein. In: Berk Z. editor. Technology of production of edible flours and protein products from soybeans. Haifa, Israel:Technion, Israel Institute of Technology.
Bernardi Don LS, Pilosaf AMR, Bartholomai GB. 1991. Enzymatic modification of soy protein concentrates by fungal and bacterial proteases. J Am Oil Chem Soc 68:102-105.
Birk Y. Saponins. 1969. Toxic constituents of plant food stuffs. In: Liener IE, editor. Academic Press, New York. p 161-182.
Boatright WL, Kim KS. 2000. Effect of electron microscopy fixation pH on the ultrastructure of soybean protein bodies. J Agric Food Chem 48:302-304.
Boye JI, Ma CY, Harwalkar VR. 1997. Thermal denaturation and coagulation of proteins. In: Damodaran S, Paraf A, editors. Food proteins and their applications. New York, Marcel Dekker. p 25-56.
Briggs DR, Mann RL. 1950. An electrophoretic analysis of soybean protein. Cereal Chem 27:243-257.
Bringe, NA, inventor; Monsanto Company, assignee. 2001 Jan 9. High beta-conglcyinin products and their use. U.S. Patent 6,171,640.
Brown JP. 1988. Hydrolysis of glycosides and esters. In: Roland JR, editor. Role of the gut flora in toxicity and cancer. San Diego: Academic Press. p 109-144.
Brzezinski A, Aldercreutz H, Shaoul R, Rosler A, Shmueli A, Tanos V, Schenker JG. 1997. Short-term effects of phytoestrogen-rich diet on postmenopausal women. Menopause 4:89-94.
Business Communications Co. Inc. 2001. Soyfoods: trends and developments. Norwalk, Connecticut.
Cantani A, Lucenti P. 1997. Natural history of soy allergy and/or intolerance in children, and clinical use of soy protein formulas. Pediatr Allergy Immunol 8:59-74.
Cassidy A, Bingham S, Setchell K. 1995. Biological effects of isoflavones in young women: importance of the chemical composition of soyabean products. Br J Nutr 74:587-601.
Catsimpoolas N, Ekenstam C. 1969. Isolation of alpha, beta, and gamma conglycinins. Arch Biochem Biophys 129:490-497.
Cheeke PR. 1996. Biological effects of feed and forage saponins and their impacts on animal production. In: Waller GR, Yamasaki K, editors. Saponins used in food and agriculture. New York, Plenum Press. p 377-380.
Chove BE, Grandison AS, Lewis MJ. 2001. Emulsifying properties of soy protein isolate fractions obtained by isoelectric precipitation. J Sci Food Agric. 81:759-763.
Circle SJ, Meyer EW, Whitney RW. 1964. Rheology of soy protein dispersions. Effect of heat and other factors on gelation. Cereal Chem 41:157-172.
Cook HJ. 1986. Neurobiology of the intestinal mucosa. Gastroenterol 90:1057-1081.

Constantinou A, Lantvit D, Lim E. Xu H, Pezzuto JM. 2001. Consumption of soy products may enhance tamoxifen's breast cancer preventative effects. Proc Am Assoc Cancer Res 42:826.

Coward L, Kirk M, Albin N, Barnes S. 1996. Analysis of plasma isolfavones by reverse-phase HPLC-reaction ion monitoring-mass spectrometry. Clin Chem 247:121.

Coward L, Smith M, Kirk M, Barnes S. 1998. Chemical modification of isoflavones in soyfoods during cooking and processing. Am J Clin Nutr 68:1486-1491S.

Creeke RI, Wilkinson AP, Lee HA, Morgan MRA, Price KR, Rhodes MJC. 1998. Development of ELISAs for the measurement of the dietary phytoestrogens daidzein and equol in human plasma. Food Agric Immunol 10:325-327.

Curl CL, Price KR, Fenwick GR. 1988. Isolation and structural elucidation of a new saponin ('soyasaponin v') from haricot bean. J Sci Food Agric 43:101-107.

Dagorn-Scaviner C, Gueguen J, Lefebvre J. 1987. Emulsifying properties of pea globulins as related to their adsorption behaviors. J Food Sci 52:335-341.

Dalluge JJ, Eliason E, Frazer S. 2003. Simultaneous identification of soyasaponins and isoflavones and quantification of soyasaponin B_b in soy products, using liquid chromatography/electrospray ionization-mass spectrometry. J Agric Food Chem 51:3520-3524.

Davidson RM, Sand RE, Johnson RE, inventors; Anderson, Clayton & Co., assignee. 1979 Oct 30. Method for processing soy protein and composition of matter. U.S. patent 4,172,828.

De Kanterewicz RJ, Elizalde BE, Pilosof AMR, Bartholomai GB. 1987. Water-oil absorption index (WOAI): A simple method for predicting the emulsifying capacity of food proteins. J Food Sci 52:1381-1383.

De Kleijn MJJ, van der Schouw YT, Wilson PWF, Grobbee DE, Jacques PF. 1999. Intake of dietary phytoestrogens in postmenopausal women: the Framingham heart study. Abstract Third international symposium on the role of soy in preventing and treating chronic disease.

De Weck-Gaudard D, Liardon R, Finot PA. 1988. Steriomeric composition of urinary lysinoalanine after ingestion of free or protein-bound lysinoalanine in rats. J Agric Food Chem 36:717-721.

deOliviera JE, de Souza N, Jordao, Jr AA, Marchin JS. 1998. Methionine supplementation of soy products: effects on nitrogen balance parameters. Arch Latinoam Nutr 48:35-40.

Dewick PM.1994. Isoflavanoids. In: Harborne JB, editor. The flavanoids: advances in research since 1986. New York; Chapman & Hall. p 117-232.

Dobbins T, inventor; Wiley Organics, Inc., assignee. 2002 March 12. Process for isolating saponins from soybean-derived materials. U.S. patent 6,355,816.

Eldridge AC, Wolf WJ. 1967. Purification of the 11S component of soybean proteins. Cereal Chem 44:645-652.

Fang N, Yu S, Badger TM. 2002. Characterization of isoflavones and their conjugates in female rat urine using LC/MS/MS. J Agric Food Chem 50:2700-2707.

Finot PA. 1997. Effects of processing and storage on the nutritional value of food proteins. In: Damodaran S, Paraf A, editors. Food proteins and their applications. New York:Marcel Dekker, Inc.

Franke AA, Hankin JH, Yu MC, Maskarinec G, Low SH, Custer LJ. 1999. Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. J Agric Food

Chem 47:977-986.
Friedman M. 1982. Chemically reactive and unreactive lysine as an index of browning. Diabetes 31:5-14.
Friedman M. 1999. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histadinoalanine in food and other proteins. J Agric Food Chem 47:1307-1318.
Friedman M, Brandon DL. 2001. Nutritional and health benefits of soy proteins. J Agric Food Chem 49:1069-1086.
Gestetner B, Assa Y, Henis Y, Tencer Y, Royman M, Birk Y, Bondi A. 1972. Interaction of Lucerne saponins with sterols. Biochim Biophys Acta 270:181-187.
Gibson GR. 2002. Human gut microbiology: the end of the food chain or the start of good health? Microbiology Today 29:4-6.
Gibson GR, Macfarlane GT. 1995. Human colonic bacteria: role in nutrition, physiology, and pathology. Boca Ration: CRC Press.
Gotoh T, Yamada K, Ito A, Yin H, Kataoka T, Dohi K. 1998. Chemoprevention of N -nitroso-N-methylurea-induced rat mammary cancer by miso and tamoxifen, alone and in combination. Jpn J Cancer Res 89:487-495.
Grady D, Cummings SR. 2001. Postmenopausal hormone therapy for prevention of fractures. How good is the evidence? J Am Med Assoc 285:2909-2910.
Graf E, Eaton JW. 1993. Suppression of colonic cancer by dietary phytic acid. Nutr Cancer 19:11-19.
Grün IU, Adhikari K, Li C, Li Y, Lin B, Zhang J, Fernando LN. 2001. Changes in the profile of genistein, daidzein, and their conjugates during thermal processing of tofu. J Agric Food Chem 49:2839-2843.
Gu L, Tao G, Gu W, Prior RL. 2002. Determination of soyasaponins in soy with LC-MS following structural unification by partial alkaline degradation. J Agric Food Chem 50:6951-6959.
Gugger E, Grabiel D, inventors; Archer Daniels Midland Company, assignee. 2001 Jan 9. Production of isoflavone enriched fractions from soy protein extracts. U.S. Patent 6,171,638.
Harborne JB. 1994. The flavanoids: advances in research since 1986. New York: Chapman Hall. 676 p.
Hasegawa H, Sung J, Matsumiya S, Uchiyama M. 1996. Main ginseng saponin metabolites formed by intestinal bacteria. Planta Med 62:453-457.
Hatta H, Kitabatake N, Doi E. 1986. Turbidity and hardness of a heat-induced gel of hen egg ovalbumin. Agric Biol Chem 50:2083-2089.
Hayakawa K, Mizutani J, Wada K, Masa T, Yoshihara I, Mitsuoka T. 1990. Effects of soybean oligosaccharides on human faecal flora. Microbiol Ecol Health Dis 3:293.
Hayashi K, Hayashi H, Hiraoka N, Ikeshiro Y. 1997. Inhibitory activity of soyasaponin II on virus replication *in vitro*. Plant Med 63:102-105.
Hendrich S, Murphy PA. 2001. In: Wildman REC, editor. Handbook of nutraceuticals and functional foods. Boca Raton, FL: CRC Press. p 55-75.
Henkel J. 2000. Soy: Health claims for soy protein, questions about other components. FDA Consumer May-June http://www.cfsan.fda.gov/~dms/fdsoypr.html.

Herman EM, Larkins BA. 1999. Protein storage bodies and vacuoles. The Plant Cell. 11:601 -613.
Hettiarrachachy N, Kalapathy K. 1997. Soybean protein products. In; Liu K., editor. Soybeans: chemistry, utilization, and technology. New York: Chapman and Hall.
Hill SE. 1996. Solubility. In: Hall GM, editor. Methods of testing protein functionality. New York:Blackie Academics & Professional. p 152-185.
Hirose M, Ozaki K, Tabaka K, Fukushima S, Shiarai T, Ito N. 1991. Modifying effects of the naturally occurring antioxidants γ-oryzanol, phytic acid, tannic acid and η-tririacontane-16,18-dione in a rat wide-spectrum organ carcinogenesis model. Carcinogenesis 12:1917 -1921.
Holder CL, Churchwell MI, Doerge DR. 1999. Quantification of soy isoflavones, genistein and daidzein, and conjugates in rat blood using LC/ES-MS. J Agric Food Chem 47:3764-3770.
Hostettmann K, Marston A. 1995. Saponins. In: Phillipson JD, Ayres DC, Baxter H, editors. Chemistry and pharmacology of natural products. Cambridge University Press:New York.
Howard PA, Lehnhardt WF, Orthoefer FT, inventors; A. E. Staley Manufacturing Company, assignee. 1983 Jan 11. 7S and 11S vegetable protein fractionation and isolation. U.S patent 4,368,151.
Hu J. 2000. Isolation and quantitative analysis of soybean saponins by high performance liquid chromatography. [MS Thesis] Ames, IA: Iowa State University. 96 p.
Hu J, Lee SO, Hendrich S, Murphy PA. 2002. Quantification of the Group B saponins by high performance liquid chromatography. J Agri Food Chem 50:2587-2594.
Illinois Specialty Farm Products. 2003. High sucrose soybeans – updated for 2003. http://web.aces.uiuc.edu/value/factsheets/soy/fact-sucrose-soy.htm.
Iowa State University Extension. 2000. What you need to know about new food words – phytochemicals, functional foods, nutraceuticals. PM 1846.
Ireland PA, Dziedzic SZ. 1985. Analysis of soybean saponins by high-performance liquid chromatography. J Chromatogr 325:275-281.
Ireland PA, Dziedzic SZ, Kearsley MW. 1986. Saponin content of soya and some commercial soya products by means of high-performance liquid chromatography of the sapogenins. J Sci Food Agric 37:694-698.
Irvine C, Fitzpatrcik M, Robertson I, Woodhams D. 1995. The potential adverse effects of soybean phytoestrogens in infant feeding. N. Z. Med J. 108:208-209.
Ishaaya I, Birk Y, Bondi A, Tencer Y. 1969. Soybean saponins IX. Studies of their effect on birds, mammals and cold-blooded organisms. J Sci Food Agric 20:433-436.
Iwabuchi S, Yamauchi F. 1987. Determination of glycinin and β-conglycinin in soybean proteins by immunological methods. J Agric Food Chem. 35:200-205.
Jariwalla RJ, Sabin R, Lawson S, Herman ZS. 1990. Lowering of serum cholesterol and triglycerides and modulation of divalent cations by dietary phytate. J Appl Nutr 42:18-28.
Jun HS, Kim SE, Sung MK. 2002. Protective effect of soybean saponins and major antioxidants against aflatoxin B1-induced mutagenicity and DNA-adduct formation. J Med Food 5:235-240.
Karikura M, Miyase T, Tanizawa H, Taniyama T, Takino Y. 1991. Studies on absorption, distribution, excretion and metabolism of ginseng saponins. VI. the decomposition products of ginsenoside Rb_2 in the stomach of rats. Chem Pharm Bull 39:400-404.

Kato A, Fujishige T, Matsudomi N, Kobayashi K. 1985. Determination of emulsifying properties of some proteins by conductivity measurements. J Food Sci. 50:56-58, 62.

Kato A, Nakai S. 1980. S Biochim Biophys Acta. 624:13-20.

Kato A, Tsutsumi N, Matsudomi N, Kobayashi K, Nakai S. 1981. Agric Biol Chem 45:2755 -2760.

Kelly GE, Huang JL, Deacon-Shaw MG, Waring MA, inventors; Novogen, Inc., assignee. 2000 Nov 14. Preparation of isoflavones from legumes. U.S. Patent 6,146,668.

Kennedy AR. 1995. The evidence for soybean products as cancer preventative agents. J Nutr 125:733S-743S.

Khalil AH, El-Adaway TA. 1994. Isolation, identification and toxicity of saponin from different legumes. Food Chem 50:197-201.

Khatib KA, Herald TJ, Aramouni FM, MacRitchie F, Schapaugh WT. 2002. Characterization and functional properties of soy β-conglycinin and glycinin of selected genotypes. J Food Sci 67:2923-2929.

Kim HJ, Chun YJ, Park JD, Kim SI, Roh JK, Jeong TC. 1997. Protection of rat liver microsomes against carbon tetrachloride-induced lipid peroxidation by red ginseng saponin through cytochrome P_{450} inhibition. Planta Med 63:415-418.

Kim JS, Kwon CS. 2001. Estimated dietary isoflavone intake of Korean population based on National Nutrition Survey. Nutr Res 21:947-953.

Kinjo J, Imagire M, Udayama M, Arao T, Nohara T. 1998. Structure-hepatoprotective relationships study of soyasaponins I-IV having soyasapogenol B as aglycone. Planta Med 64:233-236.

Kinney AJ, Jung R, Herman EM. 2001. Cosuppression of the α subunits of β-conglycinin in transgenic soybean seeds induces the formation of endoplasmic reticulum-derived protein bodies. The Plant Cell. 13:1165-1178.

Kinsella JE. 1976. Functional properties of proteins in foods: a survey. CRC Crit Rev Food Sci Nutri 7:219.

Kinsella JE. 1979. Functional properties of soy proteins. J Am Chem Soc 56:242-258.

Kinsella JE, Damodaran S, German B. 1985. Physicochemical and functional properties of oilseed proteins with emphasis on soy proteins. In: Altschul AM, Wilke HL, editors. New Protein Foods. New York, Academic Press. p 108-180.

Kirk P, Patterson RE, Lampe JL. 1999. Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults. Am J Diet Assoc 99:558-563.

Kitagawa I, Yoshikawa M, Hayashi T, Taniyama T. 1984. Quantitative determination of soyasaponins in soybeans of various origins and soybean products by means of high perfomance liquid chromatography. Yakugaku Zasshi 104:275-279.

Klita PT, Mathison GW, Fenton TW, Hardin RT. 1996. Effects of alfalfa root saponins on digestive function in sheep. J Anim Sci 74:1144-1156.

Klump S, Alfred MC, MacDonald JL, Ballam JM. 2001. Determination of isoflavones in soy and foods containing soy by extraction, saponification and LC: Collaborative study. J AOAC Int. 84:1865-1883.

Koo M, Rao AV. 1991. Long-term effect of Bifidobacteria and neosugar on precursor lesions of colonic cancer in CF1 mice. Nutr Cancer 16:249.

Koratkar R, Rao AV. 1997. Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions on the colon of mice. Nutr Cancer 27:206-209.

Kulling SE, Rosenberg B, Jacobs E, Metzler M. 1999. The phytoestrogens coumesterol and genistein induce structural chromosomal aberrations in cultured human peripheral blood lymphocytes. Arch Toxicol 73:50-54.

Kuo TM, VanMiddlesworth JF, Wolf WJ. 1988. Content of raffinose oligosaccharides and sucrose in various plant seeds. J Agric Food Chem 36:32.

Lacaille-Dubois MA. 2000. Biologically and pharmacologically active saponins from plants: recent advances. In: Oleszek W, Marston A, editors. Saponins in food, feedstuffs and medicinal plants. p 205-218.

Lakemond CMM, de Jongh HHJ, Hessing M, Gruppen H, Voragen AGJ. 2000. Soy glycinin: influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. J Agric Food Chem. 48:1985-1990.

Lamartiniere CA, Zhang JX, Cotroneo MS. 1998. Genistein studies in rats: potential for breast cancer prevention and reproductive and developmental toxicity. Am J Clin Nutr 68:1400S-1405S.

Langhendries JP, Hurrell RF, Furniss DE, Hirschenhuber C, Finot PA, Bernard A, Battisti O, Bertrand JM, Senterre J. 1992. Maillard reaction products and lysinoalanine: urinary excretion and the effects on kidney function of preterm infants fed heat-processed milk formula. J Pediatr Gastroentorol Nutri 14:62-70.

Lehnhardt WF, Gibson PW, Orthoefer FT, inventors; A. E. Staley Manufacturing Company, assignee. 1983 Jan 25. Fractionation and isolation of 7S and 11S protein from isoelectrically precipitated vegetable protein mixtures. U.S. patent 4,370,267.

Liener IE. 1986. Trypsin inhibitors: concern for human nutrition or not? J Nutr 116:920-923.

Liggins J, Bluck LJC, Coward WA, Bingham SA. 1998. Extraction and quantification of daidzein and genistein in food. Anal Biochem 264:1-7.

Liggins J, Bluck LJC, Runsweick S, Atkinson C, Coward WA, Bingham SA. 2000. Daidzein and genistein contents of fruits and nuts. J Nutri Biochem 11:326-331.

Lin MJ, Humbert ES, Sosulski FW. 1974. Certain functional properties of sunflower meal products. J Food Sci. 39:368-370.

Liu K. 1997. Soybeans: chemistry, technology, and utilization. Florence, KY: International Thomson Publishing. 532 p.

Liu M, Lee DS, Damodaran S. 1999. Emulsifying properties of acidic subunits of soy 11S globulin. J Agric Food Chem 47:4970-4975.

LMC International. 1999. Soy protein market analysis. NY: LMC International.

Lovati MR, Manzoni C, Gianazza E, Sirtori CR. 1998a. Soybean protein products as regulators of liver low-density lipoprotein receptors. I. Identification of active β-conglycinin subunits. J Agric Food Chem 46:2474-2480.

Lovati C, Lovati MR, Gianazza E, Sirtori CR. 1998b. Soybean protein products as regulators of liver low-density lipoprotein receptors. II. α-α' rich commercial soy concentrate and α' deficient mutant differently affect low-density lipoprotein receptor activation. J Agric Food Chem 46:2481-2484.

Lovati MR, Manzoni C, Gianazza E, Arnoldi A, Kurowska E, Carroll KK, Sirtori CR. 2000. Soy protein peptides regulate cholesterol homeostasis in Hep G2 cells. J Nutr 130:2543 -2549.

Mahungu S, Diaz-Mercado S, Li J, Schwenk M, Singletary K, Faller J. 1999. Stability of isoflavones during extrusion processing of corn/soy mixtures. J Agric Food Chem 47:279

-284.
Malinow MR, McNulty WP, McLaughlin P, Stafford C, Burns AK, Livingston AL, Kohler GO. 1981. The toxicity of alfalfa saponins in rats. Food & Cosmet Toxicol 19:443-445.
Malinow MR, McNulty WP, Houghton DC, Kessler S, Stenzel P, Goodnight SH, Bardana EJ, Polatay JL, McLaughlin P, Livingston AL. 1982. Lack of toxicity of alfalfa saponins in monkeys. J Med Primatol 11:106-118.
Manzoni C, Lovati MR, Gianazza E, Morita P, Sirtori CR. 1998. Soybean protein products as regulators of liver low-density lipoprotein receptors. II. Alpha-alpha′ rich commercial soy concentrate and alpha′ deficient mutant differently affect low-density lipoprotein receptor activation. J Agric Food Chem 46:2481-2484.
Mao LC, Lee KH, Erbersdobler HF. 1993. Effects of heat treatment on lysine in soya protein. J Sci Food Agric 62:307-309.
Maruyama N, Sato R, Wada Y, Matsumura Y, Goto H, Okuda E, Nakagawa S, Utsumi S. 1999. Structure-physicochemical function relationships of soybean β-conglycinin constituent subunits. J Agric Food Chem 47:5278-5284.
Maruyama N, Salleh MRM, Takahashi K, Yagasaki K, Goto H, Hontani N, Nakagawa S, Utsumi S. 2002. Structure-physicochemical function relationships of soybean β-conglycinin heterotrimers. J Agric Food Chem 50:4323-4326.
Matsumura Y, Mori T. 1996. Gelation. In: Hall GM, editor. Methods of testing protein functionality. New York:Blackie Academics & Professional. p 77-109.
Mazur WM, Duke JA, Wahala K, Rasku S, Adlercreutz H. 1998. Isoflavonoids and lignans in legumes: nutritional and health aspect in humans. J Nutri Biochem 9:192-200.
Medeiros JS. 1982. Characterization of the subunits of β-conglycinin and the application of ELISA to the determination of β-conglycinin and glycinin in soybeans. Ph.D. Thesis, Purdue University, West Lafayette, Indiana.
Messina MJ. 1997. Soyfoods: Their role in disease prevention and treatment. In: Liu K, editor. Soybeans: chemistry, technology, and utilization. New York: Chapman Hall. p 442-477.
Messina MJ, Loprinzi CL. 2001. Soy for breast cancer survivors: a critical review of the literature. J Nutr 131:3095S-3108S.
Messina M, Gardner C, Barnes S. 2002. Gaining insight into the health effects of soy but a long way still to go: commentary on the Fourth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease. J Nutr 132:547S-551S.
Meyer BN, Heinstein PF, Burnouf-Radosevich M, Delfel NE, McLaughlin JL. 1990. Bioactivity-directed isolation and characterization of quinoside A: one of the toxic/bitter principles of quinoa seeds (*Chenopodium quinoa wild*). J Agric Food Chem 38:205-208.
Morton MS, Chan PSF, Cheng C, Blacklock N, MatosFerreira A, AbranchesMontiero L, Correia R, Lloyd S, Griffiths K. 1997. Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the UK. Prostate 32:122-128.
Murkies AL, Lombard C, Strauss BJG, Wilcox G, Burger HG, Morton MS. 1995. Dietary flour supplementation decreases postmenopausal hot flushes – effect of soy and wheat. Maturitas 21:189-195.
Murphy PA, Wang H. 1994. Isoflavone content in commercial soybean foods. J Agric Food Chem 42:1666-1673.

Murphy PA, Song T, Buseman G, Barua K. 1997. Isoflavones in soy-based infant formulas. J Agric Food Chem 45:4635-4638.
Murphy PA, Song TT, Buseman G, Barua K, Beecher GR, Trainer D, Holden J. 1999. Isoflavones in retail and institutional soy foods. J Agric Food Chem 47:2697-2704.
Murphy PA, Hendrich S. 2002. Phytoestrogens in foods. Advances in food and nutrition research. 44:195-246.
Murphy PA, Barua K, Hauck CC. 2002. Solvent extraction selection in the determination of isoflavones in soy foods. Journal of Chromatography B 777:129-138.
Nagano T, Hirotsuka M, Mori H, Kohyama K, Nishinari K. 1992. Dynamic viscoelastic study on the gelation of 7S globulins from soybeans. J Agric Food Chem 40:941-944.
Nagano T, Fukuda Y, Akasaka T. 1996. Dynamic viscoelastic study on the gelation properties of β-conglycinin-rich and glycinin-rich soybean protein isolates. J Agric Food Chem 44:3484-3488.
Nakai S. 1983. Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. J Agric Food Chem 31:676-683.
Nakai S, Li-Chan E, Arteaga GE. 1996. Measurement of surface hydrophobicity. In: Hall GM, editor. Methods of testing protein functionality. New York: Blackie Academics & Professional. p 152-185.
Nakamura T, Utsumi S, Mori T. 1986. Interactions during heat-induced gelation in a mixed system of soybean 7S and 11S globulins. Agric Biol Chem 50:2429
Nestel PJ, Yamashita T, Sasahara T. 1997. Soy isoflavones improve systemic arterial compliance but not plasma lipids in menopausal and perimenopausal women. Arterioscler Thromb Vasc Bio 17:3392-3398.
Nielsen NC. 1985a. Structure of soy proteins. In: Altschul AM, Wilcke HL, editors. New protein foods. Volume 5: Seed Storage Proteins. New York: Academic Press, Inc.
Nielsen NC. 1985b. Structure and complexity of the 11S polypeptides in soybeans. J Am Oil Chem Soc 62:1680-1685.
Oakenfull DG, Topping DL, Illman RJ, Fenwick DE. 1984. Prevention of dietary hypercholestrolaemia in the rat by soya bean and quillaja saponins. Nutr Rep Inter 29:1039-1041.
Oakenfull D. 1986. Aggregation of saponins and bile acids in aqueous solution. Aust J Chem 39:1671-1673.
Oakenfull D, Sidhu GS. 1990. Could saponins be a useful treatment for hypercholesteremia? Eur J Clin Nutr 44:79-88.
Ochiai K, Kamata Y, Shibasaki K. 1982. Effect of tryptic digestion on emulsifying properties of soy protein. Agric Biol Chem 46:91-96.
Oh YJ, Sung MK. 2001. Soybean saponins inhibit cell proliferation by suppressing PKC activation and induce differentiation of HT-29 human colon adenocarcinoma cells. Nutr Cancer 39:132-138.
O'Keefe SF, Wilson LA, Resurreccion AP, Murphy PA. 1991. Determination of the binding of hexanal to soy glycinin and β-conglycinin in an aqueous model system using a headspace technique. J Agric Food Chem 39:1022-1027.
Oleszek W, Jurzystra M. 1990. High-performance liquid chromatography of alfalfa root saponins. J Chromatogr 519:109-116.

Osborne TB, Campbell GF. 1898. Proteids of the soy bean. J Am Chem Soc 20:419-428.
Oste RE. 1991. Digestibility of processed food proteins. Adv Exp Med Biol 289:371-388.
Pearce KN, Kinsella JE. 1978. Emulsifying properties of proteins: Evaluation of a turbidometric technique. J Agric Food Chem 26:716-723.
Perkin JE. 1990. Appendix B. General guidelines for management of soy allergy. In: Perkin JE, editor. Food allergies and adverse reactions. Gaithersberg, Maryland: Aspen Publishers, Inc. p 249-252.
Perkins EG. 1995. Composition of soybeans and soybean products. In: Erikson DR, editor. Practical handbook of soybean processing and utilization. Champaign, IL: AOCS Press and St. Louis, MO: United Soybean Board. p 9-28.
Pernollet JC. 1978. Protein bodies of seeds: Ultrastructure, biochemistry, biosynthesis and degradation. 17:1473-1480.
Peterson G, Barnes S. 1991. Genistein inhibition on the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. Biochem Biophys Res Comm 179:661.
Petereson G, Barnes S. 1993. Isoflavones inhibit the growth of human prostate cancer cell lines without inhibiting epidermal growth factor receptor phosphorylation. Prostate 22:335 -345.
Pollard M, Luckert PH. 1997. Influence of isoflavones in soy protein isolates on development of induced prostate-related cancers in L-W rats. Nutr Cancer 28:41-45.
Potter JD, Topping DL, Oakenfull D. 1979. Soya saponins and plasma cholesterol. Lancet i:223.
Potter SM, Jimenez-Flores R, Pollack J, Lone TA, Berber-Jimenez. 1993. Protein-saponin interaction and its influence on blood lipids. J Agric Food Chem 41:1287-1291.
Potter SM. 1995. Overview proposed for the hypercholesterolemic effect of soy. J Nutr 125:606S-611S.
Price KR, Fenwick GR. 1984. Soyasaponin I, a compound possessing undesirable taste characteristics isolated from the dried pea (*Pisum sativum L.*). J Sci Food Agric 35:887-892.
Price KR, Curl CL, Fenwick GR. 1986. The saponin content and sapogenol composition of the seed of 13 varieties of legume. J Sci Food Agric 37:1185-1191.
Price KR, Johnson IT, Fenwich GR. 1987. The chemistry and biological significance of saponins in feeds and feedingstuffs. CRC Crit Rev Food Sci Nutri 26:27-135.
Puppo MC, Anon MC. 1999. Soybean protein dispersions at acid pH. Thermal and rheological properties. J Food Sci. 64:50-56.
Rackis JJ, Gumbmann MR. 1982. Protease inhibitors: physiological properties and nutritional significance. In: Ory RL, editor. Antinutrients and natural toxicants in foods. Westport, CT: Food and nutrition press. p 203.
Rao AV, Sung MK. 1995. Saponins as anticarcinogens. J Nutr 125:717S-724S.
Regenstein JM, Regenstein CE. 1984. Protein functionality for food scientists. In: Regenstein JM, Regenstein CE, editors. Food protein chemistry. Orlando:Academic Press.
Rhee KC, Choi YR. 1981. Annual progress report of Food Protein Research and Development Center. College Station, TX: Texas A&M University. p 203-233.
Ridout CL, Wharf SG, Price KR, Johnson IT, Fenwick GR. 1988. United Kingdom mean daily intakes of saponins – intestine-permeabilizing factors in legumes. Food Sci Nutr

42:111-116.
Rimbach G, Pallauf J. 1999. Effect of dietary phytate on magnesium bioavailability and liver antioxidant status in growing rats. Food Chem Toxicol 37:37-45.
Roberts RC, Briggs DR. 1965. Isolation and characterization of the 7S component of soybean globulins. Cereal Chem 42:71-85.
Saito T, Kohno M, Tsumura K, Kugimiya W, Kito M. 2001. Novel method using phytase for separating soybean β-conglycinin and glycinin. Biosci Biotechnol Biochem. 65:884-887.
Schenz TW, Morr CV. 1996. Solubility. In: Hall GM, editor. Methods of testing protein functionality. New York: Blackie Academics & Professional. p 61-75.
Schneeman B, Gallaher D. 1986. Pancreatic response to dietary trypsin inhibitor: variation among species. Adv Exp Med Biol 199:185-187.
Schwenke KD. 1997. Enzyme and chemical modification of proteins. In: Damodaran S, Paraf A, editors. Food proteins and their applications. New York, Marcel Dekker. p 393-424.
Setchell KDR, Nechemias-Zimmer L, Cai J, Heubi JE. 1997. Exposure of infants to phytoestrogens from infant soy formulas. Lancet 350:23-27.
Setchell KDR, Nechemias-Zimmer L, Cai J, Heubi JE. 1998. The isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. Am J Clin Nutr 68:1453S-1461S.
Setchell KDR, Cassidy A. 1999. Dietary isoflavones: biological effects and relevance to human health. J Nutr 129:758S-767S.
Shamsuddin AM, Baten A, Lahwani ND. 1992. Effect of inositol hexaphophate on growth and differentiation of human mammary cancer cell lines *in vivo*. Cancer Lett 64:195-202.
Shamsuddin AM, Yang GY, Vucenik I. 1996. Novel anti-cancer functions of IP6:growth inhibition and differentiation of human mammary cancer cell lines *in vitro*. Anticancer Res 16:3287-3302.
Sheehan D. 1997. Isoflavone content of breast milk and soy formulas: benefits and risks. Clin Chem 43:850-852.
Shen JL. 1976. Solubility profile, intrinsic viscosity, and optical rotation studies of acid precipitated soy protein and of commercial soy isolate. J Agric Food Chem 24:784-788.
Shen JL. 1981. Solubility and viscosity. In: Cherry JP, editor. Protein functionality in foods. Washington D.C., American Chemical Society. p.89-109.
Shimoyamada M, Kudo S, Okubo K, Yamauchi F, Harada K. 1990. Distributions of saponin constituents in some varieties of soybean plant. Agric Biol Chem 54:77-81.
Shimoyamada M, Kudou S, Harada K. 1991. Variation in saponin contents in germinating soybean seeds and effects of light irradiation. Agric Biol Chem 55:577-579.
Shiraiwa M, Harada K, Okubo K. 1991. Composition and content of saponins in soybean seed according to variety, cultivation year and maturity. Agric Biol Chem 55:323-331.
Simonne AH, Smith M, Weaver DB, Vail T, Barnes S, Wei CI. 2000. Retention and changes of soy isoflavones and carotenoids in immature seeds (edemame) during processing. J Agric Food Chem 48:6061-6069.
Singletary K, Faller J, Li JY, Mahungu S. 2000. Effect of extrusion on isoflavone content and antiproliferative bioactivity of soy/corn mixtures. J Agric Food Chem 48:3566-3571.
Sirtori CR, Lovati MR. 2001. Soy proteins and cardiovascular disease. Curr Atheroscler Rep 3:47-53.

Smith AK, Cirek SJ. 1972. Soybeans: chemistry and technology. In: Smith AK, Cirek SJ, editors. Vol. 1 Proteins. Westport, CT: AVI Publishing Co.

Song TT, Barua K, Buseman G, Murphy PA. 1998. Soy isoflavone analysis: quality control and new internal standard. Am J Clin Nutr. 68:1474S-1479S.

Sorgentini DA, Wagner JR, Anon MC. 1995. Effects of thermal treatment of soy protein isolate on the characteristics and structure-function relationship of soluble and insoluble fractions. J Agric Food Chem 43:2471-2479.

Soya & oilseed bluebook. 2003. World soybean: production & area harvested. P 352.

Soyfoods.com. 2003. New foods, new uses: How soy protein can expand your business. http://www.soyfoods.com/manufacture/market.html.

SPINS/Soyatech Study. 2002. Soyfoods: The U.S. market 2002. SPINS, Inc. & Soyatech Inc.

Strom BL, Schinnar R, Ziegler EE, Barnhart KT, Sammuel MD, Macones GA, Stallings VA, Drulis JM, Nelson SE, Hanson SA. 2001. Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood. JAMA 286:807-814.

Taniyama T, Yoshikawa M, Kitagawa I. 1988. Saponin and sapogenol. XLIV. Soyasaponin composition in soybeans of various origins and soyasaponin content in carious organs of soybean. Structure of soyasaponin V from soybean hypocotyls. Yakugaku Zasshi 108: 562-571.

Tava A, Odoardi M. 1996. Saponins from *Medicago spp.*: chemical characterization and biological activity against insects. In: Waller GR, Yamasaki K; editors. Saponins used in food and agriculture. New York, Plenum Press. p. 97-109.

Thanh VH, Okubo K, Shibasaki K. 1975. Isolation and characterization of the multiple 7S globulins of soybean. Plant Physiol 56:19-22.

Thanh VH, Shibasaki K. 1976a. Heterogeneity of β-conglycinin. Biochem Biophys Acta 439:326-338.

Thanh VH, Shibasaki K. 1976b. Major proteins of soybean seeds. A straightforward fractionation and their characterization. J Agric Food Chem 24:1117-1121.

Thanh VH, Shibasaki K. 1978. Major proteins of soybean seeds. Subunit structure of β -conglycinin. J Agric Food Chem 26:692.

Thompson LU, Zhang L. 1991. Phytic acid and minerals effect on early markers of risk for mammary and colon carcinogenesis. Carcinogenesis 12:2041-2045.

Timbekova AE, Isaev MI, Abubakirov NK. 1996. Chemistry and biological activity of triterpenoid glycosides from *Medicago sativa*. In: Timbekova AE, editor. Saponins used in food and agriculture. New York: Plenum Press p. 171-182.

Tombs MP. 1974. Gelation of globular proteins. Faraday Discuss Chem Soc 57:158-164.

Topping DL, Storer GB, Calvert GD, Illman RJ, Oakenfull DG, Weller RA. 1980. Effects of dietary saponins on fecal bile acids and neutral sterols, plasma lipids, and lipoprotein turnover in the pig. Am J Clin Nutr 33:783-786.

Tsukamoto C, Kikuchi A, Harada K, Kitamura K, Okubo K. 1993. Genetic and chemical polymorphisms of saponins in soybean seeds. Phytochem 34:1351-1356.

Tsukamoto, C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K. 1995. Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem 43:1184-1192.

United Soybean Board. 2000. World soybean production 2000. www.united soybean.org/soystats2001/page_34.htm.

Utsumi S, Matsumura Y, Mori T. 1997. Structure-function relationships of soy proteins. Food proteins and their applications. In: Damodaran S, Paraf A, editors. New York: Marcel Dekker, Inc.

van der Schouw YT, Grobbee DE. 2001. HRT and heart disease: Dr. Jekyl or Mrs. Hyde? Maturitas 38:213-217.

Vojdani F. 1996. Solubility. In, Hall GM. Methods of testing protein functionality. New York: Blackie Academics & Professional. p 11-60.

Waggle DH, Bryan BA, inventors; Protein Technologies International, Inc, assignee. 2002 Dec 17. Recovery of isoflavones from soy molasses. U.S. patent 6,495,141.

Wagner JR, Gueguen J. 1999. Surface functional properties of native, acid-treated, and reduced soy glycinin. 1. Foaming properties. J Agric Food Chem 47: 2173-2180.

Wagner JR, Sorgentini DA, Anon MC. 2000. Relation between solubility and surface hydrophobicity as an indicator of modifications during preparation processes of commercial and laboratory-prepared soy protein isolates. J Agric Food Chem 48:3159-3165.

Wakai K, Egami I, Kato K, Katamura T, Tamakoshi A, Lin Y, Nakayama T, Wada M, Ohno Y. 1999. Dietary intake and sources of isoflavones among Japanese. Nutr Cancer 33:139 -145.

Wang C, Ma Q, Pagadala S, Sherrard MS, Krishnan PG. 1998. Changes of isoflavones during processing of soy protein isolates. J Am Oil Chem Soc 75:337-341.

Wang CH, Damodaran S. 1991. Thermal gelation of globular proteins: Influence of protein conformation on gel strength. J Agric Food Chem 39:433-438.

Wang H, Murphy PA. 1994a. Isoflavone content in commercial soybean foods. J Agric Food Chem 42:1666-1673.

Wang H, Murphy PA. 1994b. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J Agric Food Chem 42:1674-1677.

Wang H, Murphy PA. 1996. Mass balance study of isoflavones during soybean processing. J Agric Food Chem 44:2377-2383.

Wang J, Sporns P. 2000. MALDI-TOF MS analysis of isoflavones in soy products. J Agric Food Chem 2000 48:5887-5892.

Wilde PJ, Clark DC. 1996. Foam formation and stability. In: Hall GM, editor. Methods of testing protein functionality. New York: Blackie Academics & Professional. p 110-152.

Wolf WJ, Briggs DR. 1959. Purification and characterization of the 11S component of soybean proteins. Arch Biochem Biophys 85:186-199.

Wolf WJ, Sly DA. 1967. Cryoprecipitation of soybean 11S protein. Cereal Chem 44:653-668.

Wolf WJ. 1969. Soybean protein nomenclature: a progress report. Cereal Sci Today 14:75, 76, 78, 129.

Wrick K. 2003. The U.S. soy market: an update & outlook. http://www.nutraceuticals world.com/JanFeb031.htm.

Wu S, Murphy PA, Johnson LA, Fratzke AR, Reuber MA. 1999. Pilot-plant fractionation of soybean glycinin and β-conglycinin. J Am Oil Chem Soc 76:286-293.

Wu S, Murphy PA, Johnson LA, Reuber MA, Fratzke AR. 2000. Simplified process for soybean glycinin and β-conglycinin fractionation. J Agric Food Chem 48:2702-2708.

Wu WU, Hettiarchchy NS, Qi M. 1998. Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. J Am Oil Chem Soc 75:845-849.

Yamashita U, Kawada S, Nakano H. 1990. Induction of mammalian topoisomerase II dependent DNA cleavage by noninteractive flavonoids, genistein and equol. Biochem Pharm 39:737-744.

Yamauchi F, Sato M, Sato W, Kamata Y, Shibasaki K. 1981. Isolation and purification of a new type of β-conglycinin in soybean globulins. Agric Biol Chem 45:2863.

Yamauchi F, Yamagishi T, Iwabuchi S. 1991. Molecular understanding of heat-induced phenomena of soybean protein. Food Reviews International 7:283-322.

Yoshiki Y, Okubo K. 1995. Active oxygen scavenging activity of DDMP saponins in soybean seeds. Biosci Biotech Biochem 59:156-1557.

Yoshiki Y, Kudou S, Okubo K. 1998. Relationship between chemical structures and biological activities of triterpenoid saponins from soybean. Biosci Biotechnol Biochem 62:2291-2299.

Young VR. 1991. Soy protein in relation to human protein and amino acid nutrition. J Am Diet Assoc 91:828-835.

Zhang YL, Song TT, Cunnick JE, Murphy PA, Hendrich S. 1999. Daidzein and genistein glucuronides *in vitro* are weakly estrogenic and activate human natural killer cells in nutritionally relevant concentrations. J Nutr 129:399-405.

CHAPTER 2. SOY PROTEIN, ISOFLAVONE, AND SAPONIN PARTITIONING DURING PILOT-SCALE FRACTIONATION OF GLYCININ AND β-CONGLYCININ

A paper to be submitted to the *Journal of the American Oil Chemists' Society*

David A. Rickert, Vanessa Smith, Lawrence A. Johnson, Patricia A. Murphy

Abstract

A pilot-scale process for fractionation of the major soy storage proteins, glycinin and β-conglycinin, was scaled up from 15 kg of defatted soy flakes to 50 kg and evaluated for protein yield. The partitioning of isoflavones and saponins, protein-associated soy phytochemicals, was assessed. The glycinin and β-conglycinin fractions, and an intermediate fraction containing a mixture of the two proteins, contained 9, 17, and 3% of the total starting protein, respectively. The insoluble fraction, a waste stream, retained 59, 67, and 75% of the protein, isoflavones, and saponins, respectively. The whey, another waste stream, contained 6% of the protein, 12% of the isoflavones, and less than 0.2% of the saponins. The three product fractions, when combined, contained 18 and 22% of the isoflavones and saponins, respectively. The saponin to isoflavone ratio was more than 2:1 for the β-conglycinin, but only about 1:1 for the glycinin. Distribution of the various isoflavone and saponin forms among process streams was altered during processing. The scaled-up process was less efficient for protein recovery. Additional processing of the insoluble and whey fractions may improve protein and phytochemical recovery.

Introduction

The efforts to elucidate the effects of soy protein and associated phytochemicals on human health, to understand how physicochemical characteristics of the soy storage proteins influence functional properties, and to develop new soy protein ingredients with unique or enhanced functional properties continue. These efforts are driven by consumer demand for dietary soy products and food industry desire to meet that demand with relatively

inexpensive soy ingredients that provide necessary protein functionality characteristics. Pilot-scale fractionation of glycinin and β-conglycinin is capable of producing kilogram quantities of these proteins, therefore facilitating their use in clinical human feeding studies, functionality testing, and ingredient application in food systems.

In addition to the role of soy as a highly digestible protein with an excellent amino acid profile, consumption of soy foods and ingredients may provide several health benefits, such as improved cardiovascular health as part of a low-fat diet, anti-osteoporotic effects, and anti-carcinogenic activity. The component or components of soy imparting these benefits, such as protease inhibitors, isoflavones, saponins, and soy protein itself, have been the subject of considerable debate (1-6), especially concerning cardiovascular health. Additional research in the form of clinical trials is needed. One such recent study endeavored to elucidate the respective roles of isoflavones and β-conglycinin on serum cholesterol and lipid profiles in humans. Pilot-scale production of β-conglycinin was necessary to produce adequate quantities of for the study. Understanding the partitioning of these co-products during pilot-scale fractionation may suggest means for future modification of the system to direct partitioning or improve recovery of the co-products.

The considerable effort to understand the functionalities of the major soy storage proteins in regard to protein structure has been reviewed (7). The process to understand structure-function relationship continues with regard to genotype effect on functional properties and product applications (8-10). Limited evaluations of functionalities of pilot-scale produced glycinin and β-conglycinin fractions have been published (11-13). Additional information concerning functionalities of process fractions will be of interest as process improvement or modification occurs. Pilot-scale production will yield protein fractions in sufficient quantity to conduct food system evaluations.

Development of an efficient, sequential method to prepare glycinin and β-conglycinin fractions for characterization and functionality studies and to produce kilogram quantities of the proteins on a pilot-scale has evolved over several decades. In 1976, Thanh and Shibasaki (14) described one of the first sequential bench-scale methods for separating glycinin and β-

conglycinin fractions. This method was later modified by others after 1990 (15,16). Patents for pilot-scale processes producing enriched glycinin, β-conglycinin fractions, as well as mixtures of these proteins, have been disclosed (17-19). Wu *et al.* (11, 20) employed two methods for pilot-scale fractionation of glycinin and β-conglycinin. The first method was a modification of a bench-scale process. Three fractions, glycinin, an intermediate mixture, and β-conglycinin, were separated by sequential precipitation controlled by pH and ionic strength. The second method was a simplified method resulting in only glycinin and β-conglycinin fractions, yielding a larger β-conglycinin fraction at the expense of purity.

The objective of this research was to evaluate the effect on protein yield as scale up from 15 to 50 kg of defatted soy flake material was implemented for Wu's three-product fractionation process. The partitioning of protein and protein-associated phytochemicals, the isoflavones and saponins, was evaluated to gain understanding of their partitioning and insight concerning process improvement for recovery of these co-products.

Materials and Methods

Materials. Defatted, soybean white flakes were purchased from Cargill (Minneapolis, MN). Flakes were stored in sealed containers at 4 °C. HPLC-grade acetonitrile, methanol, trifluoroacetic acid, and glacial acetic acid for HPLC mobile phase and sample preparation were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade water for mobile phase and sample preparation was produced using a Milli-Q water system (Millipore Co., Bedford MA).

Protein fractionation process. The pilot-scale fractionation process was a modification of that used by Wu *et al.* (11). Figure 1 displays a flow chart of the process. Fifty kg of defatted soy flakes were suspended in 400 kg of 20 °C tap water in a jacketed 800-L tank (Walker Stainless Equipment Company, New Lisbon, WI) stirred at 13 rpm. The pH of the suspension was adjusted to 8.5 with 2N NaOH and stirred at 22 rpm for 60 min. A Moyno-type transfer pump (Electric Pump, Model IFFCA SSE SAA, Des Moines, IA) at 250 rpm

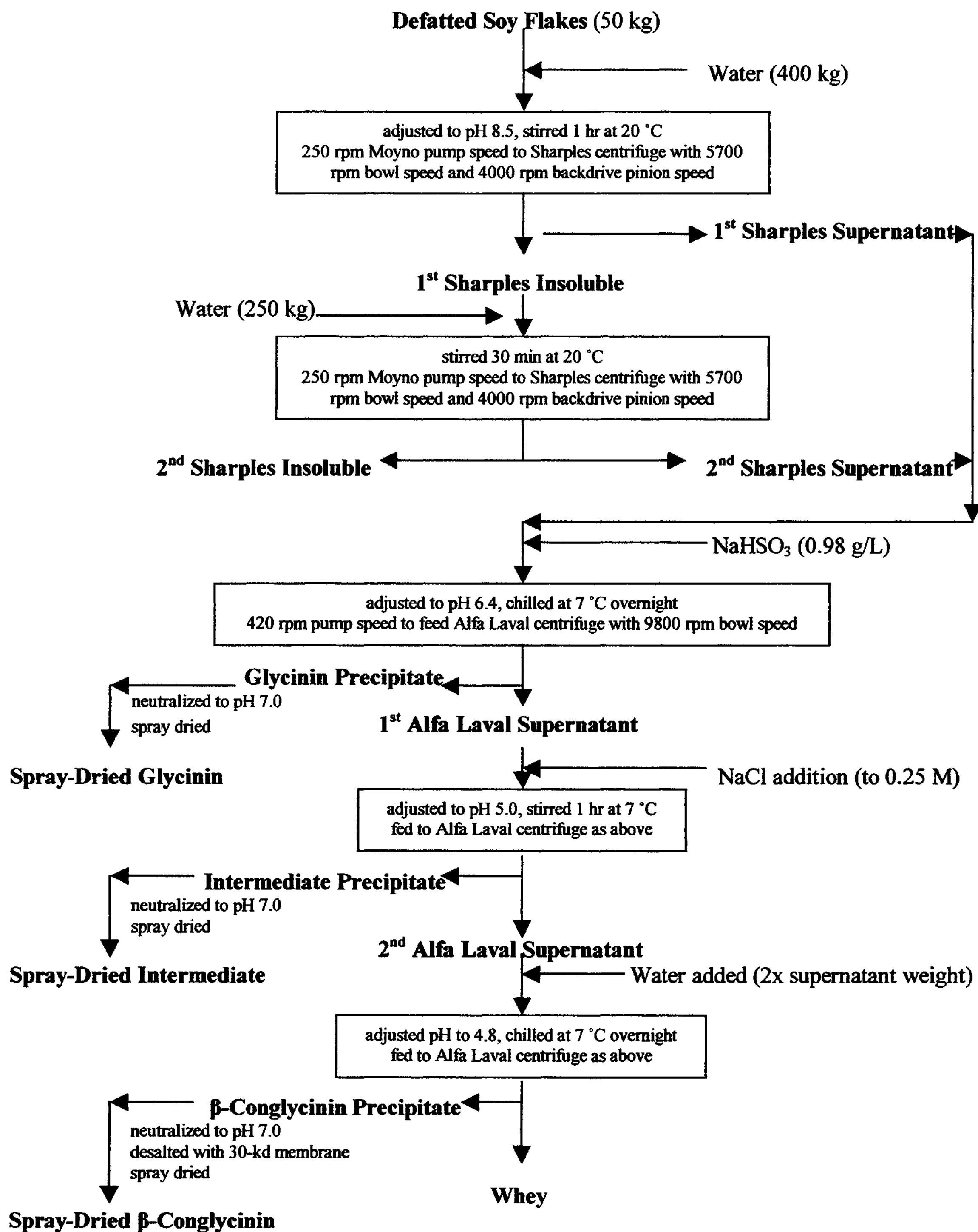


FIG. 1. Soy storage protein fractionation process.

pump speed (approximately 1.7 L/min) was used to feed the slurry to a Sharples P660 horizontal decanting centrifuge (Alfa Laval Separation Inc., Warminster, PA) set at 5700 rpm bowl speed and 4000 rpm backdrive pinion speed, separating the slurry into extract and residue fractions. To improve total recovery of solubilized proteins, the residue fraction was returned to the 800-L tank, suspended in 250 kg water (1:5 ratio based on 50 kg starting material), and stirred at 13 rpm for 30 min. The slurry was then centrifuged as above. The residue fraction from the second centrifuge pass was a waste stream and received no further processing. The extract fractions from the first and second centrifuge passes were combined in the 800-L tank, continuously stirred at 13 rpm, treated with solid sodium bisulfite to produce 10 mM SO_2 (about 0.98g/L $NaHSO_3$), adjusted to pH 6.4 using 2N HCl, chilled to approximately 7 °C, and maintained at this temperature overnight.

A 420 rpm pump speed was used to feed the chilled, treated extract to an Alfa Laval BTPX 205 disc stack centrifuge with a 9800 rpm bowl speed, producing a glycinin precipitate fraction and a supernatant fraction. The glycinin precipitate was neutralized to pH 7.0 with 2N NaOH and dried with an Anhydro Compact Spray Dryer (APV Crepaco Inc., Attleboro Falls, MA) with an air inlet temperature of 160 °C and outlet temperature of 85 °C. The supernatant fraction was returned to the 800-L tank, treated with solid NaCl to produce a 0.25 M concentration, and then adjusted to pH 5.0. This treated supernatant was stirred for 60 min at 13 rpm, and then fed to the Alfa Laval centrifuge as above. The resulting precipitate, termed the intermediate precipitate, was neutralized to pH 7.0 with 2N NaOH and spray dried as above. The supernatant was returned to the tank, combined with an amount of water equal to two times the supernatant weight, and adjusted to pH 4.8. The treated supernatant was stirred at 13 rpm and maintained at approximately 7 °C overnight.

The chilled, treated supernatant was passed through the Alfa Laval centrifuge as above, producing β-conglycinin precipitate and whey. The whey waste stream received no further processing. The β-conglycinin precipitate was neutralized to pH 7.0 with 2N NaOH, measured for volume, and then desalted with a Feed and Bleed Membrane Filtration System (Model SRT-50; North Carolina SRT Inc., Cary, NC) and a 30-KD regenerated cellulose

membrane (North Carolina SRT Inc.). Diafiltration was performed until an amount of permeate equal to five times the volume of the pH-adjusted β-conglycinin precipitate had been collected. The retentate was then spray dried as above.

Modifications to Wu's method (11) were as follows. A water-to-flake ratio of 8:1 was used instead of 15:1 or 10:1, producing an insoluble fraction with a higher solids content. Chill steps were at 7 °C instead of 4 °C because that was as low as the chilling system could provide for the configuration used. Neutralization pH of the glycinin, intermediate, and β-conglycinin precipitate fractions was 7.0 instead of 7.5. The glycinin and intermediate fractions were not desalted for this study because no functionality or clinical trial uses were planned for these products.

Sampling. For a given process stream, samples of uniform weight were collected at pre-determined intervals and then combined into a composite sample. Composites of random grab samples were prepared for each spray-dried fraction.

Proximate analyses. Moisture contents of high-moisture and spray-dried samples were determined by difference after freeze drying and oven drying (21), respectively. Ash (22) and nitrogen (23) analyses were performed in triplicate. To compensate for different nitrogen contents of the different protein fractions, conversion factors of 6.37 and 6.08 were used to calculate protein contents for the β-conglycinin and intermediate fractions, respectively. Protein contents of all other samples were calculated using 5.71 as the conversion factor.

Isoflavone and group B saponin extraction and analyses. Extractions and HPLC analyses were conducted as reported by Hu *et al.* (24) for saponins and as reported by Murphy *et al.* (25) for isoflavones with minor modifications. Approximately 4 g of freeze-dried or spray-dried material were accurately weighed into a 125-ml Erlenmeyer flask. Fourteen mL of distilled water and 20 mL of acetonitrile were added to the flask, as well as a stir bar. The flask was capped, and then mixed on a rotary shaker (Innova Model 2050, New Brunswick Sci., Edison, NJ) at 300 rpm for 2 h at ambient temperature.

The modified extraction method differed as follows from the methods described by Murphy (24) and Hu (25). For optimal isoflavone extraction from a particular food matrix, Murphy used 10 mL of acetonitrile, 2 mL of 0.1 N HCl, and water as needed (approximately 7 mL). Hu used 100 mL of 70% ethanol and 2.5 h of extraction time for saponin extraction. The modified method did not give significantly different (P<0.05) phytochemical concentrations compared to the referenced methods when ground, defatted soy flakes were analyzed.

The extract was vacuum filtered (No. 42 filter paper, Whatman, Hillsboro, OR), and the filtered extract was quantitatively transferred to a 500-mL round-bottomed flask, using acetonitrile to facilitate the transfer, and evaporated to dryness using a rotary evaporator (Brinkmann, Westbury, NY) at ≤30 °C. Isoflavones and saponins were recovered from the round-bottomed flask with 80% HPLC-grade methanol in water to a final volume of 10.0 mL. The sample was passed through a 0.45 μm polytetrafluoroethylene filter (Alltech Associates, Deerfield, IL) prior to HPLC analysis.

Quantification of the isoflavones and group B saponins was performed on different HPLC systems, but using the same type of reverse-phase column, an RP-18, 5 μm, 4.6 i.d. x 250 mm YMC-ODS-AM-303 column (YMC, Inc., Wilmington, NC). Isoflavones were separated using a gradient system comprised of 0.1% glacial acetic acid in water (A) and 0.1% glacial acetic acid in acetonitrile (B). After a 20 μL injection, the system was maintained at 15% B for 5 min, increased to 29% B over 31 min, and then increased to 35% B in 8 min. The system was returned to 15% B and maintained at that concentration for 15 min before the next injection. The flow rate was 1.0 mL/min for the first 5 min, increased to 1.5 mL/min for the next 40 min, and then returned to 1.0 mL/min. The UV absorbance was recorded from 200-350 nm. Peak areas at 254 nm were used for analysis.

Saponins were separated using a gradient system consisting of 0.05% trifluoroacetic acid in water (A) and acetonitrile (B). The programmed gradient did not differ substantially from that used by Hu *et al.* (24). Solvent B was increased from 37 to 40% in 15 min, and then solvent B was further increased to 48% over 20 min. Finally, solvent B was increased to

100% in 2 min, held at 100% for 3 min, then returned to 37% over 2 min. The system was maintained at 37% B for 8 min before the next injection. The flow rate was maintained at 1 mL/min throughout. The UV absorbance was monitored from 190 to 350 nm. Peak areas at 205 and 292 nm were used for analysis of non-DDMP and DDMP saponins, respectively.

For quality control purposes, an isoflavone standard solution (genistin, genistein, and daidzein) and a saponin standard solution (saponin I) were injected on the respective systems prior to analyzing samples. Preparation of isoflavone and saponin standards and calibration curves is described elsewhere (24, 25). Isoflavone recovery from ground soy flake and freeze-dried soy protein isolate material similar to the fractionated proteins was evaluated using a concentrated isoflavone extract produced from soy flakes and containing 7.5 μmol/mL isoflavones in 80% methanol. Saponin recovery from these matrices employed a mixture of saponin I and II at concentrations of 4.8 and 3.9 μmol/mL, respectively, in 80% methanol. Samples were spiked, mixed thoroughly, and then allowed to air dry overnight. Extraction was performed as above.

Statistical analysis. Means, least significant differences, and ANOVA results were calculated using the SAS system (version 6, SAS Institute Inc., Cary, NC).

Results and Discussion

Effects of scale up. Wu *et al.* (11) developed a pilot-scale soy protein fractionation process to prepare glycinin- and β-conglycinin-rich fractions, as well as an intermediate fraction that contained both glycinin and β-conglycinin. These protein fractions were separated sequentially, employing acid precipitation, chilling, and ionic strength manipulation of the protein extract and supernatant fractions. The intermediate fraction was precipitated between the glycinin and β-conglycinin fractions to improve the purity of the β-conglycinin fraction, removing substantial quantities of glycinin remaining in the supernatant after the glycinin precipitation step. Wu reported that most of the glycinin and β-conglycinin proteins comprising the intermediate fraction were no longer in their native state. Scale-up of Wu's process was required to meet demands for β-conglycinin, which was needed for a

clinical study to evaluate the impact of isoflavones and β-conglycinin on serum cholesterol and lipid profiles in humans. Scale up from Wu's 15-kg process to the 50-kg level enabled an evaluation of the effects of scale up.

In addition to the desired products, glycinin, intermediate, and β-conglycinin, the fractionation process produced two waste stream fractions, the insoluble residue and the whey. One impact of scale up was the decrease in protein extraction rate from the starting material, demonstrated by the dry mass and protein remaining in the insoluble fraction. The percentage fraction yield for the insoluble fraction, i.e. dry basis insoluble fraction weight divided by the dry basis weight of the starting material and expressed as a percentage, for the 15-kg process reported by Wu *et al.* (11) was 49.1%, whereas that of the 50-kg process was 73.1% (Table 1). The percentage protein recovery in the insoluble fraction, i.e. dry-basis protein weight divided by total dry-basis protein and expressed as a percentage, was 38.8% for the 15-kg process compared to 58.6 % for the 50-kg process, demonstrating a significant portion of the high fraction weight observed for the 50-kg process was due to decreased protein recovery from the starting material. The protein content of the insoluble fraction was similar for the two processes.

The protein concentrations of the 50-kg process product fractions were lower than reported by Wu *et al.* (11) for the 15-kg process. One reason for this difference was that Wu *et al.* used diafiltration with a 30-KD regenerated cellulose membrane to desalt the protein fractions prior to spray drying, whereas only the β-conglycinin fraction of the 50-kg process was desalted. The effect of not desalting the intermediate fraction was dramatic, with protein concentrations for the 15- and 50-kg processes being 85.8 and 54.4%, respectively. To precipitate the intermediate fraction, the supernatant recovered after glycinin precipitation was adjusted to 0.25 M NaCl and pH 5.0 with 2 N HCl. Consequently, the ash content of the intermediate fraction was higher than that of the other product fractions. This difference in ash concentrations between product fractions is demonstrated in Table 2. Data are means of two process runs. The ash concentrations for the glycinin and β-conglycinin precipitate fractions were 3.5 and 5.1%, respectively, compared to 19.6% for the intermediate

TABLE 1
Effects of Process Scale Up on Yield, Content, and Recovery

Fractionation Process	Process Fractions			
	Glycinin	Intermediate	β-Conglycinin	Insolubles
Fraction Yield (%, db[a])				
Wu pilot-plant process[b]	9.4 ± 0.2	4.9 ± 0.2	9.4 ± 1.6	49.1 ± 4.4
Scaled-up process [c]	5.8 ± 2.1	3.0 ± 0.2	10.3 ± 0.2	73.1 ± 0.5
Protein Content (%, db)				
Wu pilot-plant process	92.8 ± 0.6	85.8 ± 0.6	97.6 ± 0.1	42.4 ± 1.0
Scaled-up process	81.2 ± 0.9	54.4 ± 0.1	91.3 ± 0.7	43.3 ± 0.2
Protein Recovery (%)				
Wu pilot-plant process	16.2 ± 0.3	7.9 ± 0.4	17.2 ± 2.8	38.8 ± 2.7
Scaled-up process	8.7 ± 3.2	3.0 ± 0.2	17.4 ± 0.2	58.6 ± 0.2

[a]db, dry basis.
[b]n=3, 15 kg of defatted soy flake starting material, data from Wu *et al.* (11).
[c]n=2, 50 kg of defatted soy flake starting material.

TABLE 2
Mass Balance Data for the Scaled-Up Fractionation Process

	Weight (kg, as is)[a]	Weight (kg, db[b])	Ash (%, db)	Ash (kg, db)	Protein (%, db)	Protein (kg, db)	Isoflavones (μmol/g, db)	Isoflavones (mmol, db)	Saponins (μmol/g, db)	Saponins (mmol, db)
Flakes	50.00_{ef}	47.29_{a}	6.3_{fg}	3.0_{b}	53.9_{de}	25.5_{a}	8.25_{cd}	390_{a}	5.39_{cd}	255_{a}
1SE[c]	125.43_{de}	11.44_{e}	7.9_{ef}	0.9_{d}	57.4_{cd}	6.6_{d}	10.18_{a}	117_{d}	5.90_{bc}	68_{d}
1SR	305.63_{c}	38.50_{b}	6.9_{efg}	2.7_{b}	45.2_{f}	17.4_{b}	7.78_{de}	300_{b}	5.81_{bc}	224_{h}
2SE	81.00_{ef}	4.64_{f}	8.6_{de}	0.4_{de}	57.5_{cd}	2.7_{f}	10.14_{a}	47_{f}	6.47_{ab}	30_{e}
2SR	435.00_{b}	34.54_{c}	6.8_{efg}	2.4_{b}	43.3_{f}	15.0_{c}	7.59_{de}	262_{c}	5.57_{bcd}	193_{c}
1ALS	223.95_{cd}	14.86_{d}	10.8_{d}	1.6_{c}	50.9_{e}	7.6_{d}	9.08_{abc}	135_{d}	4.74_{d}	71_{d}
GLY P	16.00_{ef}	2.83_{fg}	3.5_{h}	0.1_{e}	83.0_{b}	2.4_{fg}	9.73_{ab}	28_{fg}	4.78_{d}	14_{g}
GLY SD	2.85_{f}	2.72_{fg}	5.5_{gh}	0.2_{e}	81.1_{b}	2.2_{fg}	9.56_{ab}	26_{fg}	4.60_{d}	13_{g}
2ALS	253.38_{c}	16.92_{d}	29.6_{b}	5.0_{a}	38.9_{g}	6.6_{d}	5.29_{gh}	90_{e}	1.36_{e}	23_{ef}
INT P	16.10_{ef}	1.53_{g}	19.6_{c}	0.3_{de}	58.5_{c}	0.9_{h}	8.50_{bcd}	13_{g}	5.88_{bc}	9_{g}
INT SD	1.48_{f}	1.42_{g}	21.1_{c}	0.3_{de}	54.2_{de}	0.8_{h}	7.39_{def}	11_{g}	4.58_{d}	7_{g}
Whey	735.25_{a}	10.01_{e}	43.5_{a}	4.4_{a}	14.8_{h}	1.5_{gh}	4.80_{h}	48_{f}	0.05_{f}	0.5_{g}
BCON P	26.30_{ef}	4.91_{f}	5.1_{gh}	0.3_{de}	89.1_{a}	4.4_{e}	6.72_{ef}	33_{fg}	7.23_{a}	36_{e}
BCON SD	5.08_{f}	4.86_{f}	5.1_{gh}	0.3_{de}	91.2_{a}	4.4_{e}	6.28_{fg}	31_{fg}	7.41_{a}	36_{e}
LSD	114.62	2.37	2.1	0.7	3.9	1.2	1.20	25	2.14	13

[a]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[b]db, dry basis.
[c]Abbreviations: 1SE, first Sharples centrifuge pass extract; 1SR, first Sharples pass residue (insoluble fraction); 2SE, second Sharples pass extract; 2SR, second Sharples pass residue; 1ALS, first Alfa Laval supernatant; P, precipitate; SD, spray dried; 2ALS, second Alfa Laval supernatant; INT, intermediate; BCON, β-conglycinin; LSD, least significant difference.

precipitate fraction. The ash content of the β-conglycinin precipitate samples did not reflect the effects of desalting because the samples were collected prior to the desalting step. The dry-basis ash concentration of the whey solids was about 44%, demonstrating that most of the ash was carried through the process in the aqueous fraction and expelled in the whey waste stream.

An interesting difference between the 15- and 50-kg processes was the distribution of proteins among the three product fractions. Table 1 shows that, on a percentage basis, the intermediate fraction of the 15-kg process was larger relative to the glycinin and β-conglycinin fractions compared to the 50-kg process. The percentage recoveries of protein in the β-conglycinin fractions were essentially the same for both processes, while recovery in the glycinin fraction of the 50-kg process was about one-half that of the 15-kg process. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) data was not available for both processes, so the effect of this distribution difference on product fraction purity cannot be discussed. It is possible that the β-conglycinin purity for the 50-kg process was lower than that of the 15-kg process. A larger percentage of total protein was lost into the whey fraction for the 15-kg process, 19.9%, than for the 50-kg process, 12.3%.

The 50-kg process was not as efficient as the 15-kg process with regard to protein extraction and recovery. When the three product fractions were summed, the data indicate that the percentage protein recovery was about 12% less for the 50-kg process. However, the 50-kg process recovered about 13.4 kg of total protein, compared to 5.7 kg for the 15-kg process. Additionally, 4.7 kg of β-conglycinin fraction were produced per process run for the 50-kg process, about 3.6 times more than for the 15-kg process. Thus, scale-up greatly decreased the number of process runs needed to supply the desired amount of β-conglycinin.

Several factors may have led to the yield differences between the two processes. The defatted soy flake starting material was from different crop years and probably from different varieties and growing locations. Flake storage time may have been different, and defatted soy flake preparation was certainly different. Any of these factors may have impacted

protein solubilization. Finally, the processing equipment, which was the same for both processes, may have operated at different efficiencies due to wear and other factors.

It should be noted that both protein fractionation processes had lower fraction mass yields than was observed for soy protein isolate, a protein product of a least 90% dry-basis protein recovered by isoelectric precipitation of extracted soy proteins. The soy protein isolate process used at the Iowa State University Center for Crops Utilization Research pilot plant, employing the equipment to produce the fractionated proteins described here, generally produces a protein isolate yield of 33%. This compares to a combined glycinin, intermediate, and β-conglycinin fraction yield of 19 and 24% for the 50- and 15-kg fractionation processes, respectively. Industry may experience soy protein fraction yields as high as 44%. One reason for the superior fraction yield received during soy protein isolate production is the higher protein extraction temperature, reported to be as high as 80 °C (26). The 20 °C extraction temperature used for the fractionation processes minimizes loss of native state protein, promoting the efficient fractionation of the proteins (11).

Mass balance and partitioning. The fractionation process employed a two-pass system to increase protein recovery. The flake slurry was centrifuged to produce an extract, the first Sharples extract, and a residue fraction, the first Sharples residue. The first Sharples residue was extracted with more water and then centrifuged, producing the second Sharples extract and the second Sharples residue, which was a waste stream. The first and second Sharples extracts were combined, treated with sodium bisulfite and acid, precipitating the glycinin. The glycinin was recovered by the Alfa Laval centrifuge, also producing the first Alfa Laval supernatant. Further treatment of supernatants and two subsequent passes through the Alfa Laval centrifuge yielded the intermediate and β-conglycinin fractions.

As described above, Table 2 shows that most of the ash from the salt, sodium bisulfite, acid, and base treatments was deposited in the intermediate fraction (0.3 kg, dry basis) or carried through with the whey (4.4 kg, dry basis). The ash content of the intermediate fraction, compared to the other product fractions, may need to be considered when evaluating protein functionality, as functionality is affected by ionic strength. The protein

concentrations of the product fractions were different, about 83, 59, and 89% for the glycinin, intermediate, and β-conglycinin precipitate fractions, respectively. The β-conglycinin was very close to the 90% dry basis protein concentration required of soy protein isolates. The glycinin produced would have met this 90% requirement if a nitrogen conversion factor of 6.25, used by industry for soy, had been employed instead of 5.71, as suggested by Wu *et al.* (11), to calculate protein concentration.

The total protein content of the starting flake material was about 25.5 kg. The total protein in the Sharples extracts was 6.6 and 2.7 kg for the first and second Sharples extracts, respectively, while total protein content of the first Sharples residue was 17.38 kg. The first protein extraction pass recovered 26% of the total protein in the starting material, whereas the second pass recovered about 15% of the total protein in the first Sharples residue or 10% of original total protein in the starting material. Diminishing returns suggest that it might not economically feasible to add additional extraction passes to improve protein recovery.

The isoflavone concentration in the extracts was about 35-40% higher than the saponin concentrations, but there were also about 50% more total isoflavones in the starting material. Although isoflavones are less hydrophobic than the saponins, both of these phytochemicals have very low solubility in water, largely being pulled into the extract through mass action and possibly by association with the proteins. Table 2 data show that about 30 and 26% of the isoflavones and saponins, respectively, were recovered from the starting material during the first extraction. The second extraction pass recovered an additional 12% for each, based on total isoflavones and saponins in the starting material. This recovery relationship was very similar to that observed for the protein.

The data in Table 3 show that the mass balance recoveries for protein, isoflavones, and saponins were 94, 97, and 97%, respectively. Mass balance recoveries for the daidzein, glycitein, and genistein families, each family being comprised of an aglucon, β-glucoside, acetylglucoside, and malonylglucoside form, were 105, 107, and 102%, respectively. For the non-DDMP and DDMP saponin families, V and αg, I and βg, and II and βa, mass balance recoveries were 52, 116, and 88%, respectively. The low recovery for the V and αg saponin

TABLE 3
Scaled-Up Process Co-Product Recovery and Distribution

	Insoluble	Glycinin	Intermediate	β-Conglycinin	Whey	Total Recovery
Protein (weight %, db[a])	59	9	3	17	6	94
Isoflavones (mole %, db)	67	7	3	8	12	97
Saponins (mole %, db)	75	5	3	14	0.2	97

[a]db, dry basis.

family was likely due to small peak areas leading to quantification inaccuracies. Table 3 also shows that 59, 67, and 75% of the protein, isoflavones, and saponins were not recovered from the second Sharples residue fraction, a waste stream. The low recovery of saponins from the insoluble fraction, which still contained the majority of the protein, supports a limited body of literature suggesting that saponins associate with glycinin and β-conglycinin through hydrophobic interaction, hydrogen bonding, or ionic bonding (27). It may be possible that the isoflavones also associate with the proteins, though to a lesser extent. The whey fraction contained 12% of the total isoflavones, while a negligible quantity of saponins were found in the whey. This again demonstrates a difference in hydrophobicity or suggests a difference in affinity for protein or other components remaining in the whey. About 6% of the total protein was recovered in the whey fraction.

The high protein and phytochemical contents of the insoluble fraction suggest that the fraction might be economically exploited with further processing, other than additional aqueous extraction steps. Organic solvent extraction might be utilized to recover the phytochemicals. Enzymatic treatment of the fraction with cellulases and/or proteinases may enhance solubilization of the remaining proteins, making it then feasible to attempt additional aqueous extraction passes. The whey is another potential stream for protein and phytochemical recovery. It may be possible to precipitate and recover whey proteins through heating, acid precipitation, or a combination of these. Recovery of isoflavones from the whey by column chromatography or membrane filtration may be possible, although probably not economical.

Migration of isoflavones into the aqueous phase and their persistence in that fraction is not unusual and has been demonstrated previously by Wang and Murphy (28), who conducted mass balance studies for tempeh, tofu, and soy protein isolate production. By difference, about 12% of the total isoflavones were lost from raw whole soybeans to the soak water during soybean processing for tempeh production. During soymilk production for tofu manufacture, the isoflavone content of the soaking water was found to contain 1% of the total isoflavones. About 44% of total isoflavones were present in the whey after tofu processing.

Mass balance of the bench-scale soy protein isolate process showed that about 60% of the total isoflavones were removed from the starting material into the extract, approximately twice as much as removed during the first extraction in our 50-kg fractionation process. They recovered 48% of the isoflavones in the isolate and 11% in the whey. The total isoflavone recovery from all product and waste fractions of the soy isolate process was about 114% compared to the starting material. Achieving accurate mass balance accounting can be problematic. For the 50-kg fractionation process evaluated here, total recovery of isoflavones and saponins from product and waste stream fractions was about 97% for each.

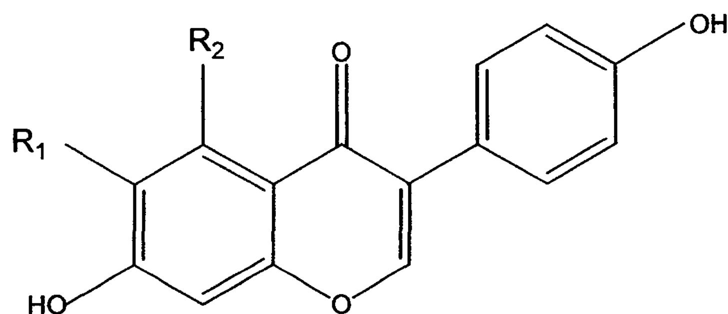
Some mass balance accounting issues can be attributed to the sample matrix and the efficiency of phytochemical recovery by the solvent extraction process prior to analysis. A small quality control recovery study with ground, defatted soy flakes and freeze-dried soy protein isolate revealed considerable recovery differences between these matrices. Recovery of saponin I and II was about 99% for the flake material, but only 60-70% for the isolate. Isoflavone recoveries were 75-92% for the flake material, with the aglucons exhibiting the lowest recoveries. Isoflavone aglucon recovery from the isolate material was 65-75%, while recoveries for the glucosides and malonylglucosides were 78-93%.

Isoflavone and saponin partitioning during protein fractionation affected the relative proportion of their concentrations in the glycinin and β-conglycinin fractions compared to the soy flake starting material. The isoflavone-to-saponin ratio for the flakes was about 1.5:1, as it was for the intermediate fraction. The ratios for the glycinin and β-conglycinin fractions were about 0.8:1 and 2.1:1, respectively. Using the 8-anilino-1-naphthalene sulfonic acid (ANS) fluorescence method, Wu et al. (11) demonstrated that β-conglycinin had more surface hydrophobicity than did glycinin, about 370 compared to 260, though much lower than that demonstrated by a soy protein isolate, 640. Based on the isoflavone and saponin partitioning differences observed for glycinin and β-conglycinin, in addition to Wu's hydrophobicity data, it may be suggested that physicochemical characteristics of proteins affect partitioning of the phytochemicals. Limited data are available concerning soy

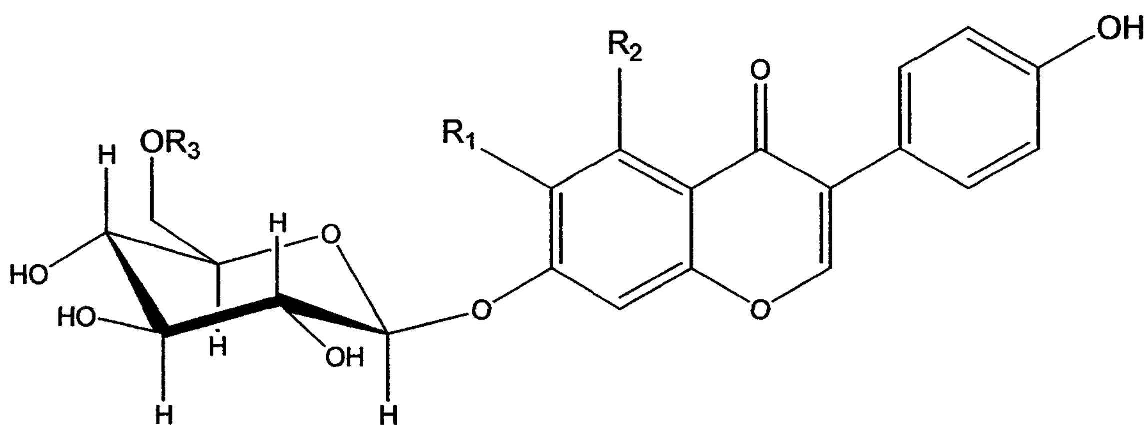
isoflavone and saponin proportions in soy products. Hu (29) reported isoflavone:saponin concentration ratios in soy protein isolates ranging from about 0.4:1 to 0.7:1.

Effect of processing on phytochemical profile. All 12 common soybean isoflavones and 6 forms of group B soyasaponins were quantified. The isoflavones are comprised of three aglucon forms, daidzein, genistein, and glycitein, and their respective malonylglucoside, acetylglucoside, and β-glucoside forms (Fig. 2). The malonylglucosides are the predominant form in raw soy flour, comprising about 80% of the mole isoflavone mass (30). The β-glucosides comprise another 15%, with negligible amounts of aglucons and virtually no acetylglucosides. High temperature processing, such as toasting or frying in oil causes decarboxylation of the malonylglucosides to acetylglucosides and β-glucosides. Toasted soy germ has a mole mass distribution of 50% β-glucoside, 38% acetylglucoside, and 5% malonylglucoside (30). Milder processing conditions, such as the heating of soy milk during tofu manufacturing, converts at least a portion of the malonylglucosides to aglucons and β-glucosides. The isoflavone mole distribution in tofu is about 37% malonylglucoside, 25% β-glucoside, and 37% aglucon (30). Presence of glucosidase enzymes, e.g. during soaking steps, converts the three glucoside forms to their respective aglucon forms (30). During tempeh production, fermentation organisms produce β-glucosidases that alter the isoflavone mole distribution to 50% aglucon, 35% malonylglucoside, and 17% β-glucoside (30).

Table 4 shows the concentration of each type of isoflavone for each process fraction. There were no significant differences for the individual isoflavones before and after spray drying. The temperature treatment was probably too mild and brief to cause conversions. The data suggest that there was β-glucosidase activity occurring in the soy slurry prior to the first Sharples centrifuge pass. The genistein concentration of the flakes was 0.15 μmol/g, but the concentration in the first Sharples extract was 0.31 μmol/g. This trend was obvious, approximately doubling the genistein concentration while the total isoflavone concentration in the first Sharples extract was only about 25% greater than for the flakes. These types of comparisons and conclusions are more difficult when the total isoflavone concentrations vary greatly between fractions. For instance, the total isoflavone concentration of the first



Isoflavone	R_1	R_2
Daidzein	H	H
Genistein	H	OH
Glycitein	OCH3	H



Isoflavone	R_1	R_2	R_3
Genistin	H	OH	H
Glycitin	OCH3	H	H
Daidzin	H	H	H
Malonylgenistin	H	OH	COCH2COOH
Malonylglycitin	OCH3	H	COCH2COOH
Malonyldaidzin	H	H	COCH2COOH
Acetylgenistin	H	OH	COCH3
Acetylglycitin	OCH3	H	COCH3
Acetyldaidzin	H	H	COCH3

FIG. 2. Isoflavone structures.

TABLE 4

Distribution of Isoflavones in Process Fractions (μmol/g, dry basis)

	MDIN[a,b]	DIN	AcDIN	DEIN	MGLY	GLY	AcGLY	GLYEIN	MGIN	GIN	AcGIN	GEIN	Total
Flakes	2.31_b	0.91_d	0.09_a	0.14_h	0.47_{bcd}	0.39_c	0.06	0.04_h	2.46_{cd}	1.13_{de}	0.09_{abc}	0.15_h	8.25_{cd}
1SE[c]	2.75_a	1.27_b	0.03_b	0.32_{gh}	0.57_a	0.49_{ab}	nd	0.08_{gh}	2.74_{abc}	1.57_{ab}	0.06_{def}	0.31_h	10.18_a
1SR	1.92_c	1.05_{cd}	0.02_b	0.27_{gh}	0.39_{ef}	0.40_c	nd	0.07_{gh}	2.01_{ef}	1.32_{cd}	0.06_{def}	0.28_h	7.78_{de}
2SE	2.45_b	1.43_a	0.01_b	0.38_{fg}	0.50_b	0.53_a	nd	0.10_{fg}	2.51_{bcd}	1.78_a	0.05_{def}	0.39_{gh}	10.14_a
2SR	1.68_d	1.14_{bc}	0.01_b	0.31_{gh}	0.34_{fg}	0.42_{bc}	nd	0.08_{gh}	1.78_{fg}	1.44_{bc}	0.05_{def}	0.33_{gh}	7.59_{de}
1ALS	2.35_b	0.93_d	0.03_b	0.62_e	0.48_{bc}	0.38_{cd}	nd	0.16_{de}	2.31_{de}	1.10_e	0.05_{def}	0.67_{ef}	9.08_{abc}
GLY P	1.97_c	0.64_{ef}	0.04_b	0.85_{cd}	0.44_{cde}	0.29_e	0.02	0.22_c	2.87_a	1.15_{de}	0.11_a	1.01_{cd}	9.73_{ab}
GLY SD	1.97_c	0.71_e	0.04_b	0.75_{de}	0.43_{de}	0.31_{de}	0.07	0.18_{cd}	2.81_{ab}	1.27_{cde}	0.10_{ab}	0.87_{de}	9.56_{ab}
2ALS	1.01_e	0.55_f	0.02_b	0.55_{ef}	0.21_h	0.25_{ef}	0.01	0.14_{def}	1.26_{ij}	0.67_f	0.04_{ef}	0.58_{fg}	5.29_{gh}
INT P	1.01_e	0.70_e	0.02_b	1.20_{ab}	0.21_h	0.31_e	0.02	0.28_{ab}	1.67_{fgh}	1.27_{cde}	0.05_{def}	1.46_b	8.50_{bcd}
INT SD	0.92_{ef}	0.66_{ef}	0.02_b	1.01_{bc}	0.19_h	0.29_e	0.06	0.24_{bc}	1.51_{ghij}	1.13_{de}	0.06_{cde}	1.23_{bc}	7.39_{def}
Whey	1.66_d	0.27_g	0.02_b	0.40_{fg}	0.33_g	0.20_f	0.02	0.12_{efg}	1.21_j	0.25_g	0.03_f	0.24_h	4.74_h
BCON P	0.84_{ef}	0.12_h	0.02_b	1.29_a	0.19_h	0.09_g	0.06	0.30_a	1.56_{ghi}	0.32_g	0.07_{bcd}	1.94_a	6.72_{ef}
BCON SD	0.75_f	0.13_{gh}	0.03_b	1.20_{ab}	0.17_h	0.09_g	0.06	0.28_{ab}	1.40_{hij}	0.36_g	0.07_{cde}	1.81_a	6.28_{fg}
LSD	0.23	0.14	0.04	0.22	0.05	0.07	NDIFF	0.06	0.34	0.22	0.03	0.26	1.20

[a]Isoflavone abbreviations: MDIN, malonyldaidzin; DIN, daidzin; AcDIN, acetyldaidzin; DEIN, daidzein; MGLY, malonylglycitin; GLY, glycitin; AcGLY, acetylglycitin; GLYEIN, glycitein; MGIN, malonygenistin; GIN, genistin; AcGIN, acetylgenistin; GEIN, genistein.

[b]Subscript, lowercase letters with a column indicate significant difference at $P<0.05$.

[c]Abbreviations: 1SE, first Sharples centrifuge pass extract; 1SR, first Sharples pass residue (insoluble fraction); 2SE, second Sharples pass extract; 2SR, second Sharples pass residue; 1ALS, first Alfa Laval supernatant; P, precipitate; SD, spray dried; 2ALS, second Alfa Laval supernatant; INT, intermediate; BCON, β-conglycinin; LSD, least significant difference; NDIFF, not different.

Sharples extract was 10.18 μmol/g, while that of the whey was only 4.74 μmol/g. For this type of comparison it was difficult to readily determine whether the distribution of the individual isoflavones changed or whether the concentration of the individual isoflavone was low or different just because the total isoflavone concentration was so different. Consequently, all total isoflavone values were normalized to the total isoflavone concentration of the first Sharples extract, the highest concentration. Each individual isoflavone for a given process fraction was then adjusted using the same correction factor used for the total of that fraction (Table 5).

When data are presented in a normalized fashion, it is not difficult to observe a decline in malonyldaidzin as the process progressed from flakes to spray-dried β-conglycinin, 2.90 to 1.21 μmol/g. A similar trend was observed for daidzin, 1.14 to 0.22 μmol/g. These differences were significant. There was also a significant decrease in acetyldaidzin concentration, but only for the flakes compared to the rest of the fractions. The daidzein concentrations correspondingly increased from 0.17 to 1.95 μmol/g for these two fractions. The isoflavone conversion that occurred seemed to be related to the action of β-glucosidases, as daidzein was accumulating at the expense of both malonyldaidzin and daidzin. Since there was not even a mild heating step, the conversion of malonyldaidzin to daidzin did not seem to occur in a substantial fashion. There may have been a small amount of β-glucosidase action on the acetyldaidzin, as these concentrations decreased. The process was clearly not converting malonyldaidzin to acetyldaidzin. These trends observed for the daidzein family occurred for the genistein family as well, but the concentrations of the malonylgenistin did not decrease to the same extent as was observed for malonyldaidzin.

The isoflavone distributions of the whey and β-conglycinin fractions were very different. The malonyldaidzin concentration of the whey was about three times that of the β-conglycinin, whereas the daidzein concentration of the β-conglycinin was about 2.3 times that of the whey. This suggests a different affinity of the isoflavones for an aqueous environment. The malonylglucosides are more hydrophilic than the aglucons, so it stands to reason that a higher proportion of the malonyl forms might be in the whey fraction.

TABLE 5

Normalized[a] Distribution of Isoflavones in Process Fractions (µmol/g, dry basis)

	MDIN[b,c]	DIN	AcDIN	DEIN	MGLY	GLY	AcGLY	GLYEIN	MGIN	GIN	AcGIN	GEIN
Flakes	2.90_{b}	1.14_{d}	0.11_{a}	0.17_{i}	0.59_{b}	0.49_{bc}	0.07	0.05_{g}	3.09_{a}	1.41_{de}	0.12_{a}	0.19_{g}
1SE[d]	2.79_{bc}	1.29_{c}	0.03_{b}	0.32_{h}	0.58_{bc}	0.49_{bc}	nd	0.08_{f}	2.78_{b}	1.59_{cd}	0.06_{e}	0.32_{fg}
1SR	2.55_{de}	1.39_{bc}	0.03_{b}	0.36_{gh}	0.52_{de}	0.53_{ab}	nd	0.09_{f}	2.67_{bc}	1.76_{bc}	0.07_{e}	0.37_{efg}
2SE	2.50_{e}	1.46_{ab}	0.01_{b}	0.39_{gh}	0.51_{e}	0.54_{ab}	nd	0.10_{f}	2.57_{bcd}	1.81_{ab}	0.05_{e}	0.39_{efg}
2SR	2.29_{f}	1.55_{a}	0.01_{b}	0.43_{g}	0.46_{f}	0.57_{a}	nd	0.11_{f}	2.43_{cde}	1.96_{a}	0.06_{e}	0.46_{ef}
1ALS	2.68_{cd}	1.06_{de}	0.03_{b}	0.71_{f}	0.55_{cd}	0.44_{cd}	nd	0.18_{e}	2.63_{bc}	1.25_{e}	0.06_{e}	0.76_{d}
GLY P	2.11_{gh}	0.69_{hi}	0.04_{b}	0.91_{d}	0.47_{f}	0.31_{f}	0.03	0.24_{d}	3.09_{a}	1.24_{e}	0.12_{ab}	1.08_{c}
GLY SD	2.14_{g}	0.77_{gh}	0.05_{b}	0.81_{e}	0.47_{f}	0.34_{ef}	0.08	0.20_{e}	3.04_{a}	1.38_{e}	0.11_{abc}	0.94_{cd}
2ALS	1.98_{h}	1.07_{d}	0.03_{b}	1.07_{c}	0.41_{g}	0.49_{bc}	0.03	0.28_{c}	2.46_{cde}	1.31_{e}	0.07_{cde}	1.13_{c}
INT P	1.27_{i}	0.89_{fg}	0.02_{b}	1.51_{b}	0.27_{h}	0.39_{de}	0.03	0.35_{b}	2.10_{g}	1.60_{c}	0.07_{de}	1.83_{b}
INT SD	1.30_{i}	0.93_{ef}	0.02_{b}	1.43_{b}	0.27_{h}	0.40_{de}	0.09	0.33_{b}	2.13_{fg}	1.60_{c}	0.09_{abcde}	1.73_{b}
Whey	3.63_{a}	0.58_{j}	0.05_{b}	0.86_{de}	0.72_{a}	0.43_{cd}	0.05	0.25_{cd}	2.64_{bc}	0.53_{f}	0.06_{e}	0.53_{e}
BCON P	1.28_{i}	0.18_{j}	0.03_{b}	1.96_{a}	0.28_{h}	0.14_{g}	0.09	0.46_{a}	2.36_{def}	0.49_{f}	0.11_{abc}	2.95_{a}
BCON SD	1.21_{i}	0.22_{j}	0.04_{b}	1.95_{a}	0.27_{h}	0.14_{g}	0.10	0.46_{a}	2.26_{efg}	0.59_{f}	0.11_{abcd}	2.97_{a}
LSD	0.15	0.11	0.05	0.10	0.03	0.07	NDIFF	0.30	0.25	0.18	0.04	0.21

[a]Total isoflavone concentrations from Table 4 were normalized to the highest concentration, and then each isoflavone for a given total concentration was multiplied by that respective normalization factor.

[b]Isoflavone abbreviations: MDIN, malonyldaidzin; DIN, daidzin; AcDIN, acetyldaidzin; DEIN, daidzein; MGLY, malonylglycitin; GLY, glycitin; AcGLY, acetylglycitin; GLYEIN, glycitein; MGIN, malonylgenistin; GIN, genistin; AcGIN, acetylgenistin; GEIN, genistein.

[c]Subscript, lowercase letters with a column indicate significant difference at $P<0.05$.

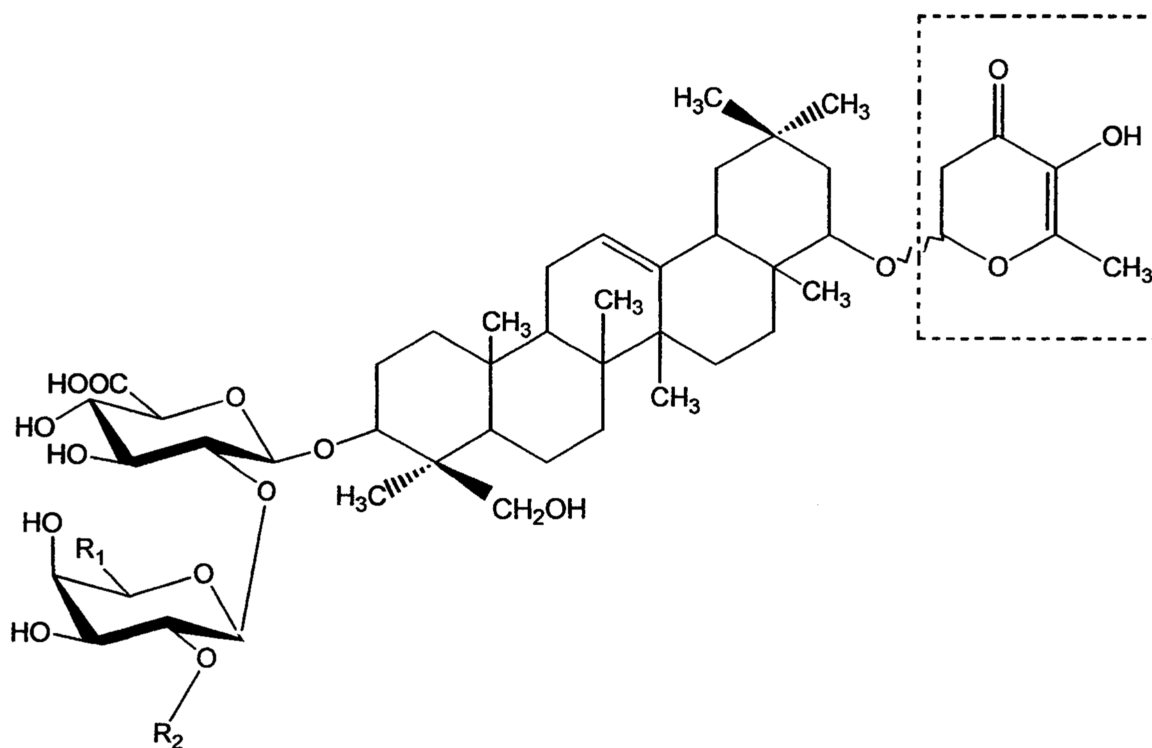
[d]Abbreviations: 1SE, first Sharples centrifuge pass extract; 1SR, first Sharples pass residue (insoluble fraction); 2SE, second Sharples pass extract; 2SR, second Sharples pass residue; 1ALS, first Alfa Laval supernatant; P, precipitate; SD, spray dried; 2ALS, second Alfa Laval supernatant; INT, intermediate; BCON, β-conglycinin; LSD, least significant difference; NDIFF, not different.

The group B saponins quantified were V, I, II, αg, βg, and βa. The latter three possess a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) moiety that is quite labile. When the moiety is liberated from αg, βg, and βa, the resulting non-DDMP forms are V, I, and II, respectively (Fig. 3).

Table 6 shows saponin distribution of the process fractions. The flakes have relatively high αg, βg, and βa concentrations, about 85% of the measured saponins, compared to their saponin V, I, and II counterparts. This is in agreement with Hu (29), reporting that the DDMP-conjugated saponins comprised about 85% of the group B saponins in raw soybean seeds. Saponin βg was present in the highest concentration, 3.36 μmol/g in our flakes, with an αg to βg ratio of 1:13. As expected, this was much different than the 1:2 ratio calculated by Hu for soy hypocotyls. Hypocotyls, compared to cotyledons, are high in saponin αg or V but very low in βa or II. The soy flakes used for the 50-kg process were not processed to remove the hypocotyls, but the cotyledon material had a dilution effect on the αg or V concentration. The saponin concentration of soy hypocotyls, as measured by Hu, was about 8 times that of soybean flour, 27.46 vs. 3.31 μmol/g on an as is basis.

The as is saponin concentration of the fractionated proteins, 4.82, 4.97, and 7.73 μmol/g for glycinin, intermediate, and β-conglycinin, respectively, were generally lower than Hu (29) observed for commercial soy isolate and concentrates on an as is basis. Two isolates had concentrations of 10.60 and 9.51 μmol/g. An acid washed soy concentrate and an ethanol-washed concentrate had concentrations of 9.41 and 0.20 μmol/g, respectively, demonstrating the extensive saponin extraction caused by the ethanol-wash concentrate process.

During the 50-kg soy protein fractionation process, a conversion from DDMP to non-DDMP saponins was observed. By the time the soy slurry, which had a βg concentration of 3.36 μmol/g, was separated into the first Sharples extract and first Sharple residue, spanning only a few hours, roughly 50% of the labile DDMP forms had lost their DDMP moiety. This rapid conversion seemed to diminish, with βg concentrations leveling off at about 1.5 μmol/g. Even by the time the β-conglycinin precipitate was recovered, about three days after



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

FIG. 3. Structures of group B soyasaponins. Rha: rhamnosyl; Glc: glucosyl; Y: yes; N: no DDMP: 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one

TABLE 6
Distribution of Saponins in Process Fractions (µmol/g, dry basis)

	V[a]	I	II	αg	βg	βa	Total
Flakes	nd	0.65_{e}	0.17_{e}	0.26	3.36_{a}	0.96_{a}	5.39_{bcdef}
1SE[b]	0.40_{bc}	2.50_{cd}	1.02_{bcd}	0.12	1.43_{bcd}	0.44_{b}	5.90_{bc}
1SR	0.40_{bc}	2.16_{d}	0.87_{d}	0.16	1.74_{b}	0.48_{b}	5.81_{bcde}
2SE	0.47_{ab}	3.08_{abc}	1.29_{abc}	0.10	1.10_{def}	0.33_{cd}	6.37_{ab}
2SR	0.42_{bc}	2.51_{bcd}	1.04_{bcd}	0.11	1.17_{cde}	0.34_{cd}	5.57_{bcdef}
1ALS	0.37_{bc}	1.98_{d}	0.82_{d}	0.10	1.13_{cdef}	0.34_{cd}	4.78_{cdef}
GLY P	0.39_{bc}	2.05_{d}	0.83_{d}	0.31	0.79_{f}	0.28_{d}	4.60_{ef}
GLY SD	0.31_{c}	1.90_{d}	0.82_{d}	0.09	1.12_{def}	0.34_{cd}	4.60_{f}
2ALS	0.18_{d}	0.50_{e}	0.25_{e}	0.02	0.30_{g}	0.13_{e}	1.36_{g}
INT P	0.41_{bc}	2.62_{bcd}	1.23_{abc}	0.09	1.16_{cde}	0.40_{bc}	5.88_{bcd}
INT SD	0.38_{bc}	2.04_{d}	0.99_{cd}	0.08	0.91_{ef}	0.32_{cd}	4.70_{def}
BCON P	0.56_{a}	3.26_{ab}	1.36_{ab}	0.14	1.44_{bcd}	0.47_{b}	7.23_{a}
BCON SD	0.55_{a}	3.38_{a}	1.42_{a}	0.13	1.50_{bc}	0.46_{b}	7.41_{a}
LSD	0.11	0.76	0.36	NDIFF	0.37	0.09	1.22

Normalized[c] Distribution of Saponins in Process Fractions (µmol/g, dry basis)

	V	I	II	αg	βg	βa	
Flakes	nd	0.93_{d}	0.25_{e}	0.37	4.83_{a}	1.39_{a}	
1SE	0.53_{b}	3.28_{ab}	1.34_{cd}	0.16	1.88_{bc}	0.58_{bcd}	
1SR	0.53_{b}	2.88_{c}	1.16_{d}	0.22	2.33_{b}	0.64_{bc}	
2SE	0.57_{b}	3.75_{a}	1.57_{abc}	0.12	1.35_{cd}	0.40_{e}	
2SR	0.58_{b}	3.49_{ab}	1.44_{abc}	0.15	1.63_{cd}	0.47_{de}	
1ALS	0.60_{b}	3.23_{bc}	1.35_{cd}	0.16	1.85_{bcd}	0.56_{cde}	
GLY P	0.65_{b}	3.42_{ab}	1.38_{bcd}	0.52	1.32_{d}	0.47_{de}	
GLY SD	0.53_{b}	3.21_{bc}	1.39_{bcd}	0.16	1.90_{bc}	0.58_{bcd}	
2ALS	1.03_{a}	2.81_{c}	1.42_{abc}	0.11	1.65_{cd}	0.73_{b}	
INT P	0.55_{b}	3.38_{ab}	1.60_{abc}	0.12	1.56_{cd}	0.55_{cde}	
INT SD	0.62_{b}	3.37_{ab}	1.63_{abc}	0.13	1.50_{cd}	0.53_{cde}	
BCON P	0.61_{b}	3.50_{ab}	1.46_{abc}	0.15	1.54_{cd}	0.50_{cde}	
BCON SD	0.57_{b}	3.53_{ab}	1.48_{abc}	0.13	1.57_{cd}	0.48_{cde}	
LSD	0.14	0.47	0.24	NDIFF	0.55	0.17	

[a]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[b]Abbreviations: 1SE, first Sharples centrifuge pass extract; 1SR, first Sharples pass residue (insoluble fraction);2SE, second Sharples pass extract; 2SR, second Sharples pass residue; 1ALS, first Alfa Laval supernatant; P, precipitate; SD, spray dried; 2ALS, second Alfa Laval supernatant; INT, intermediate; BCON, β-conglycinin; LSD, least significant difference; NDIFF, not different.
[c]Total saponin concentrations were normalized to the highest concentration, and then each saponin for a given total concentration was multiplied by that respective normalization factor.

the fractionation process was initiated, the concentration of the βg was not significantly different than it had been for the first Sharples extract and residue fractions. This was not expected. Hu (29) showed that there was not a significant conversion of βg to saponin I within 3 h at room temperature. However, this fractionation process took about three days to complete. It seemed as if there were a protective effect or an equilibrium was reached.

As was done for the isoflavones, Table 6 was normalized to the fraction that had the highest saponin concentration. The total saponin concentrations were similar for most of the fractions, so the impact of normalization was not as dramatic as observed for the isoflavones. The normalization made it very clear that the individual saponin concentrations did not change dramatically, and in most cases not significantly, from the first Sharples extract to the spray-dried β-conglycinin. An exception to this was the second Alfa Laval supernatant (2ALS) for saponin V. The saponin V concentrations also seemed high compared to the αg saponin in the starting material and the process fractions. Spray drying did not seem to affect the conversion of DDMP to non-DDMP saponins. As for the isoflavones, the heat treatment and duration of spray drying were probably too short to have a significant affect.

Acknowledgments

This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project N. 6571, was supported in part by USDA NRI grant No. 2001-35503-10814, the Iowa Soybean Promotion Board, the NASA Food Technology Commercial Space Center, the Center for Designing Foods to Improve Nutrition, and by Hatch Act and State of Iowa funds.

Kobita Barua and Jiang Hu provided technical assistance with HPLC analysis. Mark Reuber of the Center for Crops Utilization Research at Iowa State University provided pilot-plant expertise.

References

1. Messina, M.J., Soyfoods: their role in disease prevention and treatment, in *Soybeans: Chemistry, Technology, and Utilization*, edited by K.S. Liu, Chapman and Hall, New

York, 1997, pp. 443-447.
2. Friedman, M., and D.L. Brandon, Nutritional and health benefits of soy proteins, *J. Agric. Food Chem.* *49*:1069-1086 (2001).
3. Potter, S.M., Overview of proposed mechanisms for the hypocholesterolemic effect of soy, *J. Nutr.* *125*:606S-611S (1995).
4. Setchell, K.D.R., and A. Cassidy. Dietary isoflavones: biological effects and relevance to human health, *J. Nutr.* *129*:758S-767S (1999).
5. Oakenfull, D., and G.S. Sidhu, Could saponins be a useful treatment for hypercholesterolaemia?, *Eur. J. Clin. Nutr.* *44*:79-88 (1990).
6. Kennedy, A.R., The evidence for soybean products as cancer preventative agents, *J. Nutr.* *125*:733S-734S (1995).
7. Utsumi, S., Y. Matsumura, and T. Mori, Structure-function relationships of soy proteins, in *Food Proteins and Their Applications*, edited by S. Damodaran and A. Paraf, Marcel Dekker, Inc., New York, 1997, pp. 257-291.
8. Mujoo, R., D. T. Trinh, and P.K.W. Ng, Characterization of storage proteins in different soybean varieties and their relationship to tofu yield and texture, *Food Chemistry* *82*:265-273 (2003).
9. Khatib, K.A., T.J. Herald, F.M. Aramouni, F. MacRitchie, and W.T. Schapaugh, Characterization and functional properties of soy β-conglycinin and glycinin of selected genotypes, *J. Food Sci.* *67*:2923-2929 (2002).
10. Bringe, N.A., High beta-conglycinin products and their use, U.S. Patent 6,171,640 (2001).
11. Wu, S., P.A. Murphy, L.A. Johnson, A.R. Fratzke, M.A. Reuber, Pilot-plant fractionation of soybean glycinin and β-conglycinin, *J. Am. Oil Chem. Soc.* *76*:285-293 (1999).
12. Bian, Y., D. J. Myers, K. Dias, M. K. Lihono, S. Wu, and P.A. Murphy, Functional properties of soy protein fractions produced using a pilot plant-scale process, *J. Am. Oil Chem. Soc.* *80*:551-555 (2003).
13. Dias, K., D. J. Myers, Y. Bian, M. A. Lihono, S. Wu, and P. Murphy, Functional properties of the acidic and basic subunits of the glycinin (11S) soy protein fraction, *J. Am Oil Chem. Soc.* *80*:545-549 (2003).
14. Thanh, V. H., and K. Shibasaki, Major proteins of soybean seeds. A straightforward fractionation and their characterization, *J. Agric. Food Chem.* *24*:1117-1121 (1976).
15. O'Keefe, S. F., L.A. Wilson, A.P. Resurreccion, and P.A. Murphy, Determination of the binding of hexanal to soy glycinin and β-conglycinin in an aqueous model system using a headspace technique, *J. Agric. Food Chem.* *39*:1022-1027 (1991).
16. Nagano T., M. Hirotsuka, H. Mori, K. Kohyama, and K. Nishinari, Dynamic viscoelastic study on the gelation of 7S globulins from soybeans, *J. Agric. Food Chem.* *40*:941-944 (1992).
17. Davidson, R.M., R.E. Sand, and R.E. Johnson, Method for processing soy protein and composition of matter, U.S. Patent 4,172,828 (1979).
18. Howard, P.A., W.F. Lehnhardt, and F.T. Orthoefer, 7S and 11S vegetable protein fractionation and isolation, U.S. Patent 4,368,151 (1983).
19. Lehnhardt, W.F., P.W. Gibson, and F.T. Orthoefer, Fractionation and isolation of 7S and 11S protein from isoelectrically precipitated vegetable protein mixtures, U.S. Patent 4,370,267 (1983).

20. Wu, S., P.A. Murphy, M.A. Reuber, and A.R. Fratzke, Simplified process for soybean glycinin and β-conglycinin fractionation, *J. Agric. Food Chem. 48*:2702-2708 (2000).
21. AACC. Approved Methods of the American Association of Cereal Chemistry, 8th ed.; AACC: St. Paul, MN, 1983a; Method 44-15A.
22. AACC. Approved Methods of the American Association of Cereal Chemistry, 8th ed.; AACC: St. Paul, MN, 1983b; Method 08-03.
23. AOAC. Official Methods of Analysis of Association of Official Analytical Chemists, 15th ed.; AOAC: Arlington, VA, 1990; methods 988.05 and 960.52.
24. Hu, J., S.O. Lee, S. Hendrich, and P.A. Murphy, Quantification of the Group B soyasaponins by high-performance liquid chromatography, *J. Agric. Food Chem. 50*:2587-2594 (2002).
25. Murphy, P.A., T. Song, G. Buseman, K. Barua, G.R. Beecher, D. Trainer, and J. Holden, Isoflavones in retail and institutional soy foods, *J. Agric. Food Chem. 47*:2697-2704 (1999).
26. Berk, Z., *Technology of production of edible flours and protein products from soybeans*, FAO, Rome, 1992, p. 89.
27. Shimoyamada, M., S. Ikedo, R. Ootsubo, and K. Watanabe, Effects of soybean saponins on chymotryptic hydrolyses of soybean proteins, *J. Agric. Food Chem. 46*:4793-4797 (1998).
28. Wang, H.J., and P.A. Murphy, Mass balance study of isoflavones during soybean processing, *J. Agric. Food Chem. 44*:2377-2383 (1996).
29. Hu, J., Isolation and quantitative analysis of soybean saponins by high performance liquid chromatography, Iowa State University, Ames, IA, 2000, p. 74.
30. Murphy, P.A., K. Barua, and C.C. Hauck, Solvent extraction selection in the determination of isoflavones in soy foods, *J. Chrom. B 777*:129-138 (2002).

CHAPTER 3. ISOFLAVONE AND SAPONIN PARTITIONING DURING BENCH-SCALE SOY ISOLATE PRODUCTION: EFFECTS OF TEMPERATURE AND pH

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

David A. Rickert, Melissa A. Meyer, Jiang Hu, Patricia A. Murphy

Abstract

Temperature and pH affect protein solubilization from soy flakes. Varying these parameters during the solubilization step of bench-scale soy protein isolate production affected isoflavone and saponin recovery, partitioning, and profile. Discrepancies in the phytochemical mass balance led to investigation of soy slurry prepared at 25 or 60 °C and pH 8.5, 9.5, or 10.5. The total saponin concentration in the slurry was not affected by these conditions, but increasing temperature and pH decreased total isoflavone concentrations. This observation led to modification of phytochemical extraction conditions. Sample neutralization prior to extraction increased quantified isoflavone concentration of the soy slurry material, while neutralizing the isolate fraction dramatically increased quantified saponin concentrations. Phytochemical extraction pH should receive careful consideration when analyzing soy samples for isoflavones and saponins.

Introduction

Soy isoflavones have received considerable attention as plant phytoestrogens that may favorably impact human health. The potential effects of soy isoflavones on bone integrity (Murphy and Hendrich 2002), colon and breast cancer (Messina and Bennick 1998, Messina 1999), and cardiovascular health (Setchell and Cassidy 1999) have been reviewed. Although they have received less scrutiny, soy saponins may also provide health benefits. Oakenfull and Sidhu (1990) have reviewed the ability of saponins to treat hypercholesterolemia. Soy saponins seem to have an anticarcinogenic effect against colon cancer (Koratkar and others 1997, Sung and others 1995). Due to their potential health impact, the contents of these soy

phytochemicals in foods and their fate during soy food and soy ingredient processing are of considerable interest.

The isoflavone contents of soybean varieties (Wang and Murphy 1994a), commercial soybean foods (Wang and Murphy 1994b), ethnic soy foods (Franke and others 1999), retail and institutional soy foods (Murphy and others 1999) and infant formulas (Murphy and others 1997) have been published. Wang and Murphy (1996) have detailed the partitioning of isoflavones and the mass distribution or profile of isoflavone isomers during soy protein isolate, soymilk, tofu, and tempeh processing. Isoflavone profile is important since the glucoside forms require enzymatic modification to the aglycone forms for gut absorption in humans (Xu and others 1995). Profile changes have been described for isoflavones during soy protein isolate processing (Wang and others 1998), corn/soy mixture extrusion (Mahungu and others 1999), β-glucosidase treatment (Matsuura and Obata 1993), thermal processing of tofu (Grün and others 2001), and edamame processing (Simonne and others 2000).

Absence of good analytical methodology and limited interest have hindered quantification and reporting of soy saponin data. An efficient HPLC method to measure group B saponins, comprising approximately 83% of total soyasaponins in defatted soy meal (Gu and others 2002), was recently developed by Hu and others (2002). This method was used to quantify saponin concentrations in nearly 50 soybean varieties (Hu and others 2002), as well as soymilk, tempeh, textured vegetable protein, tofu, soy protein isolate, and soy concentrates. Tsukamoto and others (1995), Ireland and others (1986), and Kitagawa and others (1984) have measured soy saponin concentrations in soybeans, soy ingredients, and soy foods, respectively.

Another group of soy saponins, group A saponins, have also been quantified by HPLC analysis. Group A and group B saponins exhibit structural similarities, and each group has labile forms that undergo structural changes under certain processing conditions. These numerous, structurally similar saponins have complicated quantification of each specific soy saponin. Shimoyamada and others (1990) and Shiraiwa and others (1991) have described HPLC methods to quantify group A and group B saponins. Evidence suggests that when soy

is heated in an aqueous environment above 80 °C and at least pH 9, the structure of several group A forms becomes so similar to several group B forms that they share the same HPLC retention time and cannot be resolved (Meredith 2002). These processing conditions may be encountered during the manufacture of some soy protein isolates, influencing the quantification of group B saponins in these isolates.

Soy protein isolate is a popular soy ingredient with useful protein functionality and may be the most significant source of soy protein, and thus soy phytochemicals, for those not consuming more traditional soy foods, such as tofu or soy milk. Soy protein isolate is the isoelectric precipitate of the alkaline protein extract from defatted soy flakes. The protein is generally extracted at temperatures of up to 80 °C and pH 7.5 to 9.0 (Berk 1992), although 60 °C and pH 8.5 are more typical of conditions employed commercially. Conditions of approximately 25 °C and pH 8.5 have been used to extract soy proteins in their native state for efficient fractionation of the soybean storage proteins, glycinin and β-conglycinin (Wu and others 1999).

The objective of the current research was to determine whether isoflavone and saponin partitioning, recovery, and profile during soy protein isolate production are affected by extraction temperature (25 and 60 °C) and pH (8.5-10.5). An additional interest was whether group A saponins could be observed to influence group B concentrations under one or more of the treatment conditions.

Materials and Methods

Materials

The starting material for soy slurry and soy protein isolate production was defatted soy white flakes from Cargill (Minneapolis, MN). Flakes were stored in sealed containers at 4 °C. HPLC-grade acetonitrile, methanol, trifluoroacetic acid, and glacial acetic acid for HPLC mobile phase and sample preparation were purchased from Fisher Scientific (Fair

Lawn, NJ). HPLC-grade water for mobile phase and sample preparation was produced using a Milli-Q water system (Millipore Co., Bedford, MA).

Soy Slurry and Soy Isolate Production

Soy slurries were prepared by combining defatted soy flakes with de-ionized water (6 g flakes and 60 g water) at 25 or 60 °C with continuous stirring in 125-mL Erlenmeyer flasks. The pH of each slurry was then adjusted to 8.5, 9.5, or 10.5, as measured with an automatic temperature control (ATC) pH meter and probe, using 2 N NaOH. The pH was readjusted as needed at 15 and 30 min, and the processing was ended at 30 min. Slurries were then freeze-dried. Each treatment was performed in duplicate.

Soy protein isolates were prepared as follows. Defatted soy flakes were combined with de-ionized water (80.0 g flakes to 800 g water) at 25 or 60 °C and stirred continuously. The pH of each slurry was adjusted to 8.5, 9.5, or 10.5, as measured with an ATC pH meter and probe, using 10 N NaOH. The pH was readjusted as needed at 15 and 30 min, and the protein solubilization was ended at 30 min. Slurries were passed through a 60-mesh screen. The recovered liquid fractions were centrifuged at 14,000 x *g* for 30 min at 15 °C. The resulting precipitates and the screenings from the previous step were combined to comprise the respective insoluble fractions, weighed, frozen, and freeze dried. Supernatants at 20 °C were adjusted to pH 4.5 with 2 N HCl, refrigerated at 4 °C for 1 h, and then centrifuged at 14,000 x *g* for 30 min at 4 °C. Resulting supernatant (whey) and precipitate (isolate) fractions were weighed and freeze-dried. Each treatment was performed in duplicate.

Isoflavone and Group B Saponin Extraction and Analysis

Extractions and HPLC analyses were conducted as reported by Hu and others (2002) for saponins and Murphy and others (1999) for isoflavones with modifications. Approximately 2.5 g of freeze-dried slurry, insoluble fraction, or protein isolate, or 4.0 g of freeze-dried whey fraction, was accurately weighed into a 125-mL screw-capped Erlenmeyer flask, and 14 mL of water and 20 mL of acetonitrile were added to the flask to extract the isoflavones

and saponins. Extractions were performed with and without neutralization to pH 7, adjusted using 2N HCl or 2N NaOH, and flasks were shaken at 300 rpm for 2 h at room temperature.

This modified extraction method differed as follows from the methods described by Murphy and others (1999) and Hu and others (2002). For optimal isoflavone extraction from a particular food matrix, Murphy and others (1999) used 10 mL of acetonitrile, 2 mL of 0.1 N HCl, and water as needed (approximately 7 mL). Hu and others (2002) used 100 mL of 70% ethanol and 2.5 h of extraction time for saponin extraction. The modified method did not give significantly different ($P<0.05$) phytochemical concentrations compared to the referenced methods when ground, defatted soy flakes were analyzed.

The extract was vacuum filtered (Whatman 42 paper, Hillsboro, OR), and the extract was rotary evaporated to dryness at <30 °C. The residue was dissolved and recovered in 80:20 methanol:water to a volume of 10.0 mL. Samples for isoflavone analysis were filtered through a 0.45-µm polytetrafluoroethylene filter (Alltech Associates Inc., Deerfield, IL) prior to HPLC analysis. Samples for saponin analysis were further purified using a disposable C18 cartridge (Waters Corporation, Milford, MA.). Cartridges were preconditioned by sequentially passing 3 mL of de-ionized water, 2 mL of 100% methanol, and 4 mL of de-ionized water through the cartridges. Exactly 2.0 mL of the sample was combined with 7 mL of de-ionized water, mixed vigorously, and then passed through the cartridge. The cartridge was then washed with 5 mL of 5% methanol. The saponins were finally eluted from the cartridge with 2.0 mL of 100% methanol. This purified extract was filtered with a 0.45-µm filter as above prior to HPLC analysis. Quantification of the isoflavones and group B saponins was performed on different HPLC systems, but using the same type of column, an RP-18, 5 µm, 4.6 i.d. x 250 mm YMC-ODS-AM-303 column (YMC, Inc., Wilmington, NC).

For quality control purposes, an isoflavone standard solution (genistin, genistein, and daidzein) and a saponin standard solution (saponin I) were injected on the respective systems prior to analyzing samples. Preparation of isoflavone and saponin standards and calibration curves is described by Murphy and others (1999) and Hu and others (2002). Isoflavone recovery from ground soy flake and freeze-dried soy protein isolate material was evaluated

using a concentrated isoflavone extract produced from soy flakes and containing 7.5 μmol/mL isoflavones in 80% methanol. Saponin recovery from these matrices employed a mixture of saponin I and II at concentrations of 4.8 and 3.9 μmol/mL, respectively, in 80% methanol. Samples were spiked, mixed thoroughly, and then allowed to air dry overnight. Extraction and analysis were performed as above.

Protein Quantification

Nitrogen content of the defatted soy flakes and process fractions was measured using the combustion or Dumas method (AOAC 990.03) with a Rapid NIII analyzer (Elementar Americas, Inc., Mt Laurel, NJ). These values were converted to Kjeldahl nitrogen concentrations using a conversion formula (Jung and others 2003). A conversion factor of 6.25 was used to convert nitrogen to protein content.

Statistical Analysis

The data was analyzed using ANOVA. Both Dunnetts and Tukeys adjustments were made in comparing the LS Means for different response variables depending on the situation for which they were compared. Means were also compared using least squares differences. These results were calculated using SAS (version 8.2, SAS Institute Inc., Cary, NC).

Results and Discussion

Protein Partitioning during Isolate Production

Traditional extraction conditions employed in soy protein isolate production are pH 7.5 - 9 and temperatures up to 80 °C (Berk 1992), although U.S. patents have disclosed a much broader range of extraction conditions with regard to pH and temperature. Proprietary concerns limit specific information about commercial extraction conditions, but pH 8.5 and 60 °C are commonly accepted extraction conditions for isolate production and are used at the Iowa State University Center for Crops Utilization Research (CCUR) pilot plant. CCUR

reports their dry weight basis isolate yields are typically 33%, but industry yields may be as high as 44%. Conditions of pH 8.5 and 25 °C have been used in the CCUR pilot plant when extracting protein for soy protein fractionation to prepare glycinin- and β-conglycinin enriched fractions (Wu and others 1999, Wu and others 2000). This relatively low extraction temperature was employed for the fractionation process to maximize the amount of native state storage proteins, which have been hypothesized to fractionate more efficiently (Wu and others 2000). Compared to soy protein isolate production, fraction weight yields have been low for the pilot-scale fractionation process, typically less than 30%. This probably was due primarily to the low protein extraction temperature.

Protein solubilization conditions of 25 °C at pH 8.5 and 60 °C at pH 8.5-10.5 were chosen for bench-scale isolate production. These selections were made to simulate extraction conditions employed in the CCUR pilot plant for the soy protein isolate production and soy protein fractionation, and to evaluate the effects of increasing the extraction pH above 8.5. Increased extraction temperature and increasing extraction pH significantly increased fraction weight and total protein recovery in the isolate fraction (Table 1). The dry weights of the insoluble fractions were inversely related to those for the isolate. The protein concentration of the isolates was not significantly different among treatments.

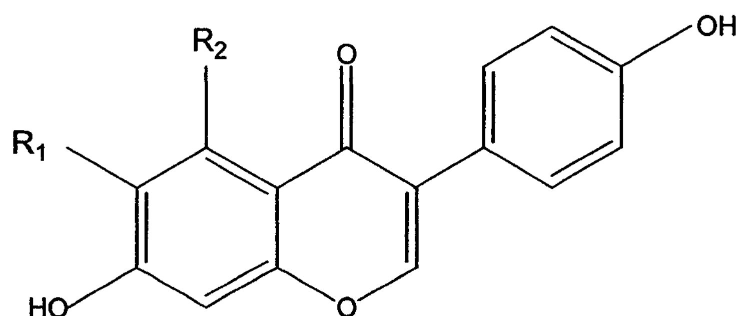
Phytochemical Partitioning and Distribution During Isolate Production

Soy isoflavones are comprised of three aglycone forms, daidzein, genistein, and glycitein, and their respective glucoside forms. There are three glucoside forms for each aglycone: 6"-O-malonylglucoside, 6"-O-acetylglucoside, and the simple β-glucoside (Figure 1). The malonylglucoside of each aglycone family is the predominant form in raw soybeans (Wang and Murphy 1994a), with prevalence of combined forms generally being genistein≈daidzein >>glycitein on a mole basis. When processed under mild conditions, malonylglucosides will gradually convert to the β-glucoside forms (Murphy and others 1999, Wang and Murphy 1996). Conversion of malonylglucosides to acetylglucosides occurs during more abusive conditions, such as toasting and extrusion (Farmakalidis and Murphy 1985, Mahungu and

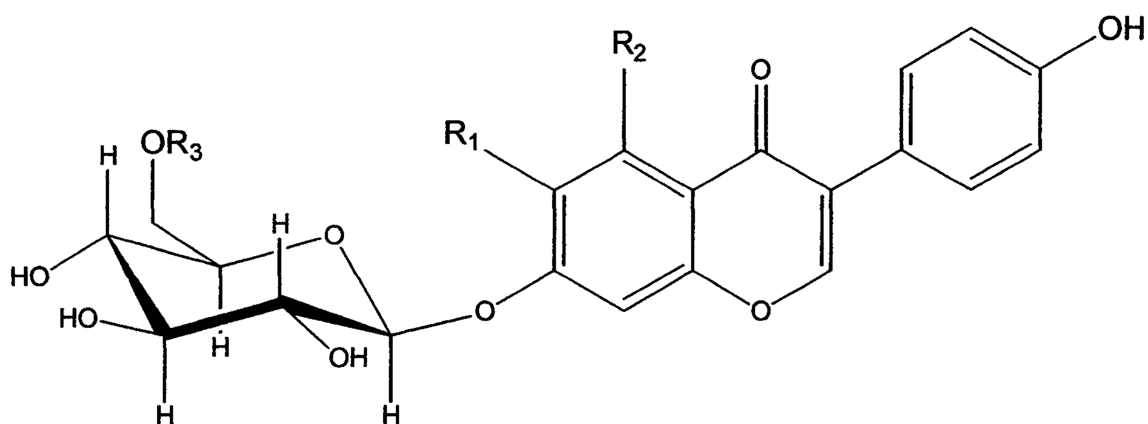
Table 1 – Effect of protein solubilization temperature and pH on fraction weight and protein partitioning during bench-scale soy protein isolate production

	Insoluble[a,b,c]	Isolate	Whey
Fraction weight (g, db[d])			
25 °C pH 8.5	35.19_a	22.70_c	18.01_{ab}
60 °C pH 8.5	32.34_b	26.21_b	17.32_{bc}
60 °C pH 9.5	33.62_b	25.41_b	16.66_c
60 °C pH 10.5	30.73_c	28.34_a	18.56_a
LSD[e]	1.47	1.25	0.89
Protein concentration (%, db)			
25 °C pH 8.5	43.0_a	90.0	18.8_a
60 °C pH 8.5	40.5_b	89.4	14.8_c
60 °C pH 9.5	39.4_b	89.6	16.3_b
60 °C pH 10.5	35.5_c	87.1	15.3_c
LSD	1.6	Not Different	0.8
Total protein (g, db)			
25 °C pH 8.5	15.1_a	20.4_c	3.39_a
60 °C pH 8.5	13.1_b	23.4_b	2.56_c
60 °C pH 9.5	13.2_b	22.8_b	2.72_{bc}
60 °C pH 10.5	10.9_c	24.7_a	2.83_b
LSD	0.8	1.1	0.18

[a]Dry basis weight, protein concentration, and total protein of the starting material were 73.88 g, 50.09 %, and 41.23 g, respectively.
[b]n=2.
[c]Values within a column with different subscript letters were significantly different at $P < 0.05$.
[d]db, dry basis.
[e]LSD, least significant difference.



Isoflavone	R1	R2
Daidzein	H	H
Genistein	H	OH
Glycitein	OCH3	H



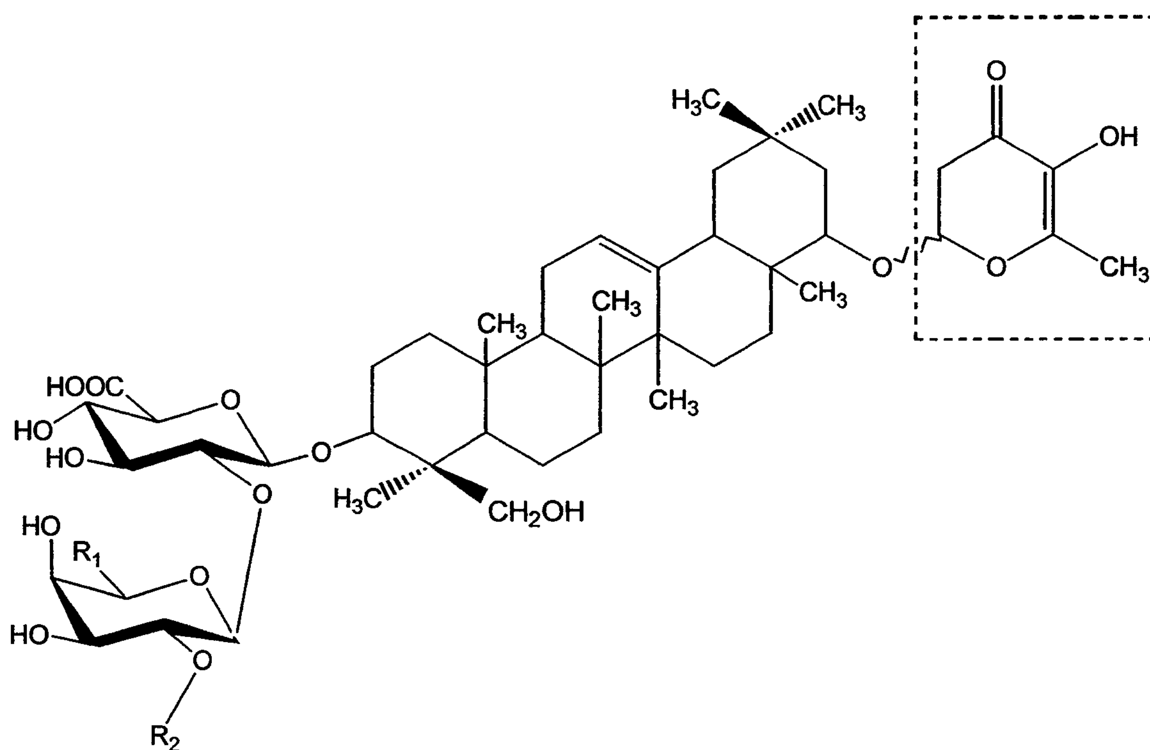
Isoflavone	R1	R2	R3
Genistin	H	OH	H
Glycitin	OCH3	H	H
Daidzin	H	H	H
Malonylgenistin	H	OH	COCH2COOH
Malonylglycitin	OCH3	H	COCH2COOH
Malonyldaidzin	H	H	COCH2COOH
Acetylgenistin	H	OH	COCH3
Acetylglycitin	OCH3	H	COCH3
Acetyldaidzin	H	H	COCH3

Figure 1 - Isoflavone structures

others 1999). In the presence of β-glucosidases, glucoside isoflavones will be converted to aglycones (Wang and Murphy 1996).

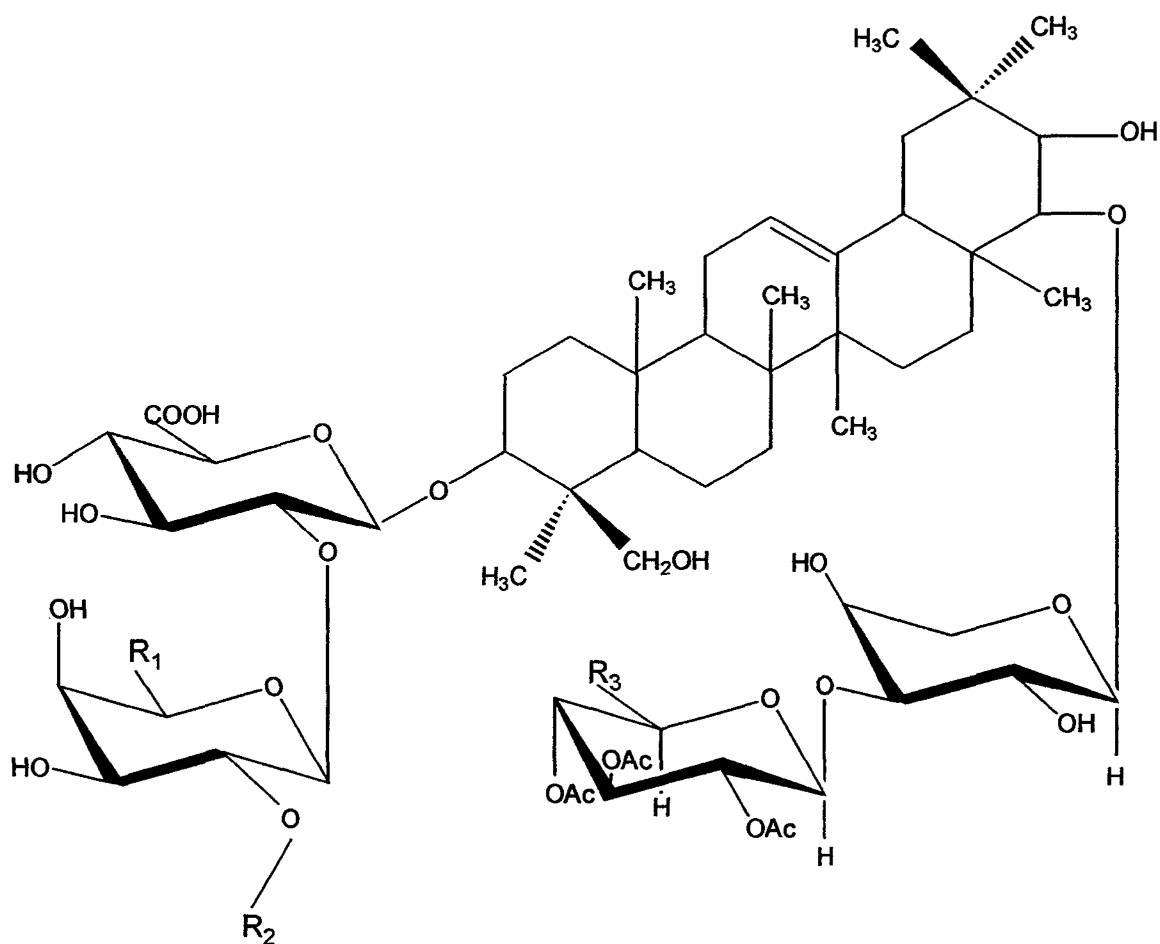
Soyasaponin composition of whole soybeans has been reported to be about 75% group B saponins and 25% group A saponins (Ireland and others 1986). The group B saponins that could be measured by the method used for our research (Hu and others 2002) were V, I, II, αg, βg, and βa. The latter three group B saponins have a 2,3-dihyro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) moiety with a labile bond, and these forms readily convert to V, I, and II, respectively (Figure 2). Group B saponins III, IV, γg, and γa are also present in soybeans, but only in very small quantities. It is difficult to isolate adequate quantities of these saponins to prepare standard curves. Group A saponins (Figure 3) have a sugar group at normal environmental temperatures that is labile at pH 9 and temperatures above 80 °C (Meredith 2002). When these sugar groups are removed, group A saponins A_4 and A_1, A_5 and A_2, and A_6 and A_3, are structurally very similar to group B saponins V, III, and IV, respectively. Unpublished data (Meredith 2002) suggest these altered, group A saponin pairs elute simultaneously with their group B counterparts, causing overestimation of group B saponins and an overall increase in measured saponins, provided only group B saponins are being quantified. Our method did not permit quantification of group B saponins III and IV. Consequently, co-elution of modified A_5 and A_2 with III and A_6 and A_3 with IV could not be effectively evaluated. Dramatic increases would need to be observed in saponin V concentrations to demonstrate that a conversion of A_4 and A_1 and co-elution with saponin V had occurred under various treatment conditions.

Soy protein isolate, recovered by precipitating protein from the protein extract by adjusting the pH to the isoelectric point (pH 4.5), may be neutralized from pH 4.5 to pH 6.8-7.5 prior to spray drying to help disperse the isolate curd and to enhance dispersion of the spray-dried product in aqueous applications, if that is desired. For this study, the isolate was not neutralized prior to freeze drying. Our typical phytochemical extraction method demonstrated discrepancies in the phytochemical mass balance data. In an attempt to correct this situation, neutralization of sample slurry just prior to phytochemical extraction was



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

Figure 2 - Structures of group B soyasaponins
Rha: rhamnosyl; Glc: glucosyl; Y: yes; N: no
DDMP: 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one



Soyasaponin	R_1	R_2	R_3
Aa = acetyl A_4	CH_2OH	β-D-Glc	H
Ab = acetyl A_1	CH_2OH	β-D-Glc	CH_2OAc
Ac	CH_2OH	α-L-Rha	CH_2OAc
Ad	H	β-D-Glc	CH_2OAc
Ae = acetyl A_5	CH_2OH	H	H
Af = acetyl A_2	CH_2OH	H	CH_2OAc
Ag = acetyl A_6	H	H	H
Ah = acetyl A_3	H	H	CH_2OAc

Figure 3 - Group A acetylated soyasaponin structures
Glc: glucosyl; Rha: rhamnosyl; Ac: acetyl

explored. Neutralization did have a favorable effect on phytochemical recovery in certain cases. Consequently, freeze-dried soy protein isolates, as well as the insoluble and whey fractions, were extracted for isoflavones and saponins both with and without neutralization of the sample slurry prior to extraction. Table 2 shows the mass balance data from soy isolate production for the isoflavones and saponins. For the insoluble fraction there was a clear, and in most cases significant, trend in total isoflavone and saponin recovery from the starting material as temperature and pH was increased, similar to that observed for the fraction weight and protein. Even though the phytochemical concentration for each insoluble fraction was multiplied by the fraction weight, which declined with increasing temperature and pH, the decrease in fraction weight did not account for all of the decrease in total phytochemical recovered in the insoluble fraction for the high temperature, high pH treatments. The treatment conditions affected total phytochemical recovery.

Saponins were not detected in the whey fraction, and total isoflavones recovered in the whey fraction were not significantly different among treatments. For both neutralized and not neutralized protein isolate samples, there were clear, significant, positive trends in quantified saponins as temperature and pH increased. Interestingly, neutralization of the sample slurry prior to phytochemical extraction significantly increased measured saponins. It is possible that as the sample slurry was neutralized, the physicochemical relationship between the saponins and proteins was altered. There is a carboxyl group on the glucuronic acid of the group B saponins that may have a pK_a of approximately 4. This could account for ionic interaction with the proteins near the isoelectric point of the proteins.

Unexpectedly, summed isoflavone and saponin fraction totals (insoluble + isolate + whey) were both low compared to the total isoflavones and saponins measured in the starting material. Soy flake and process fraction matrices were probably responsible for at least a portion of this discrepancy. A recovery study with ground, defatted soy flakes and the freeze-dried soy protein isolate revealed considerable recovery differences between these matrices. Recovery of saponin I and II was about 99% for the flake material, but only 60-70% for the isolate. Isoflavone recoveries were 75-92% for the flake material, with the more

Table 2 – Effect of protein solubilization temperature and pH on isoflavone and saponin mass of bench-scale soy protein isolate process fractions

	Isoflavones (μmol, dry basis)					Saponins (μmol, dry basis)		
Treatment	Insoluble[a]	Isolate	Whey	Totals		Insoluble	Isolate	Totals
Ground Flakes				648				515
25 °C pH 8.5[b]	196_a	227_d	135	558*		276_a	118_f	394*
25 °C pH 8.5N[c]	198_a	267_{bc}	115	580*		261_{ab}	200_{cd}	461
60 °C pH 8.5	157_b	272_{bc}	106	535*		237_{bc}	128_{ef}	365*
60 °C pH 8.5N	168_b	296_a	101	565*		238_{bc}	206_{cd}	444
60 °C pH 9.5	160_b	263_c	118	541*		235_{bc}	178_{de}	413*
60 °C pH 9.5N	169_b	265_{bc}	126	560*		223_{cd}	278_b	501
60 °C pH 10.5	100_d	277_b	108	485*		204_{cd}	244_{bc}	448
60 °C pH 10.5N	130_c	263_c	123	516*		194_d	344_a	538
LSD[d]	14	12	ND[e]			33	57	

[a]Values within a column with different subscript letters were significantly different at $P < 0.05$.
[b]n=2.
[c]N, neutralized at time of phytochemical extraction.
[d]LSD, least significant difference.
[e]ND, not different.
*Indicates that the sum of the insoluble, isolate, and whey fractions is different ($P<0.05$, Dunnetts test) from the amount of isoflavone or saponin in the ground, defatted soy flakes.

hydrophobic aglycones exhibiting the lowest recoveries. Isoflavone aglycone recovery from the isolate material was 65-75%, while recoveries for the β-glucosides and malonylglucosides were 78-93%. Neutralization of the phytochemical extraction solution increased saponin I and II recovery from the isolate samples by about 15% for saponin I and 25-45% for saponin II. Recovery of the aglycone isoflavones from the isolate samples increased by 10-15% with neutralization.

Isoflavone and saponin concentrations in the process fractions were evaluated, as well as the effect of process conditions on isoflavone and saponin profiles (Tables 3 and 4). The isoflavone content of the starting material was 8.8 μmol/g. Partitioning of the isoflavones led to concentrations of 3.26-5.63 μmol/g in the isoflavone-depleted insoluble fraction, 9.29-11.78 μmol/g in the isolate product, and 5.95-7.47 μmol/g in the whey fraction. The ranges reflect the effect of treatment and neutralization. Modest isoflavone enrichment in the isolate compared to the starting material was demonstrated. The isoflavone profile was altered by treatment conditions for the insoluble, isolate, and whey fractions. For both neutralized and non-neutralized samples, as temperature and pH were increased, malonylglucosides and acetylglucosides were converted to β-glucosides. Apparently, there was very little glucosidase activity, as conversion of glucosides to aglycones was not substantial. However, the protein solubilization time was relatively short, limiting the amount of time a glucosidase would have for isoflavone modification. The trend toward lower aglycone concentrations in the insoluble, isolate, and whey fractions as temperature and pH were increased may have been caused by reduced aglycone recovery from the sample matrices during analytical extraction. Temperature and pH affect protein native state, and as proteins unfold, additional hydrophobic areas are exposed. The aglycones are more hydrophobic than the glucosides and may have an affinity for exposed hydrophobic regions on proteins. Acetylglucoside concentrations did not increase, probably because process conditions were aqueous and still relatively mild, compared to toasting or roasting at low moisture concentrations.

Processing effects on isoflavone profile have received considerable investigation, due in part to a study by Izumi and others (2000) that demonstrated that aglycone isoflavones are

Table 3 – Effect of protein solubilization temperature and pH on isoflavone distribution in process fractions from bench-scale soy protein isolate production

	Concentration (μmol/g, dry basis)											
Treatment[a]	Din[b, c]	MDin	AcDin	Dein	Glyin	MGly[d]	Glyein	Gin	MGin	AcGin	Gein	Total
Insoluble Fraction												
25 °C pH 8.5	0.79_{d}	1.17_{a}	0.06	0.19_{a}	0.29_{d}	0.24_{a}	0.05	1.12_{d}	1.37_{a}	0.06_{b}	0.23	5.56_{ab}
25 °C pH 8.5N[e]	0.81_{d}	1.18_{a}	0.04	0.20_{a}	0.29_{d}	0.24_{a}	0.06	1.13_{d}	1.36_{a}	0.08_{ab}	0.25	5.63_{a}
60 °C pH 8.5	0.92_{c}	0.77_{b}	0.04	0.14_{b}	0.28_{d}	0.16_{b}	0.05	1.28_{cd}	0.92_{b}	0.08_{ab}	0.22	4.86_{cd}
60 °C pH 8.5N	0.96_{c}	0.85_{b}	0.06	0.15_{b}	0.30_{d}	0.17_{b}	0.05	1.32_{c}	0.99_{b}	0.10_{a}	0.26	5.20_{bc}
60 °C pH 9.5	1.50_{a}	0.08_{c}	nd	0.13_{bc}	0.44_{a}	nd	0.04	2.27_{a}	0.08_{c}	0.02_{de}	0.20	4.76_{d}
60 °C pH 9.5N	1.52_{a}	0.11_{c}	nd	0.14_{b}	0.44_{a}	0.03_{d}	0.05	2.34_{a}	0.15_{c}	0.04_{c}	0.22	5.04_{cd}
60 °C pH 10.5	0.97_{c}	0.03_{c}	nd	0.11_{c}	0.36_{c}	0.09_{c}	0.05	1.50_{b}	nd	nd	0.15	3.26_{f}
60 °C pH 10.5N	1.11_{b}	0.07_{c}	nd	0.13_{bc}	0.41_{b}	nd	0.05	2.25_{a}	nd	0.03_{cd}	0.18	4.23_{e}
LSD[f]	0.11	0.09	NDIFF	0.03	0.03	0.02	NDIFF	0.17	0.13	0.02	NDIFF	0.38
Isolate Fraction												
25 °C pH 8.5	0.84_{e}	2.40_{b}	0.07	0.46	0.31_{d}	0.49_{b}	0.10	1.52_{d}	3.19_{b}	0.12_{c}	0.52	10.02_{bc}
25 °C pH 8.5N	0.94_{d}	2.98_{a}	0.10	0.48	0.36_{d}	0.57_{a}	0.10	1.68_{d}	3.85_{a}	0.18_{ab}	0.55	11.78_{a}
60 °C pH 8.5	1.44_{c}	1.94_{c}	0.08	0.38	0.45_{c}	0.41_{c}	0.10	2.31_{c}	2.56_{d}	0.14_{bc}	0.58	10.38_{b}
60 °C pH 8.5N	1.52_{c}	2.23_{b}	0.11	0.39	0.47_{c}	0.45_{bc}	0.11	2.40_{c}	2.86_{c}	0.19_{a}	0.58	11.30_{a}
60 °C pH 9.5	2.97_{a}	0.25_{d}	nd	0.42	0.85_{b}	0.07_{d}	0.11	4.61_{ab}	0.40_{e}	0.04_{d}	0.65	10.37_{b}
60 °C pH 9.5N	2.97_{a}	0.32_{d}	nd	0.42	0.86_{b}	0.09_{d}	0.11	4.51_{b}	0.46_{e}	0.05_{d}	0.64	10.44_{b}
60 °C pH 10.5	2.68_{b}	0.26_{d}	nd	0.37	0.90_{a}	nd	0.12	4.82_{a}	0.03_{f}	0.02_{d}	0.56	9.77_{cd}
60 °C pH 10.5N	2.67_{b}	nd	nd	0.37	0.90_{a}	nd	0.13	4.64_{ab}	nd	0.03_{d}	0.54	9.29_{d}
LSD	0.09	0.17	NDIFF	NDIFF	0.03	0.06	NDIFF	0.30	0.25	0.04	NDIFF	0.56

[a]n=2.

[b]Values within a column with different subscript letters were significantly different at $P < 0.05$.

[c]Abbreviations: Din, daidzin; MDin, malonyldaidzin; AcDin, acetyldaidzin; Dein, daidzein; Glyin, glycitin; MGly, malonylglycitin; Glyein, glycitein; Gin, genistin; MGin, malonylgenistin; AcGin, acetylgenistin; Gein, genistein; nd, none detected; NDIFF, not different.

[d]Acetylglycitin was not detected for any samples and was not included in the table.

[e]N, neutralized at time of phytochemical extraction.

[f]LSD, least significant difference.

Table 3 (continued)

	Concentration (μmol/g, dry basis)											
Treatment[a]	Din[b,c]	MDin	AcDin	Dein	Glyin	MGly[d]	Glyein	Gin	MGin	AcGin	Gein	Total
Whey Fraction												
25 °C pH 8.5	1.15_{f}	2.46_{a}	0.03	0.20_{a}	0.45_{c}	0.44_{a}	0.06_{a}	0.93_{d}	2.46_{a}	0.05_{a}	0.15_{a}	7.47
25 °C pH 8.5N[e]	1.37_{ef}	1.41_{b}	0.07	0.23_{a}	0.46_{c}	0.26_{ab}	0.06_{a}	1.11_{cd}	1.41_{b}	0.05_{a}	0.17_{a}	6.41
60 °C pH 8.5	1.67_{de}	1.52_{b}	nd	0.06_{b}	0.56_{c}	0.29_{ab}	0.02_{b}	1.18_{cd}	1.52_{b}	0.04_{a}	0.04_{b}	6.12
60 °C pH 8.5N	1.89_{d}	0.88_{c}	0.06	0.07_{b}	0.56_{c}	0.18_{bc}	0.02_{b}	1.38_{c}	0.88_{c}	0.05_{a}	0.04_{b}	5.82
60 °C pH 9.5	3.12_{ab}	0.20_{d}	nd	0.07_{b}	0.96_{a}	0.03_{c}	0.02_{b}	2.51_{ab}	0.20_{d}	nd	0.06_{b}	7.09
60 °C pH 9.5N	3.47_{a}	0.04_{d}	nd	0.07_{b}	1.03_{a}	nd	0.02_{b}	2.80_{a}	0.04_{d}	nd	0.06_{b}	7.58
60 °C pH 10.5	2.46_{c}	0.12_{d}	nd	0.05_{b}	0.79_{b}	0.15_{bc}	nd	2.35_{b}	0.12_{d}	nd	0.04_{b}	5.95
60 °C pH 10.5N	2.87_{b}	0.01_{d}	0.01	0.06_{b}	0.91_{a}	0.01_{c}	0.01_{b}	2.69_{a}	0.01_{d}	0.02_{b}	0.04_{b}	6.65
LSD[f]	0.37	0.51	NDIFF	0.04	0.12	0.21	0.03	0.30	0.44	0.01	0.03	NDIFF

[a]n=2.

[b]Values within a column with different subscript letters were significantly different at $P< 0.05$.

[c]Abbreviations: Din, daidzin; MDin, malonyldaidzin; AcDin, acetyldaidzin; Dein, daidzein; Glyin, glycitin; MGly, malonylglycitin; Glyein, glycitein; Gin, genistin; MGin, malonylgenistin; AcGin, acetylgenistin; Gein, genistein; nd, none detected; NDIFF, not different.

[d]Acetylglycitin was not detected for any samples and was not included in the table.

[e]N, neutralized at time of phytochemical extraction.

[f]LSD, least significant difference.

Table 4 – Effect of protein solubilization temperature and pH on saponin distribution in process fractions from bench-scale soy protein isolate production

	Concentration (μmol/g, dry basis)						
Treatment[a]	V[b]	I	II	αg	βg	βa	Total
Insoluble Fraction							
25 °C pH 8.5	0.66	2.74_{d}	1.13	0.24	2.40_{a}	0.68_{a}	7.84_{a}
25 °C pH 8.5N[c]	0.46	2.68_{d}	1.16	0.24	2.24_{a}	0.66_{a}	7.43_{ab}
60 °C pH 8.5	0.91	3.17_{c}	1.37	0.15	1.39_{b}	0.38_{b}	7.36_{ab}
60 °C pH 8.5N	0.63	3.18_{c}	1.27	0.20	1.64_{b}	0.45_{b}	7.37_{ab}
60 °C pH 9.5	0.91	4.23_{a}	1.85	nd[d]	nd	nd	7.00_{abc}
60 °C pH 9.5N	0.76	3.93_{ab}	1.82	nd	0.10_{c}	nd	6.62_{bc}
60 °C pH 10.5	0.93	3.94_{ab}	1.77	nd	nd	nd	6.65_{bc}
60 °C pH 10.5N	0.80	3.68_{b}	1.84	nd	nd	nd	6.32_{bc}
LSD[e]	NDIFF[f]	0.42	NDIFF	NDIFF	0.54	0.17	0.87
Isolate Fraction							
25 °C pH 8.5	0.45_{de}	1.41_{f}	0.66_{e}	0.16_{b}	1.91_{b}	0.59_{b}	5.18_{d}
25 °C pH 8.5N	0.56_{cde}	2.43_{ef}	1.05_{de}	0.30_{a}	3.39_{a}	1.08_{a}	8.81_{bc}
60 °C pH 8.5	0.39_{e}	2.14_{ef}	0.94_{e}	0.10_{b}	0.98_{d}	0.35_{d}	4.90_{d}
60 °C pH 8.5N	0.63_{cde}	3.47_{de}	1.47_{cd}	0.13	1.62_{c}	0.54_{c}	7.87_{c}
60 °C pH 9.5	0.78_{bcd}	4.21_{cd}	1.93_{bc}	0.07_{b}	nd	nd	6.99_{cd}
60 °C pH 9.5N	1.13_{ab}	6.93_{ab}	2.89_{a}	nd	nd	nd	10.96_{ab}
60 °C pH 10.5	0.92_{bc}	5.43_{bc}	2.26_{b}	nd	nd	nd	8.61_{bc}
60 °C pH 10.5N	1.44_{a}	7.56_{a}	3.15_{a}	nd	nd	nd	12.15_{a}
LSD	0.37	1.60	0.47	0.12	0.19	0.04	2.37

[a]n=2.
[b]Values within a column with different subscript letters were significantly different at $P<0.05$.
[c]N, neutralized at time of phytochemical extraction.
[d]nd, none detected.
[e]LSD, least significant difference.
[f]NDIFF, not different.

absorbed faster and in greater quantities than are the glucoside forms in the gut. However, a study by Xu and others (2000) suggested that isoflavone bioavailability was not affected by isoflavone source or profile. Wang and Murphy (1996) published the first mass balance data for isoflavone partitioning and distribution during tempeh, soymilk and tofu, and soy protein isolate processing. Bean soaking prior to processing led to a 10% loss of isoflavones into the soak water. Aglycone concentration increased during soaking, probably due to the action of β-glucosidases. Cooking processes increased concentrations of acetylgenistin and acetyldaidzin. During soy protein isolate production, malonylglucosides were primarily converted to aglycones (Wang 1994). Isoflavone retention in the insoluble fraction during isolate production was about 50%, substantially higher than the 20-35% observed here.

Wang and others (1998) also evaluated the changes in isoflavones during soy protein isolate production. The primary profile change was increased aglycone content of the isolate compared to soy flakes. Singletary and others (2000) reported that the major isoflavone profile effect of extruding a mixture of corn and soy was the conversion of malonylglucosides to acetylglucosides. Grün and others (2001) reported the predominant isoflavone profile change during thermal processing of previously manufactured tofu was due to de-esterification of the malonylglucosides and acetylglucosides to the β-glucosides. All three of the preceding research groups did not prepare standard curves for malonyl- and acetylglucosides, but rather calculated these values from the corresponding glucoside forms by correcting for molecular weight differences. An additional observation by Grün and others (2001) was the decrease of aglycone isoflavones in the tofu during processing. They suggested that this was due to leaching or degradation of the aglycones. Degradation is unlikely under the relatively mild conditions described. Grün and others (2001) provided recovery data for genistein and genistin, but no recovery data was provided by Wang and others (1998) or Singletary and others (2000).

In addition to phytochemical partitioning and profile distribution, there was interest in treatment effects and interactions on phytochemical concentrations for the process fractions. Analysis of variance (ANOVA) results revealed that isoflavone concentration of insoluble

fractions were significantly affected by treatment pH and by neutralization of the samples prior to phytochemical extraction. ANOVA tables have been included in Appendix A. There was also significant interaction between pH and neutralization. Least square means are shown for both treatment temperatures (Table 5), but since the experiment was not balanced, the significance of the pH by neutralization interaction is shown only for the 60 °C treatments. The interaction was significant because neutralized and non-neutralized samples tested significantly different at pH 10.5, but not when the pH was 8.5 or 9.5. When neutralizing the insoluble fraction, the pH was decreased from 8.5, 9.5, or 10.5 to 7.0. Apparent pK_a values for the aglycone isoflavone 4' hydoxyls are 9.50, 9.55, and 9.73 for genistein, daidzein, and glycitein, respectively (McLeod and Shepherd 2000). Krol (2000) reported that the pK_a of the 4' hydroxyl for soy isoflavones was 9.9, but 9.2 for the 5 hydroxyl. Krol also reported that the pK_a of the 7 hydroxyl of the aglycones was 7.6, the pK_a of the β-glucoside was about 11, and the pK_a of the malonyl carboxyl was about 5.7. It is possible that the ionization of the isoflavones at high pH caused an increased ionic interaction between the isoflavones and the protein matrix. This interaction may have diminished as the charges on the isoflavones were altered at neutral pH.

None of the least square means (LSM) analyses for isoflavone concentrations of the whey fractions was significant, but pH, neutralization, the temperature by neutralization interaction, and the pH by neutralization interaction were all significant for the isolate. The pH by neutralization interaction tested significant because when the pH was 8.5 the LSM isoflavone concentration value was 0.92 µmol/g higher than not neutralized, but when the pH was 10.5 the neutralized value was 0.48 µmol/g less than the not neutralized (Table 6). For the soy protein isolate, the sample slurry pH was adjusted from pH 4.5, the pH at which the isolate was precipitated during processing, to 7.0 for phytochemical extraction. For the pH 8.5 treatments at 25 and 60 °C, there was still a relatively high concentration of malonylglucosides in the isolate. At pH 10.5 and 60 °C, however, the concentration of malonylglucosides in the isolate was low. The malonyl carboxyl group of the malonylglucosides has a pK_a of 5.7. These facts may explain why neutralizing the isolate

Table 5 - Least square means for isoflavone concentrations of the insoluble fractions from bench-scale soy protein isolate production

	pH 8.5[a,b]	pH 8.5 N[c]	pH 9.5	pH 9.5 N	pH 10.5	pH 10.5 N
25 °C	5.56	5.63				
60 °C	4.86_{ac}	5.20_{c}	4.76_{ac}	5.04_{c}	3.26_{b}	4.23_{a}

[a]Values are dry basis concentrations in µmol/g.
[b]Subscript, lowercase letters that are different within a row indicate significant differences ($P<0.05$) between least square means for pH by neutralization interaction. Because 60 °C was the only temperature at which every level of pH and neutralization were tested, significance was shown only for the 60 °C treatments.
[c]N, neutralized at time of phytochemical extraction.

Table 6 - Least square means for isoflavone concentrations of soy protein isolates produced by a bench-scale process

	pH 8.5[a,b]	pH 8.5 N[c]	pH 9.5	pH 9.5 N	pH 10.5	pH 10.5 N
25 °C	10.03_{bd}	11.78_{a}				
60 °C	10.38_{de}	11.30_{ae}	10.37_{de}	10.44_{de}	9.77_{cd}	9.29_{bc}

[a]Values are dry basis concentrations in µmol/g.
[b]Subscript, lowercase letters that are different within the table indicate significant differences ($P<0.05$) between least square means for pH by neutralization and temperature by neutralization interactions.
[c]N, neutralized at time of phytochemical extraction.

slurry prior to phytochemical extraction improved isoflavone recovery for the pH 8.5 treatments.

For the total isoflavone concentrations of the soy protein isolates, the temperature by neutralization interaction tested significant. This was because at 25 °C the LSM for the neutralized sample was 1.75 μmol/g larger than the not neutralized sample and they were significantly different, but when the temperature was 60 °C, the difference between neutralized and not neutralized treatments was only 0.92 μmol/g and they were no longer significantly different. This may again have been influenced by the concentration of malonylglucosides in the soy protein isolate. Temperature had an effect on malonylglucoside conversions, but temperature did not influence conversion as aggressively as did the pH at which the protein solubilization occurred.

The effect of processing on soy saponins has received much less attention than has the isoflavones, due in part to the difficulty to purify standards and efficiently quantify the saponins. Hu and others (2002) described methods to address these difficulties for several group B saponins: V, I, II, αg, βg, and βa. The concentration of these six saponins in the starting material was 7.0 μmol/g, similar to the 6.32-7.84 μmol/g concentrations of the insoluble fraction. The saponin concentration of the isolate, as discussed for the mass balance above, was dramatically affected by neutralization. The saponin profiles in the insoluble and isolate fractions were quite different across treatments and from the starting material. Table 7 shows the saponin profile of the starting material. For both the insolubles and soy protein isolate, the conversion of the group B DDMP forms αg, βg, and βa to V, I, and II, respectively, was dramatic and eventually complete as temperature and pH increased, demonstrating how labile the DDMP saponins were (Table 4).

As described above, group A saponins A_1-A_6 lose a labile sugar group at temperatures above 80 °C and a pH of at least 9. These modified group A saponins have been reported to elute at the same retention time as group B saponins V, III, or IV, with modified A_1 and A_4 eluting with saponin V. For the insoluble fraction, there was no significant difference in saponin V concentrations among treatments (Table 4). Soy protein isolates that were

Table 7 – Saponin distribution in material from soy slurry production

	Concentration (μmol/g, dry basis)						
Treatment[a]	V[b]	I	II	αg	βg	βa	Total
Ground Flakes	0.44	1.34_{i}	0.58_{h}	0.27_{a}	3.35_{a}	0.99_{a}	6.97
25 °C pH 8.5	0.31	2.05_{gh}	0.82_{gh}	0.24_{a}	2.81_{ab}	0.80_{ab}	7.04
25 °C pH 8.5N[c]	0.35	1.78_{hi}	0.66_{gh}	0.23_{ab}	2.51_{bc}	0.75_{bc}	6.29
25 °C pH 9.5	0.46	2.42_{efgh}	0.95_{fgh}	0.17_{bc}	2.05_{cd}	0.57_{cd}	6.62
25 °C pH 9.5N	0.47	2.24_{fgh}	0.99_{efgh}	0.15_{c}	1.71_{de}	0.53_{de}	6.10
25 °C pH 10.5	0.71	3.57_{cd}	1.52_{bcd}	0.04_{e}	0.35_{g}	0.14_{f}	6.34
25 °C pH 10.5N	0.65	2.78_{ef}	1.48_{cde}	0.09_{de}	1.00_{f}	0.34_{e}	6.35
60 °C pH 8.5	0.68	2.97_{de}	1.40_{cdef}	0.13_{cd}	1.25_{ef}	0.37_{e}	6.80
60 °C pH 8.5N	0.70	2.58_{efg}	1.13_{defg}	0.13_{cd}	1.43_{ef}	0.44_{de}	6.43
60 °C pH 9.5	0.99	4.34_{ab}	1.99_{ab}	nd[d]	nd	nd	7.33
60 °C pH 9.5N	0.85	4.14_{bc}	1.78_{abc}	nd	0.04_{g}	nd	6.81
60 °C pH 10.5	0.91	4.91_{a}	2.07_{a}	nd	nd	nd	7.89
60 °C pH 10.5N	0.54	4.20_{bc}	1.68_{abc}	nd	nd	nd	6.42
LSD[e]	NDIFF[f]	0.69	0.50	0.06	0.58	0.20	NDIFF

[a]n=2.
[b]Values within a column with different subscript letters were significantly different at $P<0.05$.
[c]N, neutralized at time of phytochemical extraction.
[d]nd, none detected.
[e]LSD, least significant difference.
[f]NDIFF, not different.

neutralized prior to phytochemical extraction showed that saponin V concentrations for the pH 9.5, 60 °C and pH 10.5, 60 °C treatments were significantly higher than pH 8.5 treatments at either 25 or 60 °C. Saponin αg converts to saponin V upon loss of the DDMP moiety. If saponin V and saponin αg concentrations are summed for the neutralized soy protein isolate samples, the range in combined concentrations was 0.61 to 1.44 μmol/g from the pH 8.5, 25 °C to the pH 10.5, 60 °C treatment. However, the total saponin concentrations corresponding to these two treatments were 8.81 and 12.15 μmol/g, respectively. When the combined saponin V and αg concentrations, 0.61 and 1.44 μmol/g from above, were expressed on a percentage basis relative to the total saponin concentrations for those samples, the results were 9.8 and 11.9%. Consequently, if saponins A_4 and A_1 eluted at exactly the same retention time as saponin V, their influence on the total saponin concentration was small. It is possible that our extraction conditions were too mild, 60 °C as opposed to >80 °C, for this conversion to be realized and measured.

Modified group A saponins A_5 and A_2, and A_6 and A_3 may elute at the same retention time as group B saponins III and IV. Our method does not quantify these group B saponins. A very small peak, however, was observed for neutralized samples from the insoluble and isolate fractions of 60 °C, pH 8.5, 9.5, and 10.5 treatments during a retention time region in which saponins III or IV may elute. Whether this peak was group A saponins or just conversion of group B DDMP saponins γg or γa to group B non-DDMP saponins III and IV, respectively, was difficult to determine.

The LSM for total saponin concentrations of the insoluble and isolate fractions were statistically analyzed for the effect of treatments and interactions. For the insoluble fraction, only pH tested significant. These least square means are shown in Table 8. For the isolate, pH and neutralization both tested significant. LSM are shown in Tables 9a and 9b. The protein matrix seemed to have a considerable effect on saponin extraction. A small body of evidence suggests that there is a definite interaction of saponins with glycinin and β-conglycinin proteins, the major storage proteins in soybeans, through hydrophobic interaction, hydrogen bonding, or ionic bonding (Shimoyamada and others 1998).

Table 8 - Least square means of saponin concentrations of the insoluble fractions from soy protein isolate production for various levels of pH and temperature

	pH 8.5[a,b]	pH 9.5	pH 10.5
25 °C	7.63		
60 °C	7.37_a	6.80_{ab}	6.49_b

[a]Values are dry basis concentrations in µmol/g.
[b]Subscript lowercase letters that are different within a row indicate significant differences ($P<0.05$) between least square means for pH. Because 60 °C was the only temperature at which each pH was evaluated, significance was shown only for the 60 °C means.

Table 9a - Least square means for saponin concentrations of the soy protein isolate fractions for various levels of pH and temperature

	pH 8.5[a,b]	pH 9.5	pH 10.5
25 °C	7.00		
60 °C	6.38_b	8.98_a	10.38_a

[a]Values are dry basis concentrations in µmol/g.
[b]Subscript, lowercase letters that are different within a row indicate significant differences ($P<0.05$) between least square means for pH. Because 60 °C was the only temperature at which each pH was evaluated, significance was shown only for the 60 °C means.

Table 9b - Least square means for saponin concentrations of the soy protein isolate fractions for neutralization and temperature

	Not Neutralized[a,b]	Neutralized
25 °C		
60 °C	6.83_b	10.33_a

[a]Values are dry basis concentrations in µmol/g.
[b]Subscript, lowercase letters that are different within a row indicate significant difference ($P<0.05$) between least square means for neutralization treatments. Means for the 25 °C treatments were not calculated.

Effect of Protein Solubilization Conditions and Sample Neutralization on Soy Slurry

The soy slurry or soy protein solubilization experiment was designed to eliminate mass balance discrepancies caused by fractionating soy flakes into insoluble, isolate, and whey fractions, each with potentially different protein matrix characteristics. The soy slurry study utilized a similar set of protein solubilization treatment conditions, but there was no subsequent separation of the soluble and insoluble components. This provided a uniform matrix to evaluate for each treatment condition. The soy slurry experiment was also balanced for pH (8.5, 9.5, and 10.5) and temperature treatments (25 and 60 °C), whereas the soy protein isolate experiment did not include all pH treatments at 25 °C. As done for the soy protein isolate experiment, phytochemical extraction of samples from each treatment was performed with and without neutralization.

Table 7 shows that at 25 °C the extent of conversion of DDMP saponins (αg, βg, and βa) to non-DDMP saponins (V, I, and II) increased with increasing pH. However, increasing the protein solubilization temperature from 25 to 60 °C at pH 8.5 had as much effect on this DDMP conversion as increasing pH from 8.5 to 10.5 at 25 °C, suggesting that temperature had a more profound effect on the extent of conversion. The saponin V concentrations were not significantly different across treatments (Table 7). When saponin V and αg were summed, differences among these sums were even smaller than differences among saponin V concentrations. This suggests that if any group A saponins were eluting at the saponin V retention time, the concentrations were extremely low.

Statistical analysis using least square means for treatment effects and treatment interactions on saponin concentrations showed that only neutralization produced a significant difference ($P<0.05$) across treatment conditions. LSM were 6.40 and 7.00 for neutralized and not neutralized treatments, respectively.

For the soy slurry experiment, extraction of isoflavones from the dried slurry material declined as the protein solubilization pH increased (Table 10). As seen for the insoluble fraction of the soy protein isolate experiment (Table 3), isoflavone extraction was enhanced by neutralizing the samples prior to extraction. Again, the pKa and ionization of the

Table 10 - Isoflavone distribution in material from soy slurry production

	Concentration (µmol/g, dry basis)											
Treatment[a]	Din[b,c]	MDin	AcDin	Dein	Glyin	MGly[d]	Glyein	Gin	MGin	AcGin	Gein	Total
Ground Flakes	1.04_{e}	2.40_{a}	0.09	0.16	0.41_{e}	0.48_{a}	0.05	1.27_{e}	2.57_{a}	0.13_{a}	0.17	8.77_{a}
25 °C pH 8.5	1.09_{e}	2.17_{bc}	nd	0.16	0.37_{e}	0.40_{bc}	0.05	1.37_{e}	2.27_{b}	0.09_{d}	0.18	8.15_{abc}
25 °C pH 8.5N[e]	1.09_{e}	2.21_{ab}	0.07	0.18	0.39_{e}	0.42_{b}	0.05	1.37_{e}	2.35_{ab}	0.10_{c}	0.20	8.43_{ab}
25 °C pH 9.5	1.34_{cd}	1.78_{d}	nd	0.15	0.42_{de}	0.32_{d}	0.04	1.74_{d}	1.91_{c}	0.07_{e}	0.17	7.95_{bcd}
25 °C pH 9.5N	1.16_{cde}	1.99_{c}	0.06	0.17	0.38_{e}	0.36_{cd}	0.05	1.48_{e}	2.18_{b}	0.09_{d}	0.20	8.14_{abc}
25 °C pH 10.5	1.97_{b}	0.85_{f}	nd	0.15	0.56_{bc}	0.18_{f}	0.04	2.56_{c}	0.92_{e}	0.04_{f}	0.17	7.45_{cde}
25 °C pH 10.5N	1.76_{b}	1.22_{e}	0.03	0.16	0.52_{cd}	0.24_{e}	0.05	2.34_{c}	1.42_{d}	0.07_{e}	0.18	8.01_{bcd}
60 °C pH 8.5	1.41_{c}	1.67_{d}	nd	0.24	0.43_{de}	0.32_{d}	0.07	1.75_{d}	1.78_{c}	0.09_{d}	0.36	8.12_{abc}
60 °C pH 8.5N	1.27_{cde}	1.73_{d}	0.07	0.24	0.40_{e}	0.33_{d}	0.07	1.53_{de}	1.91_{c}	0.11_{b}	0.37	8.06_{abcd}
60 °C pH 9.5	2.32_{a}	0.17_{gh}	nd	0.24	0.69_{a}	0.05_{g}	0.07	3.04_{b}	0.22_{fg}	0.04_{f}	0.35	7.18_{e}
60 °C pH 9.5N	2.40_{a}	0.26_{g}	nd	0.25	0.68_{a}	0.07_{g}	0.07	3.29_{b}	0.34_{f}	0.03_{g}	0.38	7.80_{bcde}
60 °C pH 10.5	1.92_{b}	0.04_{h}	nd	0.18	0.62_{ab}	nd	0.06	2.52_{c}	0.02_{g}	0.03_{g}	0.26	5.64_{f}
60 °C pH 10.5N	2.25_{a}	0.10_{gh}	nd	0.20	0.70_{a}	0.10	0.06	3.66_{a}	0.02_{g}	0.03_{g}	0.29	7.36_{de}
LSD[f]	0.25	0.21	NDIFF	NDIFF	0.10	0.05	NDIFF	0.37	0.24	0.01	NDIFF	0.73

[a]n=2.

[b]Values within a column with different subscript letters were significantly different at $P<0.05$.

[c]Abbreviations: Din, daidzin; MDin, malonyldaidzin; AcDin, acetyldaidzin; Dein, daidzein; Glyin, glycitin; MGly, malonylglycitin; Glyein, glycitein; Gin, genistin; MGin, malonylgenistin; AcGin, acetylgenistin; Gein, genistein; nd, none detected; NDIFF, not different.

[d]Acetylglycitin was not detected in any of the samples.

[e]N, neutralized at time of phytochemical extraction.

[f]LSD, least significant difference

isoflavones may affect their extraction at high pH. The isoflavone profile suggests that one or more of the glucoside forms were being hydrolyzed to the aglycone forms, but this conversion was small compared to conversion of malonylglucosides to their respective β-glucosides. The conversion to β-glucosides seemed to be affected primarily by increased pH rather than increased temperature. For instance, the concentration of genistin for the 25 °C, pH 8.5 treatment was 1.37 μmol/g, whereas that of the 25 °C, pH 10.5 and 60 °C, pH 8.5 treatments were 2.56 and 1.75 μmol/g , respectively. Analysis of least square means for total isoflavones indicated that temperature, pH, and neutralization, as well as temperature by pH and neutralization by pH were all significant. Tables 11a and 11b display least square means comparisons for the pH by temperature interaction and pH by neutralization interaction, respectively. The pH by neutralization interaction was significant because the difference between means for neutralized samples with respect to not neutralized samples were significantly different at pH 10.5. The temperature by pH interaction was significant because the least square means for treatments at 25 °C were significantly different than the means at 60 °C when the pH was 10.5, but were not different when the pH was 8.5 or 9.5.

Conclusions

Increased temperature and pH increased protein solubilization from the starting material and increased fraction weight and protein recovery in the isolate fraction during soy protein isolate processing. This relationship was also observed for isoflavones and saponins. The soy slurry experiment showed that changes with respect to saponin profile occurred by conversion of malonylglucosides to β-glucosides as temperature and pH were increased, although the effect of pH was more dramatic on this conversion. Conversely, the conversion of DDMP saponins to non-DDMP saponins was affected more by increased temperature than increased pH. The pH of the solvent system used to extract soy isoflavones and saponins should be taken into consideration when trying to optimize extraction of isoflavones and saponins from analytical samples. This was especially true for saponin recovery from soy protein isolate that had been precipitated at pH 4.5 but not neutralized prior to drying and for

Table 11a - Least square means of isoflavone concentrations of soy slurry materials for various levels of pH and temperature for evaluation of pH by temperature interaction

	pH 8.5[a,b]	pH 9.5	pH 10.5
25 °C	8.29_a	8.05_a	7.73_a
60 °C	8.08_a	7.49_a	6.50_b

[a]Values are dry basis concentrations in µmol/g.
[b]Subscript, lowercase letters that are different within the table indicate significant differences between least square means at $P<0.05$.

Table 11b - Least square means of isoflavone concentrations of soy slurry materials for various levels of pH and neutralization for evaluation of pH by neutralization interaction

	pH 8.5[a,b]	pH 9.5	pH 10.5
Neutralized	8.24_a	7.97_a	7.68_a
Not Neutralized	8.13_a	7.57_a	6.55_b

[a]Values are dry basis concentrations in µmol/g.
[b]Subscript, lowercase letters that are different within the table indicate significant differences between least square means at $P<0.05$.

isoflavones in soy samples with a pH near 10.5, where isoflavones are ionized and can interact more aggressively with the sample matrix. If group A saponins were modified during the higher temperature and pH treatments and did elute with saponin V, their effect on total group B saponin concentrations was negligible.

Acknowledgments

This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project N. 6571, was supported in part by USDA NRI grant No. 2001-35503-10814, NASA Food Technology Commercial Space Center, and by Hatch Act and State of Iowa funds.

References

AOAC. 1990. Official Methods of Analysis, 15th ed. Method 990.03. Arlington, VA: Association of Official Analytical Chemists.

Berk Z. 1992. Isolated soybean protein. In: Berk Z., editor. Technology of production of edible flours and protein products from soybeans. Haifa, Israel:Technicon, Israel Institute of Technology. p 83-96.

Farmakalidis E, Murphy PA. 1985. Isolation of 6"-O-acetylgenistin and 6"-O-acetyl daidzin from toasted defatted soy flakes. J Agric Food Chem 33:385.

Franke AA, Hankin JH, Yu MC, Maskarinec G, Low SH, Custer LJ. 1999. Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. J Agric Food Chem 47:977-986.

Grün IU, Adhikari K, Li C, Li Y, Zhang J, Ferenando LN. 2001. Changes in the profile of genistein, daidzein, and their conjugates during thermal processing of tofu. J Agric Food Chem 49:2839-2843.

Gu L, Tao G, Gu W, Prior RL. 2002. Determination of soyasaponins in soy with LC-MS following structural unification by partial alkaline degradation. J Agric Food Chem 50:6951-6959.

Hu J, Lee SO, Hendrich S, Murphy PA. 2002. Quantification of the Group B saponins by high performance liquid chromatography. J Agric Food Chem 50:2587-2594.

Ireland PA, Dziedzic SZ, Kearsley MW. 1986. Saponin content of soya and some commercial soya products by means of high-performance liquid chromatography of the sapogenins. J Sci Food Agric 37:694-698.

Izumi T, Piskula MK, Osawa S, Obata A, Tobe K, Saito M, Kataoka S, Kubota Y, Kikuchi M. 2000. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. J Nutr 130:1695-1699.

Jung S, Rickert DA, Deak NA, Aldin ED, Recknor J, Johnson LA, Murphy PA. 2003. Comparison of Kjeldahl and Dumas nitrogen methods for protein content determination in soy products. J Am Oil Chem Soc In press.

Kitagawa I, Yoshikawa M, Hayashi T, Taniyama T. 1984. Quantitative determination of soyasaponins in soybeans of various origins and soybean products by means of high performance liquid chromatography. Yakugaku Zasshi 104:275-279.

Koratkar R, Rao AV. 1997. Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions in the colon of mice. Nutr Cancer 27:206-209.

Krol J. 2000. Waters Corp. Personal communication.

Mahungu SM, Diaz-Mercado S, Li J, Schwenk M, Singletary K, Faller J. 1999. Stability of isoflavones during extrusion processing of corn/soy mixture. J Agric Food Chem 47:279-284.

Matsuura M, Obata A. 1993. β-Glucosidases from soybeans hydrolyze daidzin and genistin. J Food Sci 58:144-147.

McLeod GS, Shepherd MJ. 2000. Determination of the ionization constants of isoflavones by capillary electrophoresis. Phytochem Anal 11:322-326.

Meredith T. 2002. Solbar Plant Extracts, Ashdod, Israel. Personal communication.

Messina M. 1999. Soy, soy phytoestrogens (isoflavones), and breast cancer. Am J Clin Nutr 70:574-575.

Messina M, Bennink M. 1998. Soyfoods, isoflavones and risk of colon cancer: a review of the *in vitro* and *in vivo* data. Baillieres Clin Endocrinol Metab 12:707-728.

Murphy PA, Hendrich S. 2002. Phytoestrogens in foods. Advances in food and nutrition research 44:195-246.

Murphy PA, Song TT, Buseman G, Barua K. 1997. Isoflavones in soy-based infant formulas. J Agric Food Chem 45:4635-4638.

Murphy PA, Song TT, Buseman G, Barua K, Beecher GR, Trainer D, Holden J. 1999. Isoflavones in retail and institutional soy foods. J Agric Food Chem 47:2697-2704.

Oakenfull D, Sidhu G. 1990. Could saponins be a useful treatment for hypercholesterolaemia? Eur J Clin Nutr 44:79-88.

Setchell KDR, Cassidy A. 1999. Dietary isoflavones: biological effects and relevance to human health. J Nutr 129:758S-767S.

Shimoyamada M, Ikedo S, Ootsubo R, Watanabe K. 1998. Effects of soybean saponins on chymotryptic hydrolyses of soybean proteins. J. Agric. Food Chem. 46:4793-4797.

Shimoyamada M, Kudo S, Okubo K. 1990. Distributions of saponin constituents in some varieties of soybean plant. Agric. Biol. Chem. 54:77-81.

Shiraiwa M, Harada K, Okubo K. 1991. Composition and content of saponins in soybean seed according to variety, cultivation year and maturity. Agric Biol Chem 55:323-331.

Simonne AH, Smith M, Weaver DB, Vail T, Barnes S, Wei CI. 2000. Retention and changes of soy isoflavones and carotenoids in immature soybean seeds (edamame) during processing. J Agric Food Chem 48:6061-6069.

Singletary K, Faller J, Li JY, Mahungu S. 2000. Effect of extrusion on isoflavone content and antiproliferative bioactivity of soy/corn mixtures. J Agric Food Chem 48:3566-3571.

Sung M, Kendall CWC, Koo MM, Rao AV. 1995. Effect of soybean saponins and gypsophilla saponin on growth and viability of colon carcinoma cells in culture.

Nutr Cancer 23:259-270.
Tsukomoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K. 1995. Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem 43:1184-1192.
Wang C, Ma Q, Pagadala S, Sherrard MS, Krishnan PG. 1998. Changes of isoflavones during processing of soy protein isolates. J Am Oil Chem Soc 75:337-341.
Wang HJ. 1994. Quantitation of potentially anticarcinogenic isoflavones in soy foods and soybeans and the effect of processing on the composition of isoflavones. [Dphil dissertation]. Ames, IA:Iowa State University. 124 p.
Wang HJ, Murphy PA. 1994a. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J Agric Food Chem 42:1674-1677.
Wang HJ, Murphy PA. 1994b. Isoflavone content in commercial soybean foods. J Agric Food Chem 42:1666-1673.
Wang HJ, Murphy PA. 1996. Mass balance study of isoflavones during soybean processing. J Agric Food Chem 44:2377-2383.
Wu S, Murphy PA, Johnson LA, Fratzke AR, Reuber MA. 1999. Pilot-plant fractionation of soybean glycinin and β-conglycinin. J Am Oil Chem Soc 76:285-293.
Wu S, Murphy PA, Johnson LA, Reuber MA, Fratzke AR. 2000. Simplified process for soybean glycinin and β-conglycinin fractionation. J Agric Food Chem 48:2702-2708.
Xu X, Harris K, Wang HJ, Murphy P, Hendrich S. 1995. Bioavailability of soybean isoflavones depends upon gut microflora in women. J Nutr 125:2307-2315.
Xu X, Wang WJ, Murphy PA, Hendrich S. 2000. Neither background diet nor type of soy food affects short-term isoflavone bioavailability in women. J Nutr 130:798-801.

CHAPTER 4. PILOT-SCALE FRACTIONATION OF GLYCININ AND β-CONGLYCININ: PROCESS IMPROVEMENT

A paper to be submitted to the *Journal of Agricultural and Food Chemistry*

David A. Rickert, Lawrence A. Johnson, Patricia A. Murphy

Abstract

Extraction pH from 7.5 to 9.0, ethanol concentration of 0 to 10% (v/v), water-to-flake ratio of 6:1 to 15:1, and temperature from 25 to 65 °C were investigated for their abilities to improve protein yield and phytochemical recovery from 80.0 g of defatted soy white flakes during bench-scale soybean glycinin and β-conglycinin fractionation, with improved process conditions to be employed for pilot-scale fractionation. Extraction parameters of 45 °C and 10:1 water-to-flake ratio were considered optimum conditions. A pilot-scale comparison using 20 kg of starting material was conducted for the optimized method and the modified Nagano method, used previously by our research group for protein fractionation and wherein extraction conditions of 20 °C and 15:1 water-to-flake ratio were employed. The optimized method yielded significantly more β-conglycinin (2.10 vs. 1.29 kg), lower β-conglycinin purity (67.8 vs. 79.1%), higher glycinin purity (89.9 vs. 85.3%), more isoflavones in the protein extract (63.3 vs. 48.6 mmol), higher isoflavone concentration (0.67 vs. 0.31 μmol/g) and saponin concentration (3.66 vs. 1.13 μmol/g) in the β-conglycinin, lower saponin concentration in the glycinin precipitate (0.57 vs. 1.17 μmol/g), and lower total saponin (13.5 vs. 17.0 mmol) in an intermediate protein fraction containing both glycinin and β-conglycinin.

Introduction

Glycinin and β-conglycinin are the two major storage proteins in soybean seeds. There have been substantial efforts to understand the amino acid profile, subunit composition of the native-state proteins, and especially the physicochemical characteristics of these proteins as

they affect protein functionality (1, 2). Consumption of soy proteins and associated phytochemicals, such as isoflavones and saponins, may provide health benefits, such as improved cardiovascular health as part of a low-fat diet, anti-osteoporotic effects, and anti-carcinogenic activity (3). Research efforts to define the health impact of each of these soy components continue.

Bench-scale soy protein fractionation has been utilized to produce enriched glycinin and β-conglycinin fractions for protein characterization purposes (4-6). Pilot-scale fractionation processes for producing glycinin and β-conglycinin fractions, as well as mixtures of these proteins, have been patented (7-9). Wu et al. (10-11) developed two pilot-scale methods for soy protein fractionation using defatted soy white flakes as the starting material. One method was the scale-up of a modified bench-scale process, termed the modified Nagano method, yielding an intermediate fraction comprised of a mixture of glycinin and β-conglycinin, a glycinin-rich fraction, and a β-conglycinin-rich fraction. Sequential protein fractionation was controlled by pH and ionic strength adjustment of solubilized protein extracted from defatted soy white flakes. The other method, referred to as a simplified method, yielded only glycinin and β-conglycinin fractions. This method produced a larger β-conglycinin fraction, but at the expense of purity.

The protein solubilization temperature utilized for both of the methods of Wu et al. (10, 11) was 20 °C, much lower than temperatures of up to 80 °C that are employed for soy protein isolate production (12). The 20 °C was used to maximize the amount of native state storage proteins, which have been hypothesized to fractionate more efficiently (11). Soy protein isolate production, similar to Wu's modified-Nagano fractionation process, is accomplished by extraction of protein from defatted soy flakes under alkaline conditions, followed by isoelectric precipitation. Dry-basis mass recovery has typically been about 33% for isolate produced at the Center for Crops Utilization Research (CCUR) pilot plant at Iowa State University. Wu reported mass recoveries of only 24 and 29% for the three-fraction process and simplified process, respectively.

Isoflavone content and profile of several soy foods (13, 14) and information about the fate of isoflavones during soy protein isolate processing (15, 16) have been reported. Effect of processing on isoflavone profile may be important because glucoside forms require enzymatic modification in the gut to the aglucon forms for absorption in humans (17). Saponin contents of soybeans, soy foods, and soy ingredients have also been reported (18-21). However, partitioning of saponins during soy protein fractionation and soy protein isolate production, as well as mass balance and profile data, has not been reported. Extraction and partitioning differences between isoflavones and saponins have not been described for these processes.

The primary objective of the current research was to improve pilot-scale protein solubilization from the defatted soy flake starting material without decreasing mass and protein recovery in the glycinin and β-conglycinin fractions or substantially decreasing purity of these fractions. A second objective was to enhance isoflavone and saponin recovery from the starting material in order to potentially increase their recovery in product fractions. An inspection of phytochemical partitioning among the process fractions was also desired. These objectives were first addressed through bench-scale experimentation. A range of pH values (7.5-9.0), ethanol concentrations (0-10%), temperatures (25-65 °C), and water-to-flake ratios (6:1 to 15:1) were each evaluated for their effect on protein solubilization, and for some conditions, protein and phytochemical partitioning. Conditions that offered process improvement were combined to develop an optimized process. Pilot-scale fractionation was then conducted to compare the optimized process with the Wu modified Nagano process (10). Protein and phytochemical partitioning observed for the two protein fractionation processes were also compared to protein and phytochemical partitioning observed for the pilot-scale soy protein isolate process.

Materials and Methods

Materials. Defatted soy white flakes for bench-scale and pilot-scale work were from Cargill (Minneapolis, MN) and the Research Oil Mill at Texas A&M University,

respectively. Texas A&M flakes were year 2000 harvest, IA 2042 variety, and had an 84% protein dispersibility index. Flakes were stored in sealed containers at 4 °C until use. HPLC-grade acetonitrile, methanol, trifluoroacetic acid, and glacial acetic acid for HPLC mobile phase and sample preparation were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade water for mobile phase and sample preparation was produced using a Milli-Q water system (Millipore Co., Bedford, MA).

Effect of pH on Bench-Scale Protein Solubilization. Soy slurry was prepared at ambient temperature by combining water and 80.0 g defatted soy white flakes (15:1 ratio) and stirring as rapidly as possible with a magnetic stir bar. Slurry was adjusted to pH 7.5, 8.0, 8.5, or 9.0 using 2N NaOH, maintained at that pH, and stirred for 1 h. Large particulate insolubles were removed using a 60-mesh screen, whereas smaller insolubles were removed by centrifugation at 14,000 x *g* for 30 min at 20 °C. Supernatant protein concentration was determined by the biuret method (22) with bovine serum albumen as the standard (Fraction V, Sigma Chemical Co., St. Louis, MO). The product of the protein concentration and supernatant volume was considered the mass of protein solubilized and recovered.

Effect of Ethanol on Bench-Scale Protein Solubilization and Fractionation. Bench-scale fractionation for this study (Figure 1), as well as for the water-to-flake and temperature studies below, was a modification from Wu et al. (10), where 50 g of flakes, a 120-mesh stainless-steel screen for removal of large insolubles, and a 30-min protein solubilization period were used.

Soy slurry was prepared at ambient temperature by combining water, containing ethanol concentrations of 0, 0.1, 1.0, or 10%, and 80.0 g defatted soy flakes (15:1 ratio) and stirring as rapidly as possible with a magnetic stir bar. Slurry was adjusted to pH 8.5 using 2N NaOH, maintained at that pH, and stirred for 1 h. Insolubles were removed by screening and centrifugation as for the pH study. Sodium bisulfite was added to the supernatant to produce a 10 mM SO_2 concentration, and the pH was adjusted to 6.4 using 2N HCl. The protein dispersion was chilled overnight at 4 °C. The glycinin-rich precipitate was recovered by centrifugation at 7500 x *g* for 20 min at 4 °C. The supernatant was adjusted to 0.25 M NaCl

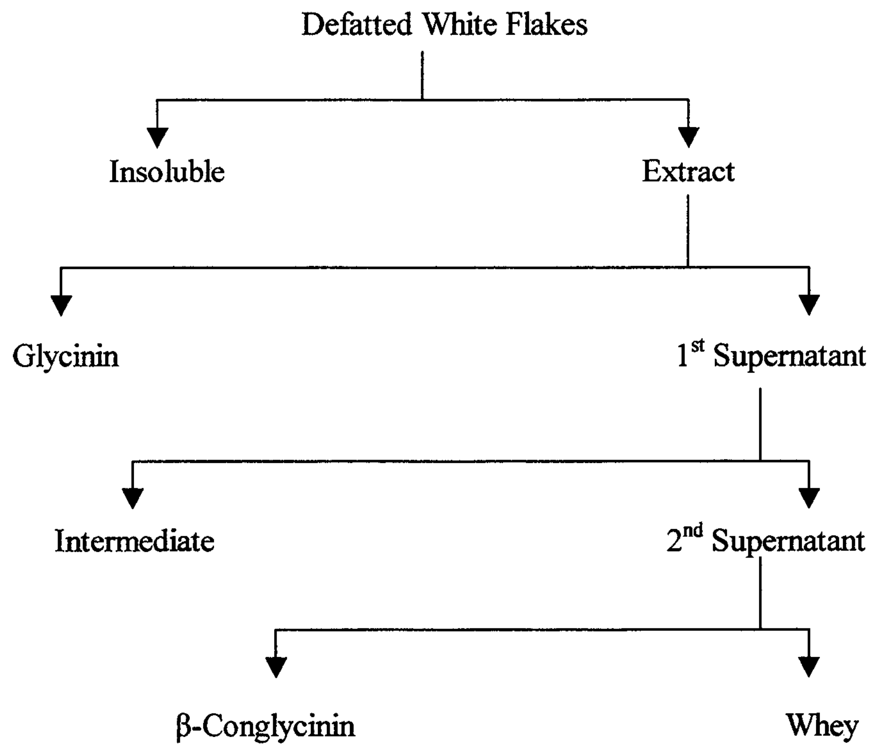


Figure 1. Soy protein fractionation process.

and pH 5.0, stirred for 1 h, and then centrifuged at 14300 x *g* for 30 min at 4 °C to recover the intermediate protein fraction, which contained glycinin, β-conglycinin, and other proteins. Water was added 2:1 to the resulting supernatant and the pH was adjusted to 4.8. The β-conglycinin-rich precipitate was removed from the whey fraction by centrifugation at 7500 x *g* for 20 min at 4 °C. The insoluble fraction and fractionated proteins were weighed, and then freeze dried. Freeze-dried weight was regarded as the dry weight and used in calculating solids content.

Effect of Water-to-Flake Ratio on Bench-Scale Protein Extraction and Fractionation. Soy slurry was prepared at ambient temperature by combining water with 80.0 g of defatted soy white flakes at 6:1, 8:1, 10:1, or 15:1 ratio and stirring as rapidly as possible with a magnetic stir bar. Slurry was adjusted to pH 8.5 using 2N NaOH, maintained at that pH, and stirred for 1 h. The storage proteins were then fractionated as described above. The insoluble fraction, whey, and fractionated proteins were weighed, and then freeze dried.

Effect of Temperature on Bench-Scale Protein Extraction and Fractionation. Soy slurry was prepared by combining 80.0 g of defatted white flakes at 10:1 water-to-flake ratio, stirring as rapidly as possible with a magnetic stir bar, and heating to 25, 35, 45, 55, or 65 °C. Slurry was adjusted to pH 8.5, maintained at that pH, and stirred for 1 h. The storage proteins were then fractionated as described above. The insoluble fraction, whey, and fractionated proteins were weighed, and then freeze dried.

Pilot-Scale Protein Fractionation. Extraction conditions for the defatted soy white flakes (20.0 kg) were 15:1 water-to-flake ratio at 20 °C, used by Wu et al. (10) for previous pilot-scale fractionation, or 10:1 water-to-flake ratio at 45 °C (optimized conditions). Flakes and temperature-adjusted tap water were combined by stirring at 22 rpm in a jacketed 800-L tank (Walker Stainless Equipment Company, New Lisbon, WI), adjusted to pH 8.5 with 2 N NaOH, maintained at that pH, and stirred at 13 rpm for 1 h. Slurry was fed to an Alfa Laval BTPX disc stack centrifuge (Alfa Laval Separation Inc., Warminster, PA) at 9800 rpm bowl speed (approximately 12,000 x *g*) at a feed rate of ~2 L/min with a Moyno transfer pump

(Electric Pump, Model IFFCA SSE SAA, Des Moines, IA) at 300 rpm. The insoluble fraction was sampled and then discarded.

The protein extract was treated with $NaHSO_3$ to provide 10 mM SO_2, adjusted to pH 6.4 with 2N HCl, and cooled overnight at ~7 °C. Glycinin-rich precipitate was recovered using the Alfa Laval centrifuge at 9800 rpm bowl speed and 420 rpm transfer pump speed (~4 L/min). The resulting supernatant was adjusted to 0.25 M NaCl and pH 5.0, stirred at 13 rpm for 1 h, and then centrifuged at 9800 rpm bowl speed with a 420 rpm transfer pump speed. The resulting intermediate fraction precipitate was comprised of a mixture of glycinin, β-conglycinin, and other proteins. The supernatant was diluted 2:1 with water, adjusted to pH 4.8, and chilled overnight at ~7 °C. The β-conglycinin-rich precipitate was removed by centrifugation at 9800 rpm bowl speed with a 420 rpm transfer pump speed. The whey fraction was sampled and discarded. Recovered protein fractions were adjusted to pH 7.5 with 2N NaOH and then desalted with a Feed and Bleed Membrane Filtration System (Model SRT-50; North Carolina SRT Inc., Cary, NC) and a 30-KD regenerated cellulose membrane (North Carolina SRT Inc.). Diafiltration was continued until permeate equaling 5X the original volume of protein dispersion was collected. The protein retentate was dried in an Anhydro Compact Spray-Dryer (APV Crepaco Inc., Attleboro Falls, MA) with an air inlet temperature of 160 °C and air outlet temperature of 85 °C. Samples of in-process supernatants, the whey, the insoluble fraction, and protein products after neutralization and after diafiltration were collected and freeze dried.

Deviations from the pilot-scale process described by Wu et al. (10) were as follow. Wu's protein extract was separated from the insoluble fraction using a two-pass system through a Sharples P660 decanting centrifuge (Alfa Laval Separation Inc., Warminster, PA) instead of a single pass through the Alfa Laval BTPX 204 disc stack centrifuge. Wu chilled overnight at 4 °C, while we chilled at ~7 °C.

Soy Protein Isolate Production. Flakes were extracted (Figure 2) with a 10:1 water-to-flake ratio at 60 °C. Flakes and temperature-adjusted water were combined with mixing as above and adjusted to pH 8.5 with 2 N NaOH. The slurry was stirred for 30 min at 13 rpm,

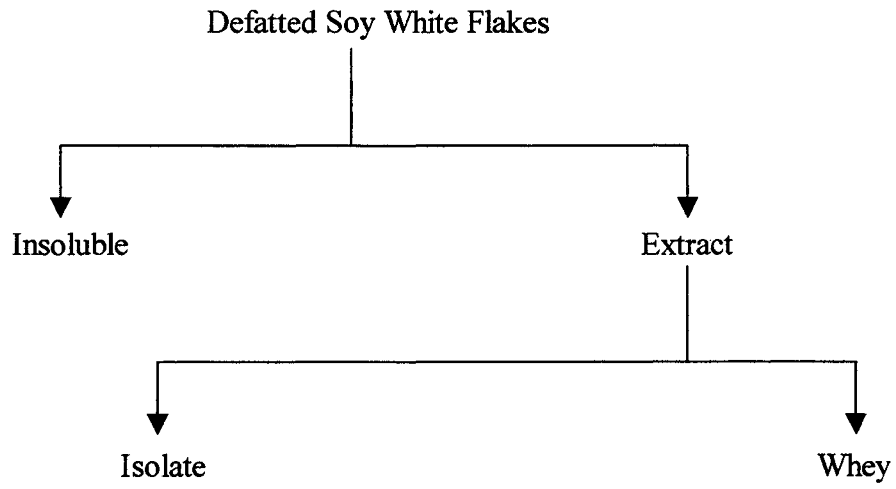


Figure 2. Soy protein isolate process.

and then the insolubles were removed by centrifugation as above. The supernatant was adjusted to pH 4.5 using 2N HCl and then chilled overnight at ~7 °C. The soy protein isolate was recovered by centrifugation, adjusted to pH 6.8, and then spray dried. Samples of in-process supernatant, the insoluble fraction, the whey, and neutralized soy isolate samples were collected and freeze-dried.

Proximate Analysis. Nitrogen contents of bench-scale samples were determined by micro-Kjeldahl (23), whereas nitrogen contents from pilot-scale samples were determined using the combustion or Dumas method (24) with a Rapid NIII analyzer (Elementar Americas, Inc., Mt Laurel, NJ). Dumas method values were converted to Kjeldahl nitrogen values using a conversion formula (25). A correction factor of 6.25 was used to convert from percent nitrogen to protein content. Moisture and ash were determined (26, 27).

Urea-SDS-PAGE. Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed using the method described by Wu et al. (10). Sample containing 25-mg of protein was dissolved in 10 mL of protein extraction buffer (50 mM 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 (125 mM THAM, pH 6.8, 5.0 M urea, 0.2% SDS, 20% glycerol, and 0.01% bromophenol blue). 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 and then destained in the same solution without the Coomassie Blue. Densitometry was determined using Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech Image Scanner (Amersham Pharmacia, Piscataway, NJ).

SDS-PAGE results were calculated as follows. Densitometry values for lipoxygenase, β-conglycinin subunit, and glycinin subunit bands were summed. This sum was then used as the denominator for a corresponding numerator comprised of a sum of subunit bands for a given protein. When multiplied by 100, this provided percentage composition for that protein with respect to the protein bands measured by densitometry analysis.

Surface hydrophobicity. Surface hydrophobicity was measured by a modification of that used by Wu et al. (10), using 1-anilino-8-naphthalene sulfonic acid magnesium salt monohydrate (ANS, ICN Biomedicals, Inc., Aurora, OH). A 1% (w/w) dry-basis sample dispersion was prepared in de-ioinized water and the pH was adjusted to 7 using 2N HCl or NaOH. The dispersions were stirred for 1 h, with pH adjusted at 15, 30, and 60 min. Undissolved material was removed by centrifugation at 10000 x *g* for 10 min at 20 °C. The protein content of the supernatant was determined by using the biuret method (22) with bovine serum albumen as the standard. Soluble protein was diluted with 0.1 M phosphate buffer (pH 7.0) to obtain concentrations ranging from 6.25 to 1000 μg/mL. ANS (40 μL, 8.0mM in 0.01 M phosphate buffer) was dispersed in 3-mL aliquots of each dilution. Fluorescence intensity units (FIU) were measured with a Turner Quantech® spectrophotometer (Barnstead Thermolyne, Dubuque, IA) using 440 nm (excitation) and 535 nm (emission) filters. FIU were standardized using a solution of 40 μL ANS in 3 mL phosphate buffer as the zero point and 15 μL ANS in 3 mL of methanol assigned a value of 80 FIU. The FIU were plotted versus percent protein concentration. The slope of the regression line was considered as the surface hydrophobicity. Samples were run in triplicate.

The conditions and parameters used by Wu et al. (10) that were different from those stated above were as follow. Protein was serially diluted in the range of 1-100 μg/mL. Excitation and emission wavelengths were 350 and 525 nm, respectively. A 100 μL aliquot of ANS was added to 5 mL of diluted dispersion.

Differential Scanning Calorimetry. Samples (15-20 mg) of 10% (w/w) dry basis protein dispersion were hermetically sealed in aluminum pans. A sealed, empty pan was used as reference. The samples were analyzed at 10 °C/min in a range of 25-120 °C using an SII Exstar 6000 (Seiko Instrument, Inc., Japan). All samples were repeated at least four times.

Isoflavone and Group B Saponin Extraction and Analysis. Extractions and HPLC analyses were conducted as reported by Hu et al. (18) for saponins and Murphy et al. (13) for isoflavones, with a few modifications. About 2.5 g of freeze-dried slurry, insoluble fraction,

or isolate, or 4.0 g freeze-dried whey fraction was accurately weighed into 125-mL screw-capped Erlenmeyer flasks, and 14 mL of water and 20 mL of acetonitrile were added to the flasks. For pilot-scale samples, this mixture was neutralized to pH 7.0 with 2N HCl or 2N NaOH prior to extraction, but bench-scale samples were not neutralized prior to extraction. Flasks were shaken at 300 rpm for 2 h at room temperature.

This extraction method differed as follows from the methods described by Murphy et al. (13) and Hu et al. (18). For optimal isoflavone extraction from a particular food matrix, Murphy et al. (13) used 10 mL of acetonitrile, 2 mL of 0.1 N HCl, and water as needed (approximately 7 mL). Hu et al. (18) used 100 mL of 70% ethanol and 2.5 h of extraction time for saponin extraction. The modified method did not give significantly different ($P<0.05$) phytochemical concentrations compared to the referenced methods when ground, defatted soy flakes were analyzed.

The sample was filtered, and the extract was rotary evaporated to dryness at < 30 °C. The residue was dissolved in 80% methanol to a volume of 10.0 mL. Sample for isoflavone and saponin quantification was filtered through a 0.45-µm polytetrafluoroethylene filter (Alltech Associates Inc., Deerfield, IL) prior to HPLC analysis. Quantifications of the isoflavones and group B saponins were performed on different HPLC systems, but using the same type of column, an RP-18, 5 µm, 4.6 i.d. x 250 mm YMC-ODS-AM-303 column (YMC, Inc., Wilmington, NC).

Saponins were separated using a gradient system consisting of 0.05% trifluoroacetic acid in water (A) and acetonitrile (B). The programmed gradient did not differ substantially from that used by Hu et al. (18). Solvent B was increased from 37 to 40% in 12 min, and then B was further increased to 48% over 25 min. Finally, B was increased to 100% in 1 min, held at 100% for 2 min, then returned to 37% over 5 min. The flow rate was maintained at 1 mL/min throughout. The UV absorbance was monitored from 190 to 350 nm. Peak areas at 205 nm (saponins V, I, and II) and 292 nm (saponins αg, βg, and βa) were used for analysis.

For quality control purposes, an isoflavone standard solution (genistin, genistein, and daidzein) and a saponin standard solution (saponin I) were injected on the respective systems

prior to analyzing samples. Preparation of saponin and isoflavone standards and calibration curves are described elsewhere (13, 18). Isoflavone recovery from ground soy flake and freeze-dried soy protein isolate material were evaluated using a concentrated isoflavone extract produced from soy flakes and containing 7.5 μmol/mL isoflavones in 80% methanol. Saponin recovery from these matrices employed a mixture of saponin I and II at concentrations of 4.8 and 3.9 μmol/mL, respectively, in 80% methanol. Samples were spiked, mixed thoroughly, and then allowed to air dry overnight. Extraction and analysis were performed as above.

Statistical Analysis. Least square means, least significant differences, and ANOVA results were calculated using the SAS system (version 6, SAS Institute Inc., Cary, NC).

Results and Discussion

Primary objectives were to improve pilot-scale protein solubilization from the defatted soy flake starting material without decreasing mass and protein recovery in the glycinin and β-conglycinin fractions or substantially decreasing purity of these fractions and to enhance isoflavone and saponin recovery from the starting material to potentially increase their recovery in product fractions. Because of the substantial cost involved with pilot-scale fractionation, process improvements were first investigated at bench scale. Protein and phytochemical solubilization parameters evaluated were pH, ethanol concentration in the solubilization medium, water-to-flake ratio, and temperature. Optimized process conditions were then evaluated at pilot-scale.

Bench Scale - Effect of pH. The effect of pH on protein solubilization is shown in Table 1. Protein in the extract increased from pH 7.5 to 9.0, although protein content was not significantly different between pH 8.5 and 9.0. Solubility profiles of fractionated glycinin and β-conglycinin have revealed that the solubilities of these two fractions are very similar from pH 8.0 to 10.0 (28). However, below pH 8.0, glycinin solubility began to decrease, diminishing steeply below pH 7.0. Decline in β-conglycinin solubility lagged by about 1 pH

Table 1. Effect of pH on Bench-Scale Protein Extraction from Defatted Soy White Flakes[a]

pH	Protein (g)[b,c]
7.5	27.4_{c}
8.0	29.8_{bc}
8.5	32.0_{ab}
9.0	33.3_{a}
LSD[d]	3.1

[a]80.0 g flakes at 6.6% moisture.
[b]Subscript, lowercase letters indicate significant differences at $P<0.05$.
[c]n=2.
[d]LSD, least significant difference.

unit. It is possible that at pH 7.5, solubilization of glycinin was more limited, accounting for the lower protein recovery at pH 7.5 observed here. Increasing solubilization pH above 9.0 increases the risk of lysinoalanine formation, although lysinoalanine formation is more extensive as temperatures are increased (29). Lysinoalanine has been shown to induce changes in kidney cells of rats, but based on non-human primate data low doses are probably safe for humans (30). Additionally, isoflavones ionize above pH 9.0 (31, 32), possibly altering their hydrophobicity, polarity, and partitioning from the soy matrix. Consequently, we decided to retain the solubilization pH of 8.5 used by Wu et al. (10).

Bench Scale - Effect of Ethanol. Improved solubilization of isoflavones and saponins from the starting material was desired, with the hypothesis that higher concentrations of these phytochemicals in the extract would translate into higher concentrations in the protein product fractions. Solvents, such as acetonitrile, ethanol, and methanol, have been used to extract soy phytochemicals from food and soy samples. The hypothesis was that if a small amount of solvent was included in the solubilization medium the phytochemical content of the protein extract might be improved. Although ethanol is not as efficient as acetonitrile in extracting isoflavones (33), it was a logical solvent choice for this potential food application.

The isoflavone and saponin concentrations in the defatted soy flakes were 8.68 and 6.51 μmol/g, respectively. Table 2 shows that increasing ethanol concentration in the solubilization medium from 0 to 10.0% significantly decreased dry-basis isoflavone concentration in the insoluble fraction, a process waste stream, from 4.53 to 4.08 μmol/g. Dry-basis saponin concentration did not change significantly, ranging from 6.19 to 6.36 μmol/g. These small differences in phytochemical concentrations in the insoluble fractions among treatments indicated that increased ethanol concentration in the solubilization medium had little impact on improving phytochemical recovery from the starting material. This limited effect on extraction was not surprising, because 70% ethanol and 59% acetonitrile solutions (18, 13) are commonly used for soy saponin and isoflavone extraction, respectively. These phytochemicals are poorly soluble or insoluble in water. Their transfer into the extract

Table 2. Ethanol Effect on Phytochemical Mass and Concentration in Insoluble Fractions Produced during Bench-Scale Soy Protein Fractionation[a]

Ethanol in extraction solution	Isoflavone concentration[b] (µmol/g, db[c])	Total isoflavones (µmol, db)	Saponin concentration (µmol/g, db)	Total saponins (µmol, db)
None[d]	4.53_{a}	135	6.28	187
0.1%	4.49_{ab}	135	6.27	188
1.0%	4.42_{b}	131	6.19	184
10.0%	4.08_{c}	138	6.36	215
LSD[e]	0.11	Not Different	Not Different	Not Different

[a]80.0 g defatted soy white flakes, 6.6% moisture.
[b]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[c]db, dry basis.
[d]n=2.
[e]LSD, least significant difference.

fraction from the starting material was likely either from a mass action effect or due to their associations with solubilized proteins.

While there apparently was a modest improvement in solubilizing isoflavones as ethanol concentration of the extraction medium increased, increasing the ethanol concentration had a detrimental effect on the weight of the insoluble fraction and on the fractionation process (Table 3). The dry-basis weight of the insoluble fraction increased significantly from 29.77 to 33.81 g as the ethanol concentration of the medium was increased to 10%, indicating reduced solubilization of some soluble soy components. The glycinin fraction weight increased with increasing ethanol concentration, but not significantly, while the intermediate fraction weight did increase significantly between 1 and 10% ethanol. These increases apparently occurred at the expense of the β-conglycinin fraction. It is possible that β-conglycinin was more readily denatured than glycinin, causing the differences in solubilizing and partitioning. However, SDS-PAGE analysis of the extract did not provide supporting evidence for the hypothesis that β-conglycinin was solubilized less efficiently by the 10% ethanol solution. Lipoxygenase, β-conglycinin, and glycinin distributions in the extract showed no obvious trends and were not significantly different, ranging from 6.8 to 8.0%, 37.9 to 40.3%, and 52.1 to 54.9%, respectively. Based on minimally improved isoflavone recovery at the expense of protein solubilization and fractionation, ethanol in the solubilization medium was rejected as a means of process improvement.

Bench Scale – Effect of Water-to-Flake Ratio. The amount of water used in the solubilization of protein and phytochemicals from defatted soy flakes is important for maximizing solubility. However, the volume of water used is also very important from a processing standpoint. The more water used, the more time required for each centrifuge pass. Wu et al. (10) used water-to-flake ratios of 15:1 and 20:1. Lehnhardt et al. (9) disclosed in their patent that a 10:1 water-to-flake ratio was optimal for glycinin and β-conglycinin recovery. With the flake material used here, a 6:1 ratio was the lowest that could be employed and still maintain the ability to effectively stir the suspension. Consequently, water-to-flake ratios of 6:1, 8:1, 10:1, and 15:1 were selected for evaluation.

Table 3. Ethanol Effect on Product Recovery during Bench-Scale Fractionation of Soy Storage Proteins[a]

Ethanol in extraction solution	Insoluble[b] (g, dry basis)	Glycinin (g, dry basis)	Intermediate (g, dry basis)	β-Conglycinin (g, dry basis)
None[c]	29.77_b	7.89	7.22_b	6.91_a
0.1%	30.03_b	8.00	7.12_b	6.92_a
1.0%	29.75_b	8.51	7.44_b	6.81_a
10.0%	33.81_a	9.04	8.92_a	2.01_b
LSD[d]	2.76	Not Different	1.12	1.02

[a]Starting material was 80.0 g ("as is" basis, 6.6% moisture) defatted soy white flakes.
[b]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[c]n=2.
[d]LSD, least significant difference.

The effect of water-to-flake ratio on process fraction weights, protein concentration, and total protein for each product or waste stream fraction is shown in Table 4. The dry basis weight of the insoluble fraction decreased significantly from 41.28 to 30.99 g for the 6:1 to 15:1 water-to-flake ratios, respectively. Higher water-to-flake ratios were clearly more efficient at extracting solubles from the starting materials. The glycinin and intermediate fraction weights increased significantly as water-to-flake ratio increased from 6:1 to 15:1, but differences between 10:1 and 15:1 water-to-flake ratios were not significant. There was no significant difference among β-conglycinin fraction weights. When the three product fraction weights were summed for each ratio, combined weights ranged from 17.73 to 21.84 g for 6:1 and 15:1, respectively. This represents only about a 4 g difference, compared to about a 10 g difference observed between the insoluble fractions for the 6:1 and 15:1 treatments. This demonstrates that while there is improved extraction of solubles from the starting material at higher water-to-flake ratios, less than 50% of that material is being recovered in the protein product fractions. The weight of the whey fraction, a process waste stream, increased significantly across treatments. This increase was due to two factors: the increased quantity of solubles liberated from the starting material but not recovered in the product fractions; and the increased addition of $NaHSO_3$ and NaCl to maintain the proper concentrations during fractionation. Total fraction weights also reflected this increased salt addition.

The protein content of the insoluble fraction decreased significantly as water-to-flake ratio increased, indicating increased extraction efficiency at higher water-to-flake ratios. The protein contents of the glycinin and intermediate fractions increased significantly as water-to-flake ratio was increased, possibly due to a higher proportion of the salts staying in solution during these protein precipitation steps. The protein content of the whey fraction decreased significantly as water-to-flake ratio increased, reflecting the increase in salt concentration of the fractions.

When the total protein from all process fractions was summed for each treatment, there was no significant difference among treatments. This indicates an excellent accounting of

Table 4. Water-to-Flake Extraction Ratio Effect on Fraction Weight and Protein Recovery during Bench-Scale Fractionation of Soy Storage Proteins[a]

	Insoluble	Glycinin	Intermediate	β-Conglycinin	Whey	Total
Process Fraction Weights (g, dry basis)[b]						
6:1[c]	41.28_a	5.45_b	5.47_b	6.81	15.04_d	74.05_c
8:1	38.18_a	7.06_a	5.85_b	7.12	21.09_c	79.29_b
10:1	33.76_b	7.87_a	7.00_a	6.61	25.24_b	80.48_b
15:1	30.99_b	7.97_a	7.35_a	6.52	32.37_a	85.21_a
LSD[d]	3.19	1.22	0.76	Not different	1.82	4.39
Protein Concentration (%)						
6:1	44.4_a	92.9_c	69.6_c	95.7	22.1_a	
8:1	41.6_b	96.9_b	73.0_b	96.2	20.8_b	
10:1	40.1_c	99.2_{ab}	78.3_a	96.2	18.3_c	
15:1	40.2_c	100.3_a	80.8_a	97.1	15.9_d	
LSD	0.8	2.4	2.8	Not different	1.8	
Total Protein (g, dry basis)						
6:1	18.3_a	5.06_c	3.79_b	6.51	3.33_c	37.1
8:1	15.9_b	6.84_b	4.27_b	6.85	4.38_b	38.2
10:1	13.6_c	7.80_{ab}	5.48_a	6.36	4.62_b	37.8
15:1	12.5_c	7.99_a	5.94_a	6.34	5.15_a	37.9
LSD	1.4	1.08	0.68	Not different	0.40	Not different

[a]Starting material was 80.0 g ("as is" basis, 6.6% moisture) defatted soy white flakes.
[b]Subscript, lowercase letters indicate significant differences at $P<0.05$.
[c]n=2.
[d]LSD, least significant difference.

protein mass balance. Total protein for the glycinin and intermediate fractions increased as water-to-flake ratio increased, but differences were not significant between 10:1 and 15:1 ratios. Total proteins for the β-conglycinin fractions were not significantly different.

Another criterion for process improvement was maintenance or improvement of fraction purity. SDS-PAGE data regarding fraction purity is shown in Table 5. There were no significant differences across treatments for glycinin fraction purity. However, purity of the β-conglycinin fraction increased from 59.4 to 66.1% as water-to-flake ratio increased, with differences being insignificant between the 10:1 and 15:1 ratios.

Improved extraction of phytochemicals from the starting material with increased deposition in the product fractions was desired. Table 6 shows the effects of water-to-flake ratio on isoflavone extraction and partitioning. The dry-basis isoflavone concentration of the insoluble, glycinin, intermediate, and β-conglycinin fractions decreased with increasing water-to-flake ratio. Differences in isoflavone concentration in the whey fraction were not significant, but total isoflavones in the whey fraction increased as water-to-flake ratio increased. Recall that the dry weight of the whey fraction increased with increasing water-to-flake ratio. These data indicated that improved isoflavone extraction from the starting material did not translate into increased isoflavone concentration in the product fractions, rather an increase in isoflavones in the whey. Shen and Bryan (34) also describe concerns about the ability of isoflavones to be lost from protein fractions through supernatant or wash fractions. Their patent discloses a method to enzymatically convert glucoside isoflavones in protein extract to aglucons prior to isoelectric precipitation of the protein. They indicate that the precipitated protein should not be washed or that washing should be quite limited to avoid loss of the aglucons from the protein.

Isoflavones were preferentially partitioned among the product fractions, with distribution being about 3.2:2.4:1 on a μmol/g basis for the intermediate, glycinin, and β-conglycinin precipitate fractions, respectively. Wu et al. (10) indicated that most of the glycinin and β-conglycinin proteins comprising the intermediate fraction were no longer in native state. One hypothesis is that as native state of the proteins is lost and protein unfolding is increased,

Table 5. Water-to-Flake Extraction Ratio Effects on Protein Distribution during Bench-Scale Soy Protein Fractionation

Water-to-flake ratio	Glycinin Fraction[a]			Intermediate Fraction			β-Conglycinin Fraction		
	LOX[b] (%)	β-Con (%)	Gly (%)	LOX (%)	β-Con (%)	Gly (%)	LOX (%)	β-Con (%)	Gly (%)
6:1[c]	nd[d]	12.9	87.1	23.0_{ab}	40.2	36.8_{c}	nd	59.4_{b}	40.6_{a}
8:1	nd	13.4	86.6	26.2_{a}	36.1	37.7_{bc}	nd	61.9_{b}	38.1_{ab}
10:1	nd	13.0	87.0	22.6_{ab}	36.2	41.2_{ab}	nd	65.7_{a}	34.3_{bc}
15:1	nd	12.4	87.6	19.4_{b}	37.0	43.6_{a}	nd	66.1_{a}	33.9_{c}
LSD[e]		Not different	Not different	4.0	Not Different	3.7		3.7	3.7

	Insoluble Fraction			Protein Extract from Flakes			Whey		
	LOX (%)	β-Con (%)	Gly (%)	LOX (%)	β-Con (%)	Gly (%)	LOX (%)	β-Con (%)	Gly (%)
6:1	9.4	52.2_{b}	38.4	7.1	36.1	56.8	16.1	37.8	46.1
8:1	10.0	52.1_{b}	37.9	7.2	35.6	57.2	16.6	34.8	48.6
10:1	9.9	54.7_{a}	35.4	7.6	36.4	56.0	19.2	33.1	47.7
15:1	10.0	55.1_{a}	34.9	7.4	36.1	56.5	18.3	33.5	48.2
LSD	Not different	2.1	Not different	Not different	Not different	Not different	Not different	Not different	Not different

[a]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.
[b]Abbreviations: LOX, lipoxygenase; β-Con, β-conglycinin; Gly, glycinin.
[c]n=2.
[d]nd, none detected.
[e]LSD, least significant difference.

Table 6. Water-to-Flake Extraction Ratio Effects on Isoflavone Concentrations and Mass in Process Fractions Produced during Bench-Scale Fractionation of Soy Storage Proteins[a]

Water-to-flake ratio	Insol[b,c] (μmol/g)	Gly (μmol/g)	Intermed (μmol/g)	β-Con (μmol/g)	Whey (μmol/g)	Insol (μmol)	Gly (μmol)	Intermed (μmol)	β-Con (μmol)	Whey (μmol)	Total (μmol)
6:1[d]	7.02$_a$	10.17$_a$	15.3$_b$	4.79$_a$	4.17	290$_a$	55.4	84$_c$	32.6$_a$	63$_b$	525
8:1	6.29$_b$	9.74$_b$	15.8$_a$	3.81$_b$	4.90	240$_b$	68.7	93$_{bc}$	27.1$_b$	103$_{ab}$	532
10:1	5.49$_c$	9.40$_c$	15.1$_b$	3.27$_{bc}$	4.81	185$_c$	74.0	105$_a$	21.7$_{bc}$	121$_a$	507
15:1	4.63$_d$	8.34$_d$	13.8$_c$	2.85$_c$	4.50	143$_d$	66.4	101$_{ab}$	18.6$_c$	146$_a$	476
LSD[e]	0.39	0.15	0.5	0.61	ND[f]	30	ND	9	5.4	43	ND

[a]Starting material was 80.0 g ("as is" basis, 6.6% moisture) defatted soy white flakes.
[b]Abbreviations: Insol, insoluble; Gly, glycinin; Intermed, intermediate; β-Con, β-conglycinin.
[c]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.
[d]n=2.
[e]LSD, least significant difference.
[f]ND, not different.

more hydrophobic areas are exposed to interact with the isoflavones. However, this does not explain the high isoflavone content of the glycinin fraction compared to the β-conglycinin fraction. The low isoflavone recoveries in the β-conglycinin fractions did not seem to be associated with depleted quantities of isoflavones, because the whey fractions still retained substantial quantities of isoflavones.

Saponin partitioning into the intermediate fraction relative to the glycinin and β-conglycinin fractions was even more substantial than for the isoflavones (Table 7). The saponins are more hydrophobic than the isoflavones. Consequently, they may have been even more attracted to the non-native state proteins in the intermediate fraction than were the isoflavones. No saponins were detected in the whey fraction. Concentration differences across treatments were significant only for glycinin, ranging from 2.67 μmol/g for the 6:1 ratio to 1.27 μmol/g for the 15:1 ratio. Saponin concentrations in the insoluble fractions were very uniform across treatments, but the total protein content of the insoluble fraction decreased by about one-third as water-to-flake ratio was increased. This may indicate that the saponins were intimately associated with another matrix component than protein in the insoluble fraction. For all process fractions, there were no significant differences in saponin concentrations between the 10:1 and 15:1 ratios.

Unexpected for the isoflavones and saponins was the incomplete accounting of these phytochemicals. The starting material contained 648 μmol of isoflavones and 486 μmol of saponins. Mass balance data (Tables 6 and 7) show that total isoflavones ranged from 507 to 531 μmol, while total saponins ranged from 340 to 406 μmol. These values translate to about 80 and 70-84% of isoflavones and saponins in the starting material, respectively. Differences in soy flake and process fraction matrices were probably responsible for at least a portion of this discrepancy.

A recovery study with ground, defatted soy flakes and freeze-dried soy protein isolate revealed considerable recovery differences between these matrices. Recovery of the saponin I and II spike was about 99% for the flake material, but only 60-70% for the isolate. Isoflavone recoveries were 75-92% for the flake material, with the more hydrophobic

Table 7. Water-to-Flake Extraction Ratio Effects on Saponin Concentrations and Mass in Process Fractions Produced during Bench-Scale Soy Protein Fractionation[a]

Water-to-flake ratio	Insoluble[b] (μmol/g)	Glycinin (μmol/g)	Intermediate (μmol/g)	β-Conglycinin (μmol/g)	Insoluble (μmol)	Glycinin (μmol)	Intermediate (μmol)	β-Conglycinin (μmol)	Total (μmol)
6:1[c]	7.05	2.67_{a}	16.5	1.51	291_{a}	14.5	90	10.2	406_{a}
8:1	7.16	2.02_{b}	18.2	1.73	273_{b}	14.2	106	12.3	406_{a}
10:1	7.05	1.62_{c}	15.8	1.97	238_{c}	12.8	110	13.0	374_{b}
15:1	6.80	1.27_{c}	14.7	1.74	211_{d}	10.1	108	11.3	340_{c}
LSD[d]	ND[e]	0.39	ND	ND	15	ND	ND	ND	12

[a]Starting material was 80.0 g ("as is" basis, 6.6% moisture) defatted soy white flakes.
[b]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.
[c]n=2.
[d]LSD, least significant difference.
[e]ND, not different.

aglucons exhibiting the lowest recoveries. Isoflavone aglucon recovery from the isolate material was 65-75%, while recoveries for the β-glucosides and malonylglucosides were 78-93%. For the recovery study (Chapter 3), neutralizing the phytochemical extraction solution increased saponin I and II recoveries from the isolate samples by about 15% for saponin I and 25-45% for saponin II. Recovery of the aglucon isoflavones from the isolate increased by 10-15% by neutralizing the phytochemical extraction medium.

Bench-scale process samples in the current study were not neutralized prior to freeze drying or phytochemical extraction. Consequently, the pH of the extraction medium used during phytochemical analysis was approximately 4.8 for β-conglycinin and 5.0 for the intermediate fractions. A glucuronic acid on the saponin aglucon has a pK_a of approximately 4, and this possibly led to ionic bonding of the saponins within the protein matrix of the product fractions and poor recovery during phytochemical extraction.

The 10:1 water-to-flake ratio was selected for use in further process improvement for the following reasons. Process fraction weights and total protein for the 10:1 and 15:1 ratios were significantly higher than for the 6:1 and 8:1 ratios, or there was no significant difference. There was not a significant difference between fraction weights or total protein for the 10:1 and 15:1 ratios. The purity of the β-conglycinin fraction was significantly higher for the 10:1 and 15:1 ratios, although there was not a significant difference between the two ratios. Saponin concentrations in the intermediate and β-conglycinin fractions were not significantly different, although the concentrations in the glycinin fraction for the 10:1 and 15:1 ratios were significantly lower. Unfortunately, isoflavone concentrations generally were significantly lower for the 10:1 ratio compared to the 6:1 or 8:1 ratios. However, the 10:1 ratio was still selected because the differences in phytochemical concentrations, though in some cases statistically significant, were small. Phytochemical recovery in product fractions was a secondary selection criterion compared to protein recovery and purity.

Bench Scale - Effect of Temperature. As process temperature was increased to as high as 80 °C during the solubilization of soy proteins, protein recovery was increased (12). Wu et al. (10) were concerned that by increasing protein solubilization temperature the native

state of the soy proteins would be affected and the fractionation process would be less efficient. Extraction temperatures of 25-65 °C were selected to test this hypothesis.

Insoluble fraction weight, protein concentration, and total protein decreased significantly with increasing temperature, indicating improved protein recovery (Table 8). Intermediate fraction weight increased significantly with increased solubilization temperature. The onset temperatures for β-conglycinin and glycinin denaturation are approximately 68 and 82 °C, respectively (35). The fraction weight of the β-conglycinin fraction began to decrease significantly at 55 °C, but dropped drastically at 65 °C. Denaturation was likely the cause of this decline, with the protein being partitioned to the intermediate fraction. The weight of the glycinin fraction was also significantly lower at 65 °C. These data support the findings of Wu et al. (10) that the intermediate fraction is primarily composed of glycinin and β-conglycinin proteins that are no longer in native state. The whey fraction weights were not significantly different, indicating that denatured protein was not being partitioned into that fraction. Protein concentrations of the product fractions were not significantly different across treatments, with the exception of glycinin at 65 °C, so total protein mirrored observations for fraction weights. On a fraction weight and protein basis, extraction at 45 °C provided the best process improvement, offering the best returns for the product fractions. The purities of the β-conglycinin fractions were not significantly different from 25 to 45 °C. The purity of the glycinin fraction was significantly better at 45 °C compared to 25 and 35 °C (Table 9).

Isoflavone concentration decreased significantly with increased protein solubilization temperature for all process fractions, except the whey fraction (Table 10). Saponin concentrations behaved similarly to the isoflavones, except there were no saponins in the whey fraction and there were no significant differences across treatments for the β-conglycinin fraction (Table 11). This decrease in phytochemical concentrations for all fractions was accounted for in the phytochemical totals of the intermediate fraction, into which increasingly more protein mass was partitioned. Isoflavone and saponin totals from all process fractions, as for the water-to-flake study, were low.

Table 8. Extraction Temperature Effects on Fraction Weight and Protein Recovery in Process Fractions Produced during Bench-Scale Fractionation of Soy Storage Proteins[a]

	Insoluble[b]	Glycinin	Intermediate	β-Conglycinin	Whey	Total
Process Fraction Weights (g, dry basis)						
25 °C[c]	32.17_a	8.41_{bc}	6.49_d	6.71_{ab}	23.22	76.99
35 °C	29.22_b	9.31_a	7.58_d	7.39_a	26.00	79.49
45 °C	28.05_{bc}	8.96_{ab}	9.53_c	7.55_a	25.29	79.36
55 °C	28.62_{bc}	8.26_c	12.91_b	6.29_b	23.64	79.70
65 °C	27.31_c	6.02_d	22.73_a	1.68_c	23.09	80.82
LSD[d]	1.46	0.60	1.41	1.07	ND[e]	ND
Protein Concentration (%)						
25 °C	40.3_a	98.8_a	75.8	94.7	18.2_a	
35 °C	37.0_b	97.0_a	72.7	94.7	18.0_a	
45 °C	35.8_c	96.8_a	73.1	94.2	17.1_{ab}	
55 °C	34.7_{cd}	97.5_a	74.5	93.6	16.3_{bc}	
65 °C	34.2_d	92.9_b	76.8	93.7	15.0_c	
LSD	1.2	3.1	ND	ND	1.4	
Total Protein (g, dry basis)						
25 °C	13.0_a	8.31_b	4.91_e	6.34_{ab}	4.23_{ab}	37.1
35 °C	10.8_b	9.03_a	5.51_d	6.99_a	4.67_a	36.9
45 °C	10.0_c	8.67_{ab}	6.96_c	7.10_a	4.32_{ab}	37.3
55 °C	9.9_{cd}	8.05_b	9.62_b	5.88_b	3.85_{bc}	37.7
65 °C	9.4_d	5.59_c	17.44_a	1.57_c	3.46_c	37.4
LSD	0.7	0.66	0.57	0.89	0.59	ND

[a]Starting material was 80.0 g ("as is" basis, 6.6% moisture) defatted soy white flakes.
[b]Subscript, lowercase letters indicate significant differences at $P<0.05$.
[c]n=2.
[d]LSD, least significant difference.
[e]ND, not different.

Table 9. Extraction Temperature Effects on Protein Distribution in Process Fractions Produced during Bench-Scale Soy Protein Fractionation

Extraction temperature	Protein Extract[a]			Glycinin Fraction			β-Conglycinin Fraction		
	LOX[b] (%)	β-Con (%)	Gly (%)	LOX (%)	β-Con (%)	Gly (%)	LOX (%)	β-Con (%)	Gly (%)
25 °C[c]	7.8	44.3	47.9	nd[d]	17.3	82.7	nd	73.5	26.5
35 °C	8.2	47.3	44.5	nd	18.3	81.7	nd	74.3	25.7
45 °C	7.3	44.2	48.5	nd	14.3	85.7	nd	73.0	27.0
LSD[e]	Not different	Not different	Not different		2.2	2.2		Not different	Not different

[a]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.
[b]Abbreviations: LOX, lipoxygenase; β-Con, β-conglycinin; Gly, glycinin.
[c]n=2.
[d]nd, none detected.
[e]LSD, least significant difference.

Table 10. Extraction Temperature Effects on Isoflavone Concentrations and Mass in Process Fractions Produced during Bench-Scale Soy Protein Fractionation[a]

Extraction temperature	Insol[b,c] (μmol/g)	Gly (μmol/g)	Intermed (μmol/g)	β-Con (μmol/g)	Whey (μmol/g)	Insol (μmol)	Gly (μmol)	Intermed (μmol)	β-Con (μmol)	Whey (μmol)	Total (μmol)
25 °C[d]	5.33_{a}	9.48_{a}	15.1_{a}	3.04_{a}	5.08	171_{a}	79.6_{ab}	98_{e}	20.5	118	487
35 °C	5.26_{ab}	9.10_{a}	14.5_{ab}	3.16_{a}	5.44	153_{b}	84.7_{a}	109_{d}	23.4	141	513
45 °C	5.04_{cd}	8.44_{b}	13.9_{b}	2.65_{ab}	5.12	141_{cd}	75.7_{bc}	132_{c}	20.0	129	498
55 °C	5.07_{bc}	8.35_{b}	12.3_{c}	2.31_{b}	5.47	145_{bc}	68.9_{c}	159_{b}	14.5	130	517
65 °C	4.85_{d}	7.86_{c}	10.4_{d}	IS[e]	5.12	132_{d}	47.3_{d}	237_{a}	IS	118	535
LSD[f]	0.20	0.47	0.7	0.55	ND[g]	10	7.2	10	ND	ND	ND

[a]Starting material was 80.0 g ("as is" basis, 6.6% moisture) defatted soy white flakes.
[b]Abbreviations: Insol, Insoluble; Gly, Glycinin, Intermed, Intermediate; β-Con, β-Conglycinin.
[c]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.
[d]n=2.
[e]IS, insufficient sample.
[f]LSD, least significant difference.
[g]ND, not different.

Table 11. Extraction Temperature Effect on Saponin Concentrations and Mass in Process Fractions Produced during Bench-Scale Soy Protein Fractionation[a]

Extraction temperature	Insoluble[b] (μmol/g)	Glycinin (μmol/g)	Intermediate (μmol/g)	B-Conglycinin (μmol/g)	Insoluble (μmol)	Glycinin (μmol)	Intermediate (μmol)	β-Conglycinin (μmol)	Total (μmol)
25 °C[c]	6.51_a	1.74_a	18.6_a	1.78	209_a	14.60_b	121_c	11.9_{ab}	357
35 °C	6.30_a	1.83_a	18.0_a	1.84	184_b	17.05_a	136_c	13.6_a	351
45 °C	6.05_a	1.09_c	17.0_b	1.90	170_{bc}	9.76_c	162_b	14.3_a	356
55 °C	5.55_b	0.80_d	13.9_c	1.44	159_c	6.61_d	179_b	9.0_b	353
65 °C	4.70_c	1.44_b	9.0_d	IS[d]	128_d	8.68_c	204_a	IS	341
LSD[e]	0.48	0.28	0.8	ND[f]	16	2.07	22	3.1	ND

[a]Starting material was 80.0 g ("as is" basis, 6.6% moisture) defatted soy white flakes.

[b]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.

[c]n=2.

[d]IS, insufficient sample.

[e]LSD, least significant difference.

[f]ND, not different.

Based on fraction and protein weights, as well as fraction purity, a protein solubilization temperature of 45 °C was selected for process improvement. Phytochemical concentrations tended to be lower for this treatment compared to the 25 and 35 °C treatments; however, differences were not substantial, and in some cases not significant.

Pilot Scale - Wu Modified-Nagano Method vs. Optimized Fractionation Process. Because bench-scale results may change as a result of scale up, the process of Wu et al. (10) (with a major modification) and an optimized process based on the bench-scale process improvement were compared at the pilot scale. The Wu modified-Nagano process, which will be referred to as the Wu process from here onward, used a 20 °C protein solubilization temperature and 15:1 water-to-flake ratio, while the optimized process used 45 °C and a 10:1 ratio. The major modification of the process as described by Wu et al. (10) was required due to equipment performance. Wu utilized a two-pass system through a Sharples P660 decanting centrifuge, but due to poor dewatering, this centrifuge was not used. A one-pass system employing an Alfa Laval BTPX 205 disc centrifuge was substituted and worked satisfactorily, producing a much drier insoluble fraction. Time savings were also realized using a one-pass system. However, a 10:1 water-to-flake ratio was the lowest ratio that could be used without plugging the centrifuge.

Soy protein isolate was also produced at the pilot scale so that protein fraction weight, protein yield, and phytochemical recovery could be evaluated against the fractionation processes. The protein solubilization temperature and water-to-flake ratio employed for soy isolate production were 60 °C and 10:1, respectively.

The wet weights of the Wu-method extract and supernatant fractions were significantly larger than the optimized method because the water-to-flake ratio was larger (Table 12). This difference also directly affected the ash content of the 2nd supernatant and whey fractions. Sodium chloride addition to alter the ionic strength of the 1st supernatant and precipitate the intermediate fraction was based on volume of the 1st supernatant. Consequently, the ash content of the 2nd supernatant and whey fractions were significantly higher. Diafiltration was used to reduce the ash concentration in product fractions to

Table 12. Mass Balance Comparisons for Pilot-Scale Fractionation Processes

Fraction	Method	Wet Weight (kg)[a]	Weight (kg, db)[b]	Ash (%, db)	Total Ash (kg, db)	Protein (%, db)	Total Protein (kg, db)	Isoflavones (μmol/g, db)	Isoflavones (mmol, db)	Saponins (μmol/g, db)	Saponins (mmol, db)
Flakes	Wu	20.00	18.06	6.6	1.19	59.1*	10.67	6.15	111.1	5.26	95.0
	Opt.	20.00	18.02	6.8	1.23	61.6	11.10	5.90	106.3	5.16	93.0
Insoluble	Wu	100.25	7.90	6.4	0.50	49.9	3.95	3.89	30.8	5.98	47.5
	Opt.	85.71	7.38	6.4	0.48	48.0	3.55	4.11	30.3	7.26	53.6
Extract	Wu	281.35**	10.04*	9.0	0.90	66.1	6.63	4.84**	48.6**	3.01	30.2
	Opt.	194.80	10.68	9.0	0.96	67.1	7.17	5.93	63.3	3.31	35.4
GLY P[c]	Wu	19.05	2.55	3.6	0.09	98.5	2.52	6.25	15.9	1.17*	3.0*
	Opt.	13.23	2.34	3.0	0.07	99.8	2.34	6.37	14.9	0.57	1.3
GLY DIA	Wu	56.80	2.34	1.9	0.04	104.1	2.44	2.28	5.3	1.01*	2.3*
	Opt.	48.15	2.13	1.6	0.03	104.3	2.22	1.80	3.9	0.35	0.7
GLY SD	Wu	2.37	2.25	1.9	0.04	104.2	2.35	2.30	5.2	0.70	1.6
	Opt.	2.15	2.04	1.7	0.03	105.6	2.16	1.83	3.8	0.36	0.8
1st Supernatant	Wu	294.10**	7.65**	13.9	1.06	52.1**	3.98**	6.86	52.4	3.46	26.5
	Opt.	208.73	8.41	12.6	1.06	55.8	4.69	6.40	53.8	3.44	28.8
INT P	Wu	15.23	1.64	13.9	0.23	75.9	1.24	8.33	13.6	14.74	24.0
	Opt.	19.55	1.64	13.7	0.22	78.3	1.29	8.57	14.0	11.09	18.0
INT DIA	Wu	36.58	1.27	2.7	0.03	91.6*	1.16	3.28	4.1	16.38	20.6**
	Opt.	46.93	1.25	2.5	0.03	94.4	1.18	2.33	2.9	13.17	16.0
INT SD	Wu	1.27	1.21	2.6	0.03	91.5	1.11	3.09	3.7	14.09	17.0**
	Opt.	1.30	1.23	2.5	0.03	95.5	1.18	2.28	2.8	11.35	13.5
2nd Supernatant	Wu	311.45**	10.18	50.4**	5.14**	26.2*	2.67	2.19	22.3	0.12	1.2
	Opt.	220.73	9.81	40.1	3.93	33.8	3.31	2.29	22.4	0.15	1.4
BCON P	Wu	21.53	1.40	9.2	0.13	89.7	1.25	1.96*	2.8*	1.50	2.3
	Opt.	24.00	2.45	7.1	0.17	91.3	2.24	3.33	8.2	5.22	13.2
BCON DIA	Wu	39.51	1.16*	2.7	0.03**	100.0*	1.16*	0.30*	0.3*	1.16**	1.4*
	Opt.	48.25	2.18	2.6	0.06	98.2	2.14	0.70	1.5	4.57	10.2
BCON SD	Wu	1.37*	1.29*	2.6	0.03**	99.4	1.28*	0.31*	0.4*	1.13**	1.5*
	Opt.	2.21	2.10	2.6	0.05	98.9	2.07	0.67	1.4	3.66	7.8
Whey	Wu	932.25**	8.77*	59.4**	5.21**	13.7	1.20*	3.42	30.1	0.24	2.1
	Opt.	673.25	7.23	53.4	3.86	14.2	1.02	4.09	29.6	0.32	2.3

[a]A single asterisk indicates significance at $P<0.10$, two indicate significance at $P<0.05$. n=2.
[b]db, dry basis.
[c]Abbreviations: GLY, glycinin; INT, intermediate; BCON, β-conglycinin; P, precipitate; DIA, after dialysis; SD, spray dried; Opt., optimized.

minimize the effect of salts during functionality testing. Dry-basis ash concentration prior to and after diafiltration ranged from 3.0 to 13.9% and 1.6 to 2.7%, respectively. Diafiltration of the protein products caused about a 4-7% loss of protein, and phytochemical losses from these fractions were substantial as well. Estimating by difference, 70-90% of the isoflavones were lost to the permeate, but only 15-45% of the saponins were lost. This indicates that the saponins may be more intimately associated with the proteins than are the isoflavones. The molecular weight cutoff of the cellulose membrane was 30 KD and the molecular weights of the isoflavones and saponins are all less than 1.1 KD.

One of the most interesting differences between the two processes was the significantly increased fraction weight of the optimized-method β-conglycinin fraction. The fraction weight of the optimized-process spray-dried β-conglycinin was 2.10 kg, while that of the Wu process was 1.29 kg. Table 13, which shows SDS-PAGE data for selected process fractions, indicates that the optimized-method β-conglycinin has a significantly lower purity, 67.8%, compared to 79.1% for the Wu process. This is lower than the 73-74% purity for β-conglycinin observed for the 25-45 °C treatments for the bench-scale comparison (Table 9). The optimized process had a higher fraction weight for the extract (Table 12). This weight was evidently not partitioned into the optimized-process glycinin and intermediate fractions, as they were not significantly different than for the Wu process, but partitioned into the β-conglycinin fraction. Total protein results for the two methods reflect what was observed for the fraction weights.

The protein extract of the optimized method contained a significantly higher isoflavone concentration and higher total isoflavones. This was not expected. Bench-scale results from the water-to-flake ratio study showed that increasing the ratio increased the amount of isoflavones recovered from the insoluble fraction (Table 6). Because the Wu method used a 15:1 ratio, compared to 10:1 for the optimized method, we predicted that the Wu-method extract would be higher in isoflavones. The bench-scale temperature study demonstrated that increased temperature significantly decreased isoflavone concentration in the insoluble fraction, but these differences were not substantial (Table 10). The isoflavone concentration

Table 13. Distribution of Proteins in the Starting Material and Selected Process Streams from Pilot-Scale Soy Protein Fractionation and Soy Protein Isolate Production

	Lipoxygenase[a,b] (%)	β-Conglycinin (%)	Glycinin (%)
Soy flake starting material	7.3_{bcd}	42.1_{fg}	50.7_{c}
Soy protein isolate (SPI)	5.2_{cde}	47.2_{de}	47.6_{cd}
Wu process glycinin	nd[c]	14.7_{h}	85.3_{b}
Optimized process glycinin	nd	10.2_{i}	89.9_{a}
Wu process intermediate	11.4_{a}	41.0_{g}	47.6_{cd}
Opt. process intermediate	10.1_{ab}	39.4_{g}	50.5_{c}
Wu process β-conglycinin	3.1_{e}	79.1_{a}	17.9_{i}
Opt. process β-conglycinin	4.2_{de}	67.8_{b}	28.1_{h}
SPI process extract	5.9_{cde}	50.8_{cd}	43.3_{efg}
Wu process extract	8.2_{abc}	51.8_{c}	40.0_{g}
Opt. process extract	6.1_{cde}	52.1_{c}	41.8_{fg}
SPI process insoluble fraction	10.2_{ab}	45.1_{ef}	44.7_{def}
Wu process insoluble fraction	7.1_{bcd}	46.4_{e}	46.5_{cde}
Opt. process insoluble fraction	9.7_{ab}	42.4_{fg}	47.9_{cd}
LSD[d]	3.5	3.6	4.2

[a]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.
[b]n=2.
[c]None detected.
[d]LSD, least significant difference.

of the insoluble fraction was not different for the Wu and optimized methods. This significant difference for isoflavones in the extract did not dramatically promote isoflavone differences in other fractions; however, the isoflavone concentrations and totals were significantly different at $P<0.10$ for the β-conglycinin fraction.

Saponin concentrations and totals for the insoluble and extract fractions were not significantly different between the Wu and optimized processes. However, in most cases there were significant differences between the processes for the product fractions. The saponin concentrations and totals for the glycinin and intermediate fractions were greater for the Wu method, while saponins were higher in the β-conglycinin fraction for the optimized method. An observation during the bench-scale studies was that a high proportion of the isoflavones and saponins were partitioned into the intermediate fraction. This was observed for the pilot-scale processes as well. One hypothesis for this partitioning was that a large proportion of the glycinin and β-conglycinin in the intermediate fraction was no longer in native state and that the relatively hydrophobic phytochemicals were more attracted to these unfolded proteins that presented more hydrophobic patches. DSC data (Table 14) suggest that very little native-state protein remained in the intermediate fractions for both processes, while the optimized process contained less native state β-conglycinin than did the Wu process. The intermediate fraction of the optimized method demonstrated high surface hydrophobicity, 183, compared to 129 and 124 of the glycinin and β-conglycinin fractions, respectively, suggesting a reason for the high saponin content of the intermediate fraction (Table 14). However, hydrophobicity values for the optimized-process glycinin and β-conglycinin do not help to explain why the saponin concentration was so high in the β-conglycinin fraction compared to the glycinin fraction. The surface hydrophobicity values for the Wu process were 114, 105, and 80 for glycinin, intermediate, and β-conglycinin, respectively. Again, these values do little to explain saponin partitioning among the product fractions, as the values do not parallel the observed saponin partitioning. With caution, it might be suggested that saponins have an affinity for denatured β-conglycinin. This would explain the high saponin concentration in the intermediate fractions. Very little β-

Table 14. Enthalpy and Surface Hydrophobicity of Pilot-Scale Soy Protein Isolate and Soy Storage Protein Fractionation Products

	Enthalpy (mJ/mg protein, dry basis)		Surface Hydrophobicity
	β-Conglycinin[a]	Glycinin	
Soy protein isolate[b]	0.08_d	7.98_b	227_a
Wu process glycinin	nd[c]	21.78_a	114_c
Optimized process glycinin	nd	22.82_a	129_c
Wu process intermediate	1.08_c	1.40_c	105_c
Opt. process intermediate	0.58_{cd}	1.98_c	183_b
Wu process β-conglycinin	7.53_a	0.52_c	80_d
Opt. process β-conglycinin	5.85_b	0.89_c	124_c
LSD[d]	0.87	3.05	25

[a]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[b]n=2.
[c]nd, none detected.
[d]LSD, least significant difference.

conglycinin is in the native state in these fractions. In addition, the β-conglycinin fraction of the optimized method had less native-state β-conglycinin in the β-conglycinin fraction and a higher concentration of saponins in the β-conglycinin fraction compared to the Wu method.

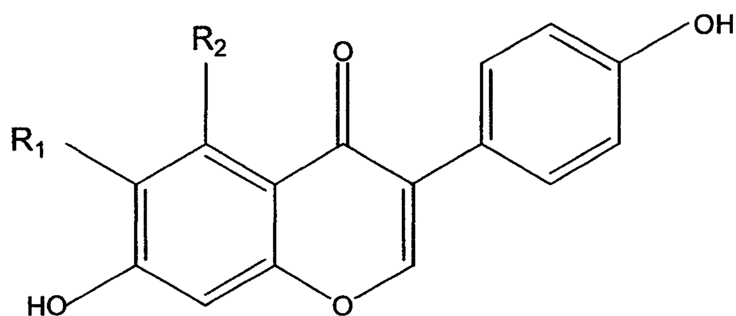
Fraction weights, total protein, and total isoflavones and saponins in the product fractions were compared to evaluate differences between the Wu, modified, and soy protein isolate processes (Table 15). Comparisons are for the precipitate fractions because the Wu and optimized processes employed diafiltration prior to spray drying. Fraction weight and total protein of the optimized process approached those for the soy protein isolate. Mass yields for the Wu, optimized, and isolate fractions (with glycinin, intermediate, and β-conglycinin fractions summed) were 31, 36, and 37%, respectively. Protein yields were 47, 53, and 54%, respectively. Total phytochemicals recovered in the soy protein isolate were significantly and substantially higher than for the other methods. Isolate used a solubilization temperature of 60 °C, which should have led to additional loss of native state of the proteins, possibly increasing phytochemical affinity for the proteins.

There are several forms of soybean isoflavones and saponins. Processing conditions can cause conversion between these forms. It has not been determined whether the saponin profile is of dietary significance, but isoflavone profile is important because glucoside forms require enzymatic modification to the aglucon forms for gut absorption in humans (17). Soy isoflavones are comprised of three aglucon forms, daidzein, genistein, and glycitein, and their respective glucoside forms. There are three glucoside forms for each aglucon: 6"-O-malonylglucoside, 6"-O-acetylglucoside, and the simple β-glucoside (Figure 3). The malonylglucoside of each aglucon family is the predominant form in soybeans (36), with prevalence of combined forms generally being genistein ≈ daidzein >>glycitein on a mole basis. When processed under mild conditions malonylglucosides will gradually convert to the β-glucoside forms (13, 15). Conversion of malonylglucosides to acetylglucosides occurs during more abusive conditions, such as toasting and extrusion (37, 38). In the presence of native soybean β-glucosidases, the glucoside isoflavones will be converted to their respective aglucon forms (15).

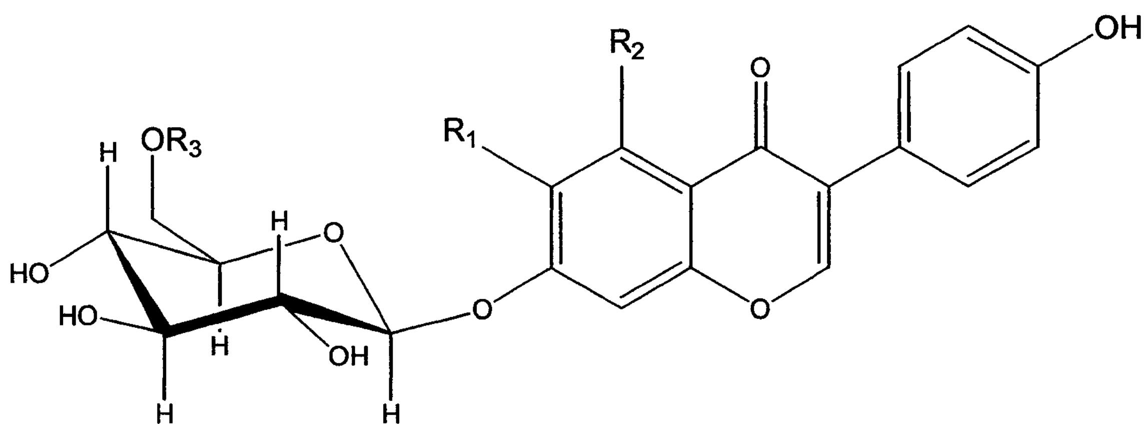
Table 15. Mass Balance Comparison of Products, Protein, and Phytochemicals of Pilot-Scale Soy Protein Isolate and Combined Glycinin, Intermediate, and β-Conglycinin Precipitates[a] from the Wu and Optimized Soy Protein Fractionation Processes

	Product Weight[b] (kg)	Total Protein (kg)	Isoflavones in Products (mmol)	Saponins in Products (mmol)
Wu[c]	5.58	5.01	32.3_b	29.3_b
Optimized	6.44	5.87	37.1_b	32.4_b
Soy Protein Isolate	6.71	5.98	53.0_a	46.0_a
LSD[d]	Not different	Not different	5.3	10.8

[a]Precipitate fractions prior to diafiltration and spray drying.
[b]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[c]n=2.
[d]LSD, least significant difference.



Isoflavone	R_1	R_2
Daidzein	H	H
Genistein	H	OH
Glycitein	OCH3	H



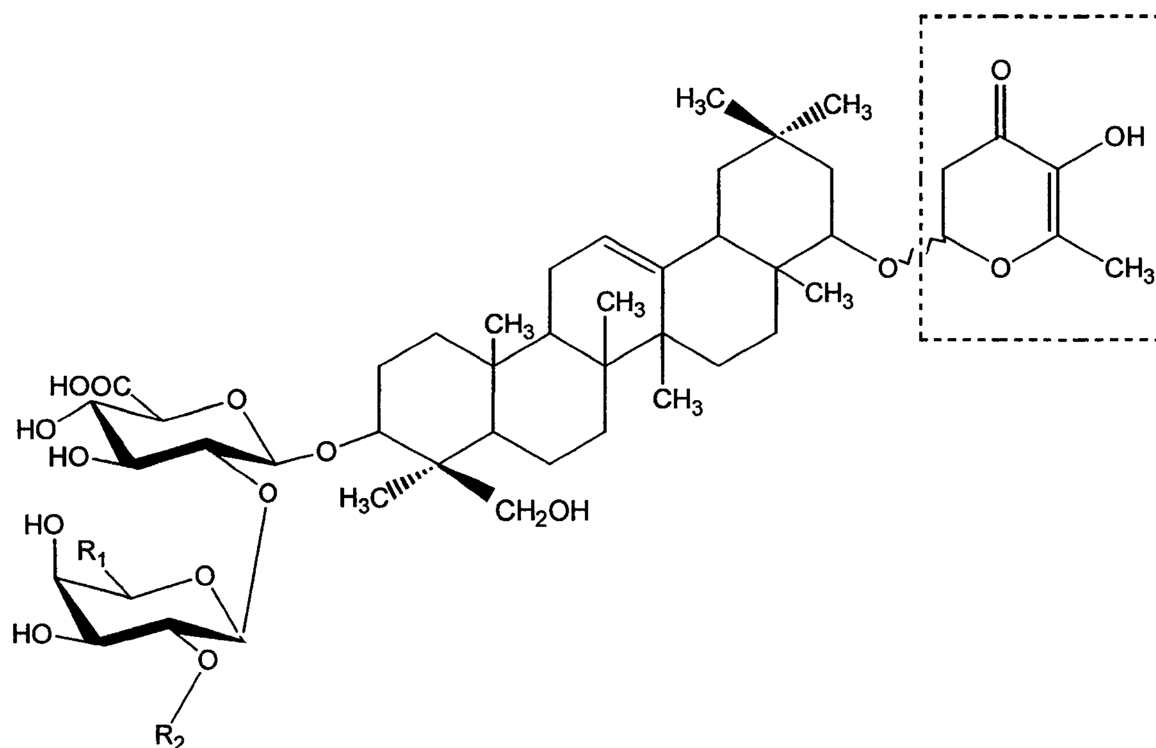
Isoflavone	R_1	R_2	R_3
Genistin	H	OH	H
Glycitin	OCH3	H	H
Daidzin	H	H	H
Malonylgenistin	H	OH	COCH2COOH
Malonylglycitin	OCH3	H	COCH2COOH
Malonyldaidzin	H	H	COCH2COOH
Acetylgenistin	H	OH	COCH3
Acetylglycitin	OCH3	H	COCH3
Acetyldaidzin	H	H	COCH3

Figure 3. Isoflavone structures.

The group B saponins that can be measured by the method of Hu and others (18) are V, I, II, αg, βg, and βa. The latter three have a 2,3-dihyro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) moiety that is labile, and these forms readily convert to V, I, and II, respectively (Figure 4). Group B saponins III, IV, γg, and γa are also present in soybeans, but only in very small quantities. It is difficult to isolate adequate quantities of these saponins to prepare standard curves.

For the Wu, optimized, and soy protein isolate processes a substantial portion, 30-35%, of the malonylglucoside isoflavones present in the defatted soy flakes were converted to the β-glucoside and aglucon forms, or for which could not be accounted (Table 16). Malonyldaidzin concentration in the soy flakes was 1.92 μmol/g compared to 1.24 and 1.36 μmol/g for the isolate precipitate and glycinin precipitate, respectively. The glycinin fraction was chosen for this comparison because processing times were similar for glycinin and soy protein isolate, and these fractions all had similar total isoflavone concentrations. Data suggest that there were two competing conversions acting on the malonylglucosides. For the isolate precipitate, malonylglucosides were converted to the β-glucosides and aglucons. There was essentially no conversion to the acetylglucoside forms because the process conditions were too mild. The protein solubilization temperature, 60 °C, drove the conversion to the β-glucosides, and this temperature probably limited the glucosidase activity responsible for conversion to the aglucons.

The conversion scheme was different between the Wu and optimized processes and also different than observed for the soy protein isolate. The data support the hypothesis that protein solubilization temperature affected the conversion process. For the glycinin precipitate of the Wu method, converted malonylglucosides were recovered as aglucons. In fact, the β-glucosides present in the starting material were also converted to aglucons. This suggests that there was considerable glucosidase activity, sufficient time for enzyme action, and insufficient effect by the process temperature to compete with this conversion and increase β-glucoside concentrations. For the optimized process, conversion of malonylglucosides to aglucons was not as substantial. Daidzin concentration decreased only



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

Figure 4. Structures of group B soyasaponins.
Rha: rhamnosyl; Glc: glucosyl; Y: yes; N: no
DDMP: 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one

Table 16. Isoflavone Profiles of Selected Pilot-Scale Soy Protein Fractionation and Soy Protein Isolate Process Fractions[a]

	Fraction	MDIN[b,c]	DIN	AcDIN	DEIN	MGLY	GLY	AcGLY	GLYEIN	MGIN	GIN	AcGIN	GEIN	Total
Wu[d]	GLY P	1.36_{b}	0.08_{de}	0.07_{abcd}	0.76_{bc}	0.15_{abc}	0.04_{cd}	nd[e]	0.13	2.42_{a}	0.18_{efg}	0.09_{ab}	0.97_{cd}	6.25_{b}
Opt.	GLY P	1.28_{b}	0.62_{c}	0.08_{abc}	0.39_{de}	0.18_{ab}	0.16_{b}	nd	0.05	2.06_{bc}	1.04_{c}	0.09_{ab}	0.41_{fghij}	6.37_{b}
Wu	GLY AD	0.23_{cd}	0.01_{e}	0.02_{cde}	0.30_{de}	0.03_{cd}	0.01_{d}	nd	0.04	0.88_{e}	0.05_{fg}	0.07_{abcdef}	0.63_{defgh}	2.27_{cde}
Opt.	GLY AD	0.22_{d}	0.09_{de}	0.02_{de}	0.13_{de}	0.05_{cd}	0.04_{cd}	nd	0.03	0.61_{ef}	0.30_{efg}	0.08_{abcd}	0.24_{ij}	1.80_{efg}
Wu	GLY SD	0.24_{cd}	0.02_{de}	nd	0.31_{de}	0.03_{cd}	0.01_{d}	0.01	0.08	0.86_{e}	0.07_{fg}	0.05_{bcdefg}	0.63_{defg}	2.30_{cde}
Opt.	GLY SD	0.21_{d}	0.09_{de}	0.01_{de}	0.14_{de}	0.05_{cd}	0.04_{cd}	0.04	0.02	0.59_{efg}	0.31_{efg}	0.08_{abcd}	0.25_{ij}	1.83_{ef}
Wu	INT P	1.45_{b}	0.14_{de}	0.05_{abcde}	1.49_{a}	0.15_{abc}	0.07_{cd}	nd	0.21	2.30_{ab}	0.41_{efg}	0.05_{bcdefg}	2.01_{a}	8.33_{a}
Opt.	INT P	1.48_{b}	0.87_{b}	0.09_{a}	0.92_{b}	0.19_{ab}	0.20_{b}	nd	0.14	2.03_{bc}	1.49_{b}	0.08_{abcd}	1.07_{c}	8.57_{a}
Wu	INT AD	0.12_{d}	0.02_{de}	nd	0.83_{b}	0.02_{cd}	0.02_{cd}	nd	0.08	0.33_{fgh}	0.13_{efg}	0.04_{defg}	1.67_{ab}	3.28_{c}
Opt.	INT AD	0.09_{d}	0.11_{de}	0.03_{cde}	0.40_{cde}	0.02_{cd}	0.04_{cd}	nd	0.05	0.25_{gh}	0.50_{de}	$0.06_{abcdefg}$	0.77_{cde}	2.32_{cde}
Wu	INT SD	0.12_{d}	0.01_{e}	nd	0.79_{b}	0.02_{cd}	0.02_{cd}	nd	0.07	0.31_{fgh}	0.08_{efg}	0.04_{defg}	1.62_{b}	3.08_{cd}
Opt.	INT SD	0.09_{d}	0.11_{de}	0.01_{e}	0.40_{de}	0.03_{cd}	0.04_{cd}	nd	0.05	0.24_{gh}	0.48_{de}	$0.06_{abcdefg}$	0.76_{cdef}	2.28_{cde}
Wu	BCON P	0.32_{cd}	0.02_{de}	nd	0.43_{cd}	0.03_{cd}	0.01_{cd}	nd	0.04	0.45_{fg}	0.06_{fg}	0.03_{efg}	0.56_{efghi}	1.96_{de}
Opt.	BCON P	0.56_{c}	0.25_{d}	0.03_{cde}	0.43_{cd}	0.08_{bcd}	0.07_{cd}	nd	0.06	0.82_{e}	0.44_{def}	0.05_{bcdefg}	0.54_{efghi}	3.33_{c}
Wu	BCON AD	0.03_{d}	nd	nd	0.06_{e}	nd	nd	nd	nd	0.05_{h}	0.02_{g}	nd	0.14_{j}	0.29_{h}
Opt.	BCON AD	0.02_{d}	0.03_{de}	nd	0.12_{de}	nd	0.02_{cd}	nd	0.01	0.06_{h}	0.12_{efg}	0.03_{efg}	0.28_{hij}	0.70_{fgh}
Wu	BCON SD	0.03_{d}	0.01_{de}	nd	0.06_{e}	0.01_{d}	nd	nd	nd	0.05_{h}	0.02_{g}	nd	0.13_{j}	0.31_{h}
Opt.	BCON SD	0.02_{d}	0.03_{de}	nd	0.12_{de}	nd	0.02_{cd}	nd	nd	0.06_{h}	0.12_{efg}	0.02_{g}	0.28_{hij}	0.67_{gh}
Isol	ISOL P	1.24_{b}	1.59_{a}	0.09_{ab}	0.22_{de}	0.23_{a}	0.40_{a}	nd	0.07	1.42_{d}	2.26_{a}	0.10_{a}	0.29_{ghij}	7.91_{a}
Isol	ISOL SD	1.23_{b}	1.55_{a}	0.02_{de}	0.22_{de}	0.21_{a}	0.39_{a}	nd	0.07	1.40_{d}	2.24_{a}	0.08_{bcde}	0.29_{ghij}	7.73_{a}
SF	FLAKES	1.92_{a}	0.71_{bc}	0.08_{ab}	0.09_{de}	0.22_{a}	0.17_{b}	0.04	0.02	1.83_{c}	0.81_{cd}	0.09_{ab}	0.07_{j}	6.01_{b}
LSD[f]		0.33	0.23	0.06	0.36	0.14	0.06	NDIFF[g]	NDIFF	0.35	0.41	0.04	0.35	1.15

[a]µmol/g, dry basis.

[b]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.

[c]Abbreviations: MDIN, malonyldaidzin; DIN, daidzin; AcDIN, acetyldaidzin; DEIN, daidzein; MGLY, malonylglycitin; GLY, glycitin; AcGLY, acetylglycitin; GLYEIN, glycitein; MGIN, malonylgenistin; GIN, genistin; AcGIN, acetylgenistin; GEIN, genistein; Wu, Wu process; Opt., optimized process; Isol, isolate; SF, soy flakes (ground); GLY, glycinin; INT, intermediate; BCON, β-conglycinin; P, precipitate fraction; AD, after dialysis; SD, spray dried.

[d]n=2.

[e]nd, none detected.

[f]LSD, least significant difference.

[g]NDIFF, not different.

slightly compared to the starting material and genistin concentrations actually increased slightly. The solubilization temperature, 45 °C, must have limited the action of the glucosidases. The intermediate and β-conglycinin precipitates also display the conversion features described for the glycinin precipitate. Isoflavone accounting problems were probably due to poor recovery of the aglucons from the glycinin fraction matrix. As described above, isoflavone aglucon recovery may be as low as 65-75% from soy protein isolate.

The effect of diafiltration on isoflavone loss into the permeate was demonstrated (Table 16). Spray drying did not cause a significant conversion among the isoflavones. The intermediate fraction had the highest concentration of aglucons. Again, if surface hydrophobicity or lack of protein native state have a role in attracting the relatively hydrophobic phytochemicals, it is predictable that the most hydrophobic isoflavones, the aglucons, would be present in higher concentrations in this fraction. The β-conglycinin fraction had the highest percentage of aglucons, >40%. Time for enzymatic activity, surface hydrophobicity, and partitioning of more hydrophilic forms into previous fractions probably all contributed to this result.

The DDMP forms of the saponins were in relatively high concentrations in the defatted soy flake starting material, similar to what was seen for the malonylglucosides of the isoflavones (Table 17). These DDMP forms, αg, βg, and βa were rapidly converted to saponin V, I, and II, respectively. One of the most surprising effects of the process on the saponins was nearly total conversion of DDMP forms to non-DDMP forms during diafiltration. This was most easily observed for the intermediate fraction precipitate (Table 16). The diafiltration process lasted only 3-4 h, yet conversion was nearly complete. Possibly the pressure of the diafiltration system was responsible for the conversion.

In conclusion, process improvement did lead to an increased overall fraction weight and protein recovery compared to the Wu method, approaching those for the soy protein isolate process. The β-conglycinin fraction was increased significantly, but at the cost of purity. Functionality testing will indicate whether decreased β-conglycinin purity is a disadvantage.

Table 17. Saponin Profiles of Selected Pilot-Scale Soy Protein Fractionation and Soy Protein Isolate Process Fractions[a]

	Fraction	V[b]	I	II	αg	βg	βa	Total
Wu[c]	GLY P	nd[d]	0.64_{fg}	0.31_{fgh}	nd	0.12_{d}	0.11_{d}	1.17_{gh}
Opt.	GLY P	nd	0.32_{g}	0.17_{gh}	nd	0.02_{d}	0.06_{d}	0.57_{h}
Wu	GLY AD	nd	0.63_{fg}	0.38_{efgh}	nd	nd	nd	1.01_{gh}
Opt.	GLY AD	nd	0.20_{g}	0.15_{h}	nd	nd	nd	0.35_{h}
Wu	GLY SD	nd	0.45_{g}	0.25_{gh}	nd	nd	nd	0.70_{h}
Opt.	GLY SD	nd	0.13_{g}	0.23_{gh}	nd	nd	nd	0.35_{h}
Wu	INT P	0.70	6.31_{cd}	3.17_{bc}	0.19	3.10_{a}	1.27_{a}	14.74_{ab}
Opt.	INT P	0.74	5.35_{d}	2.63_{c}	0.11	1.60_{b}	0.67_{b}	11.01_{d}
Wu	INT AD	0.98	10.10_{a}	5.29_{a}	nd	nd	nd	16.38_{a}
Opt.	INT AD	1.35	7.70_{bc}	3.76_{b}	nd	0.19_{d}	0.16_{d}	13.17_{bcd}
Wu	INT SD	0.81	8.61_{ab}	4.67_{a}	nd	nd	nd	14.09_{abc}
Opt.	INT SD	0.72	6.90_{c}	3.43_{bc}	nd	0.14_{d}	0.16_{d}	11.35_{cd}
Wu	BCON P	nd	0.72_{fg}	0.43_{efgh}	nd	0.23_{d}	0.11_{d}	1.50_{gh}
Opt.	BCON P	0.34	2.40_{e}	1.18_{de}	0.07	0.87_{c}	0.37_{c}	5.22_{ef}
Wu	BCON AD	nd	0.77_{fg}	0.39_{efgh}	nd	nd	nd	1.16_{gh}
Opt.	BCON AD	0.63	2.47_{e}	1.15_{def}	nd	0.20_{d}	0.12_{d}	4.57_{ef}
Wu	BCON SD	nd	0.74_{fg}	0.38_{efgh}	nd	nd	nd	1.13_{gh}
Opt.	BCON SD	0.29	2.08_{ef}	1.01_{defg}	nd	0.15_{d}	0.12_{d}	3.66_{fg}
Isol	ISOL P	0.55	3.26_{e}	1.43_{d}	0.04	1.14_{bc}	0.44_{c}	6.86_{e}
Isol	ISOL SD	0.57	3.33_{e}	1.49_{d}	0.09	1.09_{c}	0.41_{c}	6.98_{e}
SF	FLAKES	0.01	0.62_{fg}	0.35_{efgh}	0.19	2.98_{a}	1.13_{a}	5.28_{ef}
LSD		NDIFF	1.52	0.86	NDIFF	0.48	0.16	2.27

[a]μmol/g, dry basis.
[b]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[c]n=2.
[d]Abbreviations: V, I, and II are non-DDMP saponins; αg, βg, and βa are DDMP saponins; Wu, Wu process; Mod, modified process; Isol, isolate; SF, soy flakes (ground); GLY, glycinin; INT, intermediate; BCON, β-conglycinin; P, precipitate fraction; AD, after dialysis; SD, spray dried; Opt., Optimized; LSD, least significant difference; nd, none detected; NDIFF, not different.

The soy isolate contained significantly more phytochemicals than did either of the protein fractionation processes. It will be difficult to further modify the fractionation process to increase phytochemical yield in the product fractions while maintaining the ability to efficiently and effectively fractionate the proteins because different physicochemical protein properties are needed to achieve these two goals.

Acknowledgments

This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project N. 6571, was supported in part by USDA NRI grant No. 2001-35503-10814, NASA Food Technology Commercial Space Center, Hatch Act and State of Iowa funds.

Mark Reuber provided technical expertise for pilot-scale protein fractionation and soy isolate processing.

Literature Cited

(1) Utsumi, S.; Matsumura, Y.; Mori, T. Structure-function relationships of soy proteins. In *Food Proteins and Their Applications*; Damodaran S., Paraf A., Eds.; Marcel Dekker, Inc.: New York, NY, **1997**; pp 257-291.

(2) Kinsella, J.E. Functional properties of soy proteins. *JAOCS* **1979**, *56*, 242-258.

(3) Messina, M.J. Soyfoods: their role in disease prevention and treatment. In *Soybeans: Chemistry, Technology, and Utilization*; Liu, K.S., Ed.; Chapman and Hall, New York, NY, **1997**, pp 443-447.

(4) Thanh, V.H.; Shibasaki, K. Major proteins of soybean seeds. A straightforward fractionation and their characterization. *J. Agric. Food Chem.* **1976**, *24*, 1117-1121.

(5) O'Keefe, S.F.; Wilson, L.A.; Resurreccion, A.P.; Murphy, P.A. Determination of the binding of hexanal to soy glycinin and β-conglycinin in an aqueous model system using a headspace technique. *J. Agric. Food Chem.* **1991**, *39*, 1022-1027.

(6) Nagano, T.; Hirotsuka, M.; Mori, H.; Kohyama, K.; Nishinari, K. Dynamic viscoelastic study on the gelation of 7S globulins from soybeans. *J. Agric. Food Chem.* **1992**, *40*, 941-944.

(7) Davidson, R.M.; Sand, R.E.; Johnson, R.E. Method of processing soy protein and composition of matter. U.S. Patent 4,172,828, **1979**.

(8) Howard, P.A.; Lehnhardt, W.F.; Orthoefer, F.T. 7S and 11S vegetable protein fractionation and isolation. U.S. Patent 4,368,151, **1983**.

(9) Lehnhardt, W.F.; Gibson, F.T. Fractionation and isolation of 7S and 11S protein from isoelectrically precipitated vegetable protein mixtures. U.S. Patent 4,370,267, **1983**.
(10) Wu, S.; Murphy, P.A.; Johnson, L.A.; Fratzke, A.R.; Reuber, M.A. Pilot-plant fractionation of soybean glycinin and β-conglycinin. *J. Am. Oil Chem. Soc.* **1999**, *76*, 285-293.
(11) Wu, S.; Murphy, P.A.; Reuber, M.A.; Fratzke, A.R. Simplified process for soybean glycinin and β-conglycinin fractionation. *J. Agric. Food Chem.* **2000**, *48*, 2702-2708.
(12) Berk, Z. Isolated soy protein. In *Technology of Production of Edible Flours and Protein Products from Soybeans*; Berk, Z., Ed.; FAO, Rome, **1992**; pp 83-96.
(13) Murphy, P.A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G.R.; Trainer, D.; Holden, J. Isoflavones in retail and institutional foods. *J. Agric. Food Chem.* **1999**, *47*, 2697-2704.
(14) Franke, A.A.; Hankin, J.H.; Yu, M.C.; Maskarinec, G.; Low, S.H.; Custer, L.J. Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. *J. Agric. Food Chem.* **1999**, *47*, 977-986.
(15) Wang, H.J.; Murphy, P.A. Mass balance study of isoflavones during soybean processing. *J. Agric. Food Chem.* **1996**, *44*, 2377-2383.
(16) Wang, C.; Ma, Q.; Pagadala, S.; Sherrard, M.S.; Krishnan, P.G. Changes of isoflavones during processing of soy protein isolates. *J. Am. Oil Chem. Soc.* **1998**, *75*, 337-341.
(17) Xu, X.; Harris, K.; Wang, W.J.; Murphy, P.; Hendrich, S. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.* **1995**, *125*, 2307-2315.
(18) Hu, J.; Lee, S.O.; Hendrich, S.; Murphy, P.A. Quantification of the Group B saponins by high performance liquid chromatography. *J. Agric. Food Chem.* **2002**, *50*, 2587-2594.
(19) Ireland, P.A.; Dziedzic, S.Z.; Kearsley, M.W. Saponin content of soya and some commercial soya products by means of high-performance liquid chromatography of the saponins. *J. Sci. Food Agric.* **1986**, *37*, 694-698.
(20) Kitagawa, I.; Yoshikawa, M.; Hayashi T.; Taniyama T. Quantitative determination of saponins in soybeans of various origins and soybean products by means of high performance liquid chromatography. *Yakugaku Zasshi* **1984**, *104*, 275-279.
(21) Tsukamoto, C.; Shimada, S.; Igita, K.; Kudou, S.; Kokubun, M.; Okubo, K.; Kitamura, K. Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. *J. Agric. Food Chem.* **1995**, *43*, 1184-1192.
(22) AOAC. *Official Methods of Analysis of Association of Official Analytical Chemists*, 11th ed. AOAC: Washington, DC, **1970**; method 2.066.
(23) AOAC. *Official Methods of Analysis of Association of Official Analytical Chemists*, 15th ed.; AOAC: Arlington, VA, **1990**; methods 988.05 and 960.52.
(24) AOAC. *Official Methods of Analysis of Association of Official Analytical Chemists*, 15th ed.; AOAC: Arlington, VA, **1990**; method 990.03.
(25) Jung, S.; Rickert, D.A.; Deak, N.A.; Aldin, E.D.; Recknor, J.; Johnson, L.A.; Murphy, P.A. Comparison of Kjeldahl and Dumas nitrogen methods for protein content determination in soy products. *J. Am. Oil Chem. Soc.* **2003**, In press.
(26) AACC. *Approved Methods of the American Association of Cereal Chemistry*, 8th ed.; AACC: St. Paul, MN, **1983**; method 44-15A.

(27) AACC. *Approved Methods of the American Association of Cereal Chemistry*, 8th ed.; AACC: St. Paul, MN, **1983**; method 08-03.
(28) Bian, Y.; Myers, D.J.; Dias, K.; Lihono, M.A.; Wu, S.; Murphy, P.A. Functional properties of soy protein fractions produced using a pilot-scale process. *J. Am. Oil Chem. Soc.* **2003**, *80*, 551-555.
(29) Friedman, M.; Brandon, D.L. Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* **2001**, *49*, 1069-1086.
(30) Friedman, M. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. *J. Agric. Food Chem.* **1999**, *47*, 1295-1319.
(31) McLeod, G.S.; Shepherd M.J. Determination of the ionization constants of isoflavones by capillary electrophoresis. *Phytochem. Anal.* **2000**, *11*, 322-326.
(32) Krol, J. Waters Corp. Personal communication. **2000**.
(33) Murphy, P.A.; Barua, K.; Hauck, C.C. Solvent extraction selection in the determination of isoflavones in soy foods. *J. Chrom. B* **2002**, *777*, 129-138.
(34) Shen, J.L.; Bryan, B.A. Aglucone isoflavone enriched vegetable protein extract and isolate and process for producing. U.S. Patent 5,763,389, **1998**.
(35) Riblett, A.R.; Herald, T.J.; Schmidt, K.A.; Tilley, K.A. Characterization of β-conglycinin and glycinin soy protein fractions from four selected soybean genotypes. *J. Agric. Food Chem.* **2001**, *49*, 4983-4989.
(36) Wang, H.; Murphy, P.A. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. *J. Agric. Food Chem.* **1994**, *42*,1674-1677.
(37) Farmakalidis, E.; Murphy, P.A. Isolation of 6"-O-acetylgenistin and 6"-O-acetyldaidzin from toasted soy flakes. *J. Agric. Food Chem.* **1985**, *33*:385-389.
(38) Mahungu, S.; Diaz-Mercado, S.; Li, J.; Schwenk, M.; Singletary, K.; Faller, J. Stability of isoflavones during extrusion processing of corn/soy mixtures. *J. Agric. Food Chem.* **1999**, *47*, 279-284.

CHAPTER 5. PILOT-SCALE FRACTIONATION OF SOY STORAGE PROTEINS: FUNCTIONAL PROPERTIES

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

David A. Rickert, Lawrence A. Johnson, Patricia A. Murphy

Abstract

Two pilot-scale soy protein fractionation processes, differing by protein solubilization conditions, were each employed to produce glycinin, β-conglycinin, and an intermediate fraction, a mixture of the two proteins. These protein products were compared for functionality by measuring apparent viscosity, solubility, gel strength, foaming characteristics, emulsification capacity, emulsification activity, and emulsification stability. Differential scanning calorimetry, sodium dodecylsulfate-polyacrylamide gel electrophoresis, and surface hydrophobicity analyses were performed to assist in interpreting functionality characteristics. There were significant differences among protein fractions, and in some cases between the same protein fraction from the different processes. This suggests that the fractionation process and modifications to that process are capable of filling a range of functionality needs.

Introduction

The functional properties of soy proteins have been exploited in a multitude of applications, e.g. solubility in beverages, viscosity in gravies, gelling properties in meats, foaming in whipped toppings, and emulsification in hot dogs and sausages. The current popularity of soy, due to its relatively inexpensive ingredient cost and potential health benefits (Messina 1997), continues to drive soy research and commercial development of new food products.

Understanding the physicochemical properties of soy protein that translate into desirable functional properties is much more difficult than understanding soy ingredient economics and demand. Glycinin and β-conglycinin are the main storage proteins in soybean seeds, comprising nearly 70% of total seed protein (Nielsen 1985). Consequently, these proteins are largely responsible for the functional characteristics of soy ingredients, as well as those for traditional soy foods such as tofu. Glycinin and β-conglycinin are complex proteins. Under most conditions, glycinin is a hexamer of subunits, each of which contains an acidic and a basic polypeptide connected by a disulfide bond (Staswick and others 1984). There are at least five glycinin gene products leading to heterogeneity in mature glycinin macromolecules, providing multiple types of glycinin subunits. B-conglycinin is a trimer, comprised of different compositions of three possible subunits, α, α', and β (Nielsen 1985). Different forms of these subunits (α, α_2, α_3, β, β_2, and β_3) provide heterogeneity in the trimer. The glycinin and β-conglycinin contents in soybeans are different among varieties and are affected by the growing environment (Murphy and Resurreccion 1984, Fehr and others 2003). All of these factors can affect soy protein functionality.

Functionality is substantially affected by changes to the native state of the proteins during processing, because the proteins begin to unfold and hydrophobic regions of the interior are exposed. These changes in native state can be caused by heat processing during solvent removal after oil extraction, during protein solubilization for isolate production, or during the leaching process for soy protein concentrate production. For instance, ethanol extraction of oligosaccharides during soy protein concentrate production will denature soy proteins (Berk 1992). Limited proteolysis of soy proteins with enzymes has been used to alter protein native state, alter the size to charge ratio, and improve functionality characteristics such as solubility, foaming, and emulsifying capacity (Schwenke 1997, Nielsen 1997). These processes have provided soy products with different functional properties appropriate for various ingredient applications. Ionic strength and pH of the food systems in which these ingredients are used further influence their functionalities. For instance, soy protein solubility is generally lowest at pH 4-5. Ionic strength (e.g. as altered by NaCl

concentration) will affect solubility as well, but the extent of change in solubility is related to the pH of the system.

While industry has developed processes yielding a variety of soy products with useful functional properties, basic research has endeavored to elucidate how these two storage proteins influence soy protein functionality. Bench-scale fractionation procedures of Thanh and others (1976) and Nagano and others (1992), among others, have been used to produce glycinin- and β-conglycinin-rich fractions, characterized directly or with further purification, to address physicochemical and functional properties of these proteins. For instance, Utsumi and others (1984) evaluated heat-induced interactions between the two soy proteins. Fisher and others (1990) studied the effects of gelation and heat and chemical denaturation on glycinin and β-conglycinin. Wagner and Guéguen (1999a, b) evaluated the foaming and emulsifying properties of native, acid-treated and reduced soy glycinin. Lakemond and others (2000) evaluated the effects of pH and ionic strength soy glycinin. Wagner and Guéguen (1995) compared unmodified glycinin to deamidated and reduced glycinin for surface active properties. Studies have even been conducted with regard to subunit composition or the subunits themselves. Liu and others (1999) evaluated the emulsifying properties of the acidic subunits of glycinin. Maruyama and others (1999) studied the structure-physicochemical function relationships of β-conglycinin subunits with respect to solubility, surface hydrophobicity, thermal stability, heat induced association, and emulsifying ability.

Efforts to evaluate glycinin and β-conglycinin properties have influenced plant breeding. Nagano and others (1996) evaluated the gelation properties of soy protein isolates produced from low β-conglycinin and low glycinin soybean lines. Bringe (2001) disclosed that a high β-conglycinin soybean line had useful characteristics for certain food applications. Khatib and others (2002) and Riblett and others (2001) have fractionated glycinin and β-conglycinin from several soybean lines with different protein compositions and compared functional characteristics among the lines.

A substantial body of research has been accomplished using bench-scale fractionation of glycinin and β-conglycinin. Information about products from pilot-scale applications is

much more limited. Fractionation processes have been patented (Davidson and others 1979, Lehnhardt and others 1983), but it is not clear whether these are pilot-scale or bench-scale processes. Wu and others (1999) describe a pilot-scale process to produce kg quantities of glycinin, β-conglycinin, and an intermediate fraction consisting of a combination of the two proteins.

The objective of the current work was to compare protein produced by the method of Wu and others (1999), an optimization of Wu's fractionation method, and soy protein isolate, all produced at pilot scale. These data may suggest potential food system applications for the fractionated proteins.

Materials and Methods

Storage protein fractionation and soy protein isolate production

The starting material for the soy protein fractionation and soy protein isolate processes was defatted soy white flakes (from IA 2042 variety, 2000 harvest, 84% protein dispersibility index) produced at the Research Oil Mill at Texas A&M University in August 2001. The flakes were stored in sealed containers at 4 °C until use.

Extraction conditions for soy protein fractionation were 15:1 water-to-flake ratio at 20 °C, used by Wu et al. (10) for previous pilot-scale fractionation, or 10:1 water-to-flake ratio at 45 °C (optimized conditions). Flakes (20.0 kg) and temperature-adjusted tap water were combined by stirring at 22 rpm in a jacketed 800-L tank (Walker Stainless Equipment Company, New Lisbon, WI), adjusted to pH 8.5 with 2 N NaOH, maintained at that pH, and stirred at 13 rpm for 1 h. Slurry was fed to an Alfa Laval BTPX disc stack centrifuge (Alfa Laval Separation Inc., Warminster, PA) at 9800 rpm bowl speed (approximately 12,000 x *g*) at a feed rate of ~2 L/min with a Moyno transfer pump (Electric Pump, Model IFFCA SSE SAA, Des Moines, IA) at 300 rpm to separate soluble and insoluble components.

The protein extract was treated with $NaHSO_3$ to provide 10 mM SO_2, adjusted to pH 6.4 with 2N HCl, and cooled overnight at ~7 °C. Glycinin-rich precipitate was recovered using the Alfa Laval centrifuge at 9800 rpm bowl speed and 420 rpm transfer pump speed (~4

L/min). The resulting supernatant was adjusted to 0.25 M NaCl and pH 5.0, stirred at 13 rpm for 1 h, and then centrifuged at 9800 rpm bowl speed with a 420 rpm transfer pump speed. The resulting intermediate fraction precipitate was comprised of a mixture of glycinin, β-conglycinin, and other proteins. The supernatant was diluted 2:1 with water, adjusted to pH 4.8, and chilled overnight at ~7 °C. The β-conglycinin-rich precipitate was removed by centrifugation at 9800 rpm bowl speed with a 420 rpm transfer pump speed. Recovered protein fractions were adjusted to pH 7.5 with 2N NaOH and then desalted with a Feed and Bleed Membrane Filtration System (Model SRT-50; North Carolina SRT Inc., Cary, NC) and a 30-KD regenerated cellulose membrane (North Carolina SRT Inc.). Diafiltration was continued until permeate equaling 5X the original volume of protein dispersion was collected. The protein retentate was dried in an Anhydro Compact Spray-Dryer (APV Crepaco Inc., Attleboro Falls, MA) with an air inlet temperature of 160 °C and air outlet temperature of 85 °C.

Deviations from the pilot-scale process described by Wu et al. (10) were as follow. Wu's protein extract was separated from the insoluble fraction using a two-pass system through a Sharples P660 decanting centrifuge (Alfa Laval Separation Inc., Warminster, PA) instead of a single pass through the Alfa Laval BTPX 204 disc stack centrifuge. Wu chilled overnight at 4 °C, while we chilled at ~7 °C.

For soy protein isolate production, flakes (20.0 kg) were extracted with a 10:1 water-to-flake ratio at 60 °C. Flakes and temperature-adjusted water were combined with mixing as above and adjusted to pH 8.5 with 2 N NaOH. The slurry was stirred for 30 min at 13 rpm, and then the insolubles were removed by centrifugation as above. The supernatant was adjusted to pH 4.5 using 2N HCl and then chilled overnight at ~7 °C. The soy protein isolate was recovered by centrifugation, adjusted to pH 6.8, and then spray dried.

Other protein samples

Bovine serum albumin Fraction V (A-7906, Sigma, St. Louis, MO) and Supro 670 (Protein Technologies International, St. Louis, MO) were evaluated by some assays.

Proximate analysis

Nitrogen content of the defatted soy flakes and process fractions was measured using the combustion or Dumas method (AOAC 990.03) with a Rapid NIII Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). These values were converted to Kjeldahl nitrogen concentrations using a conversion formula (Jung and others 2003). A 6.25 conversion factor was used to convert percentage nitrogen to protein content. Moisture and ash were determined (AACC method 44-15A, AACC method 08-03).

Urea-SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed using the method described by Wu and others (1999). A 25-mg protein sample was dissolved in 10 mL of protein extraction buffer (50 mM 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 (125 mM THAM, pH 6.8, 5.0 M urea, 0.2% SDS, 20% glycerol, and 0.01% Bromophenol blue). Each lane was loaded with 45 μg protein. 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 and then destained in the same solution without Coomassie Blue. Densitometry was determined using Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech Image Scanner (Amersham Pharmacia, Piscataway, NJ).

SDS-PAGE results were calculated as follows. Densitometry values for lipoxygenase, β-conglycinin subunits, and glycinin subunit bands were summed. This sum was then used as 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 densitometry analysis.

Differential scanning calorimetry

Samples (15-20 mg) of 10% (w/w) dry basis protein dispersion were hermetically sealed in aluminum pans. A sealed, empty pan was used as reference. The samples were analyzed

at 10 °C/min in a range of 25-120 °C using an SII Exstar 6000 (Seiko Instrument, Inc., Japan). All samples were repeated at least four times.

Surface hydrophobicity

Surface hydrophobicity was measured by using a modification of the method used by Wu and others (1999), using 1-anilino-8-naphthalene sulfonic acid magnesium salt monohydrate (ANS, ICN Biomedicals, Inc., Aurora, OH). A 1% (w/w) dry-basis sample dispersion was prepared in de-ionized water and the pH was adjusted to 7 using 2N HCl or NaOH. The dispersions were stirred for 1 h, with pH adjusted at 15, 30, and 60 min. Undissolved material was removed by centrifugation at 10000 x *g* for 10 min at 20 °C. The protein content of the supernatant was determined using the biuret method (AOAC 1970) with bovine serum albumen (Sigma, St. Louis, MO) as the standard. Soluble protein was diluted with 0.1 M phosphate buffer (pH 7.0) to obtain concentrations ranging from 6.25 to 1000 µg/mL. ANS (40 µL, 8.0mM in 0.01 M phosphate buffer) was dispersed in 3-mL aliquots of each dilution. Fluorescence intensity units (FIU) were measured with a Turner Quantech® spectrophotometer (Barnstead Thermolyne, Dubuque, IA) using 440 nm (excitation) and 535 nm (emission) filters. FIU were standardized using a solution of 40 µL ANS in 3 mL phosphate buffer as the zero point and 15 µL ANS in 3 mL of methanol assigned a value of 80 FIU. The FIU were plotted versus percent protein concentration. The slope of the regression line was considered to be the surface hydrophobicity. Samples were run in triplicate.

The conditions and parameters used by Wu and others (1999) that were different from those stated above were as follow. Protein was serially diluted in the range of 1-100 µg/mL. Excitation and emission wavelengths were 350 and 525 nm, respectively. A 100-µL aliquot of ANS was added to 5 mL of diluted protein.

Solubility

A 1% (w/w) dry-basis sample dispersion was prepared in de-ionized water, and the pH was adjusted to 3, 4, 5, 6, 7, 9 or 11 using 2N HCl or NaOH. The dispersions were stirred for 1 h, with pH adjusted at 15, 30, and 60 min. Aliquots (30.0 g) of dispersions were portioned into 50-mL centrifuge tubes and centrifuged at 10000 x *g* for 10 min at 20 °C. Supernatant was measured for protein content using the biuret method with bovine serum albumen as the standard. Solubility was calculated as follows:

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

This method was a modification of Bian and others (2003). Bian used a 0.1% protein dispersion, 0.1 M HCl and NaOH, and centrifugation at 32,500 x *g*.

Emulsification capacity

Emulsification capacity was measured using the method of Bian and others (2003). Twenty-five g of a 2% (w/w) dry-basis sample dispersion adjusted to pH 7, 5, or 3 with 2N HCl or NaOH was transferred to a 400-mL plastic beaker. Soybean oil, dyed with Fat Red 7B (Sigma, St. Louis, MO), was continuously blended into the dispersion at a rate of 37 mL/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 at least in triplicate.

Emulsification activity and stability

This is a modification of the method of Chove and others (2001). Twenty-one mL of a 2% (w/w) dry basis sample dispersion at pH 7 was blended with 7 mL of fully refined soybean oil (Bakers and Chefs Vegetable Oil, North Arkansas Wholesale Company Inc., Bentonville, Arkansas) in a 250-mL beaker for 1 min using a Bamix wand mixer (ESGE AG

Model M133, Mettlen, Switzerland) at low speed. Immediately after mixing, the emulsion was diluted 1:1000 with 0.1% sodium dodecylsulfate. The absorbance at 500 nm was measured, and this absorbance was recorded as the emulsification activity. After 15 min the absorbance was measured again. These two absorbance readings were used to calculate the emulsification stability.

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

Where: A_0 and A_{15} are absorbance at time zero and 15 min, respectively, and t is the time interval. Samples were run in triplicate.

Modifications to the method of Chove and others (2001) were as follow. Chove and others (2001) used a 1:4000 dilution. They serially diluted samples at 0 and 10 min, rather than measuring the absorbance of the same dilution at 0 and 15 min. Chove and others (2001) measured time zero absorbance at 500 nm and used this value to calculate an emulsification activity index.

Foaming

A 0.5% (w/w) dry-basis sample dispersion was prepared at pH 7. A 95-mL aliquot was loaded into a custom-designed glass column (58.5 cm x 2 cm) with a coarse glass frit at the bottom, and N_2 was purged through the sample at 100 mL/min flow rate. Time for the foam to reach the 300-mL mark, time for one-half of the liquid incorporated into foam to coalesce to liquid, and volume of liquid incorporated into foam were measured. Three parameters were calculated:

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

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

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

Where: V_f = a fixed volume of 300 mL, f_r = the flow rate of gas in mL/min, t_f = time in min to reach V_f, V_{max} = the volume in mL of liquid incorporated into foam, and $t_{1/2}$ = the time for draining half the liquid incorporated into foam. Samples were run in triplicate.

Viscosity

A 10% (w/w) dry-basis sample dispersion was prepared at pH 7. The sample was applied to the plate of a RS-150 Rheo Stress (Haake, Karlsruhe, Germany) and shear was applied with a 60-mm 2° titanium cone (C60/2Ti) over the range of 10-500/s at 23 °C. Shear rate ($\dot{\gamma}$) and shear stress (τ) over the course of the analysis, in combination with the power law formula application, were used to determine the consistency coefficient (K) and the flow behavior index (n), where $\tau = K\dot{\gamma}^{n}$. Using k, n, and $\dot{\gamma}$, apparent viscosity (η) was estimated by the following formula: $\eta = K\dot{\gamma}^{n-1}$. Samples were run in triplicate.

Gelling

A 10% (w/w) dry-basis sample dispersion was prepared and the pH was adjusted to 3, 5, or 7 using 2N HCl. Due to foaming problems, samples were stirred slowly to reduce bubbles and an aliquot from the bottom of the container was portioned into a 30-mL No. 14000 KIMAX® beaker. Beakers were incubated in a water bath at 80 or 99 °C for 30 min, cooled in a water bath, and chilled overnight at 4°C. Gel strength was measured with a TA-XT2 Analyzer (Texture Technologies Corp., Scarsdale, NY) using Texture Expert Version 1.22 software (Stable MicroSystems Ltd., Surrey, England). The probe was a TA-11 1" diameter acrylic AOAC cylinder 35-mm tall. Test speeds were: pre-test, 2 mm/s; test, 0.9 mm/s; and post-test, 0.9 mm/s. The compression distance was 10 mm. The highest point on the analysis curve was regarded as the peak force and was expressed in N. The area under the analysis curve was regarded as the total force or work and was expressed in Nmm. Samples were run at least in triplicate.

Statistical analysis

Least square means, least significant differences, and ANOVA results were calculated using the SAS system (version 6, SAS Institute Inc., Cary, NC).

Results and Discussion

The pilot-scale protein fractionation processes employed for this study produced three distinct protein fractions: glycinin, intermediate, and β-conglycinin. The intermediate fractionation was a mixture of glycinin and β-conglycinin. Precipitation of the intermediate fraction was employed to increase the purity of the β-conglycinin fraction. In previous work Wu and others (1999) determined by rocket immunoelectrophoresis that most of the glycinin and β-conglycinin in the intermediate fraction was denatured.

The material for the current characterization and functionality comparison study was produced during a previous study (Chapter 4). The current optimized process used a lower water-to-flake ratio (10:1) and higher temperature (45 °C) during protein solubilization compared to the control, or Wu, process (15:1 water-to-flake ratio and 25 °C). For soy proteins, the most significant effect of this process modification was increased β-conglycinin fraction at the expense of decreased purity of the β-conglycinin.

Increasing protein solubilization temperature should increase protein denaturation, with extent of denaturation being directly related to the increase in temperature. This was observed in process improvement experiments with bench-scale evaluation of solubilization temperatures. A solubilization temperature of 55 °C, near the onset temperature of approximately 68 °C for β-conglycinin denaturation (Riblett and others 2001), increased the weight of the intermediate fraction at the expense of the β-conglycinin fraction, supporting Wu and others' (1999) observation of denatured proteins in the intermediate fraction. We hypothesized that if a protein solubilization temperature of 45 °C affected protein native state, there should be a difference in functionality between protein products from the two fractionation methods used here. Protein denaturation can significantly affect protein functionality by opening the protein and exposing hydrophobic regions sheltered in the interior. Exposed hydrophobic groups may decrease solubility through aggregation and increase foaming and emulsification by creating a more amphipathic molecule.

The glycinin, intermediate, and β-conglycinin fractions produced by the optimized and Wu fractionation process were desalted prior to spray drying, because protein functionality was to be evaluated. Salts and ionic strength can alter protein functionality (Kinsella 1979). Wu- and optimized-method mean ash concentrations for the glycinin, intermediate, and β-conglycinin precipitate fractions were about 3.3, 13.8, and 8.2%, respectively, but were reduced to 1.8, 2.6, and 2.7% after diafiltration.

Soy protein isolate was prepared using a pilot-scale process for further comparison purposes. The isolate was not desalted so that it would more accurately reflect commercial isolates, which may contain a maximum of 4.5% ash (Anonymous 2003). The ash content of the isolate averaged 5.7%.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

SDS-PAGE was used to compare relative amounts of glycinin, β-conglycinin, and lipoxygenase in the process fractions (Table 1). Lipoxygenase is an enzyme capable of producing undesirable flavor profiles in soy products if not inactivated, but it is used for its bleaching properties in baked products. Compared to the α, α′, and β bands of the β-conglycinin and acidic and basic bands of the glycinin fraction, it is the only other prominent band in protein extracts from soy. The soy protein isolate had approximately equal quantities of glycinin and β-conglycinin, but also a small amount of lipoxygenase. The protein distributions of the optimized- and Wu-method intermediate fractions were similar to that of the soy protein isolate, but the intermediate fractions had about twice as much lipoxygenase. The optimized process glycinin contained significantly less β-conglycinin (10.2%) than did the Wu-process glycinin fraction (14.7%), but glycinin purities were not significantly different. β-Conglycinin fraction purities for both processes were less than 80% and were significantly different. The optimized-process β-conglycinin contained significantly more glycinin compared to the Wu-process β-conglycinin.

Table 1 - Distribution of proteins in pilot-scale soy protein isolate and products of soy storage protein fractionation

	Lipoxygenase (%)[a]	β-Conglycinin (%)	Glycinin (%)
Soy protein isolate[b]	5.2_{bc}	47.2_{c}	47.6_{b}
Wu process glycinin	nd[c]	14.7_{e}	85.3_{a}
Optimized process glycinin	nd	10.2_{f}	89.9_{a}
Wu process intermediate	11.4_{a}	41.0_{d}	47.6_{b}
Optimized process intermediate	10.1_{ab}	39.4_{d}	50.5_{b}
Wu process β-conglycinin	3.1_{c}	79.1_{a}	17.9_{d}
Optimized process β-conglycinin	4.2_{c}	67.8_{b}	28.1_{c}
LSD[d]	5.5	4.5	5.0

[a]Subscript, lowercase letters within a column indicate significant differences at $P<0.05$.
[b]n=2.
[c]nd, none detected.
[d]LSD, least significant difference.

Differential scanning calorimetry (DSC)

DSC analysis was used to evaluate how much native-state glycinin and β-conglycinin were present in the protein products (Table 2). Higher enthalpies indicate more native-state protein. The soy protein isolate had almost no native-state β-conglycinin, even though SDS-PAGE revealed that about half of the protein in the fraction was β-conglycinin. The protein solubilization temperature used for the isolate process was 60 °C. Riblett and others (2001) revealed that the onset temperature for denaturation of β-conglycinin from four soybean lines was about 69 °C and the peak temperature was about 76 °C. The 60 °C solubilization temperature likely caused the loss of β-conglycinin native state.

The glycinin-fraction DSC curves made it impossible to separate β-conglycinin enthalpies from the glycinin enthalpies. Consequently, the enthalpies for the glycinin fractions were overstated, and no β-conglycinin contaminant enthalpies were reported independently. Wu and others (1999) measured native state of their process fractions using rocket immunoelectrophoresis and reported that no native-state β-conglycinin was present in the glycinin fraction. However, the enthalpy curves for our glycinin, from both the optimized- and Wu-process fractions, were quite large and started at the onset temperature for denaturation of β-conglycinin, observed during DSC analysis of the β-conglycinin fraction, suggesting some native-state β-conglycinin was present. There was no significant difference between glycinin enthalpies for the glycinin fractions of the Wu and optimized processes.

The intermediate fractions contained relatively low amounts of native-state proteins. Wu and others (1999) determined that almost no native-state proteins remained in their intermediate fractions. In the current work, the Wu process had a higher enthalpy for β-conglycinin in the β-conglycinin fraction than did the optimized process. This may have been due to the difference in protein solubilization temperatures for the two different fractionation processes. The higher solubilization temperature of the optimized process may have led to increased denaturation of the β-conglycinin and therefore lower enthalpy.

Table 2 – Enthalpies of storage proteins in pilot-scale soy protein isolate and protein fractionation products determined by differential scanning calorimetry

	Enthalpy (mJ/mg protein, dry basis)	
	β-Conglycinin[a]	Glycinin
Soy protein isolate[b]	0.08_d	7.98_b
Wu-process glycinin	nd[c]	21.78_a
Optimized-process glycinin	nd	22.82_a
Wu-process intermediate	1.08_c	1.40_c
Optimized-process intermediate	0.58_{cd}	1.98_c
Wu-process β-conglycinin	7.53_a	0.52_c
Optimized-process β-conglycinin	5.85_b	0.89_c
LSD[d]	0.87	3.05

[a]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[b]n=2.
[c]nd, none detected.
[d]LSD, least significant difference.

Surface hydrophobicity

Surface hydrophobicity contributes to the functionality of proteins. Table 3 shows the surface hydrophobicities for the process fractions. The magnitude of the fluorescence intensity is correlated with surface hydrophobicity. Soy protein isolate had the highest surface hydrophobicity. The native-state data (Table 2) indicate that there was almost no native-state β-conglycinin in the isolate, and possibly a diminished amount of native-state glycinin. Because proteins unfold during denaturation, the hydrophobic regions of the protein interior become exposed. Subsequent re-arrangement of the protein may again shelter some of these regions, but the surface hydrophobicity may be increased if the re-arrangement does not take the molecule back to native state. Wagner and others (1996) demonstrated that when soy protein isolate was acidified from pH 4.5 to pH 1.0 at RT, there was a substantial increase in denaturation of the protein as measured by DSC. The surface hydrophobicity increased as the pH was decreased, indicating a relationship between loss of native state and surface hydrophobicity.

For the Wu process, the glycinin, intermediate, and β-conglycinin hydrophobicities were 114, 105, and 80, respectively, but they were 129, 183, and 124, respectively, for the optimized process. The higher hydrophobicities for the optimized-process fractions were probably due to the extent of protein denaturation caused by the higher protein solubilization temperature. The DSC data supported this reasoning for the β-conglycinin fraction, but were not convincing for the glycinin and intermediate fractions. The hydrophobicity of the soy protein isolate was about twice that of the Wu process fractions. This is in agreement with the hydrophobicity data reported by Wu and others (1999). The hydrophobicity of their glycinin and intermediate fractions were not statistically different, in agreement with results from our Wu method, but their β-conglycinin fraction was more hydrophobic than were their glycinin and intermediate fractions. Hayakawa and Nakai (1985) also determined that β-conglycinin was more hydrophobic than glycinin. In contrast, our optimized- and Wu-process β-conglycinins had lower hydrophobicity compared to the glycinin and intermediate fractions, at least within their respective process.

Table 3 - Surface hydrophobicity of pilot-scale soy protein isolate and soy storage protein fractionation products

	Hydrophobicity[a]
Soy protein isolate[b]	227_a
Wu-process glycinin	114_c
Optimized-process glycinin	129_c
Wu-process intermediate	105_c
Optimized-process intermediate	183_b
Wu-process β-conglycinin	80_d
Optimized-process β-conglycinin	124_c
LSD[c]	25

[a]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[b]n=2.
[c]LSD, least significant difference.

Solubility

The preferred solubility of soy protein depends on its application. For meat applications, relatively low solubility is desired so that the protein is not lost during processing, providing water-holding and fat-binding capabilities. Applications such as emulsification and foaming require a very soluble protein, which moves rapidly to interfaces and stabilizes them. The isoelectric region, the range where net charge is at a minimum, is between pH 4.2 to 4.6 for soy protein. This is the pH range where they generally display the lowest solubility. At pH extremes, especially under alkaline conditions, solubility is generally improved by dissociation and disaggregation of the proteins (Ishino and Ikamato 1975). It is important to report the nature of the solvent used to evaluate solubility since ionic strength can dramatically affect solubility (Shen 1976).

The solubility profiles of the glycinin, intermediate, and β-conglycinin fractions, as well as soy protein isolate, are shown in Table 4. The point of lowest solubility differs among the products. The soy protein isolate had low solubility between pH 4 and 5, approximately the isoelectric range for soy proteins. The region of lowest solubility for glycinin was pH 5 to 6. In protein fractionation schemes, pH values around 6.4 have been used to precipitate the glycinin fraction from β-conglycinin (Wu and others 1999). These data suggest why that is a prudent choice. The isoelectric regions of the intermediate fraction curves were similar to those for soy protein isolate. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) results for the isolate and the intermediate fractions were similar in peptide band array, showing approximately equal portions of glycinin and β-conglycinin (Table 2). However, the differential scanning calorimetry (DSC) data showed that there was much more native-state glycinin in the isolate than in the intermediate fraction. The isoelectric region for the β-conglycinin fractions was very sharp, occurring near pH 5.

There were no significant differences between the Wu- and optimized-method solubility curves for the glycinin and β-conglycinin fractions. The intermediate-fraction solubility curves showed lower solubility across most of the pH range compared to the glycinin and β-conglycinin fractions, probably due to the amount of denatured proteins in the intermediate

Table 4 - Solubility of soy protein isolate and fractionated soy proteins (%)

	pH 3[a]	pH 4	pH 5	pH 6	pH 7	pH 9	pH 11
Soy protein isolate[b]	91.6_{a}	5.3_{d}	4.70_{a}	91.5_{a}	91.8_{a}	92.0_{a}	92.8
Wu-process glycinin	88.5_{a}	72.6_{ab}	0.00	1.6_{d}	84.5_{a}	88.0_{a}	88.2
Optimized-process glycinin	88.7_{a}	87.8_{a}	1.66_{bc}	9.9_{d}	87.9_{a}	89.8_{a}	90.7
Wu-process intermediate	53.4_{b}	22.1_{d}	0.49_{c}	40.3_{c}	47.9_{b}	53.8_{b}	83.7
Optimized-process intermediate	79.8_{a}	44.0_{c}	2.61_{b}	64.0_{bc}	74.4_{a}	81.6_{a}	93.9
Wu-process β-conglycinin	87.5_{a}	83.1_{ab}	0.00	84.7_{ab}	86.8_{a}	87.9_{a}	88.9
Optimized-process β-conglycinin	88.3_{a}	66.5_{b}	0.00	86.9_{ab}	89.7_{a}	89.2_{a}	88.3
LSD[c]	11.9	18.9	1.19	26.6	21.3	13.1	NDIFF[d]

[a]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.

[b]n=2.

[c]LSD, least significant difference.

[d]NDIFF, not different.

fraction. Supro 670, a commercial isolate that receives substantial high temperature treatment during processing, displayed no native-state glycinin or β-conglycinin and demonstrated somewhat lower solubility than the isolate across the pH range evaluated (data not shown).

The hydrophobicity of the pilot plant soy protein isolate was much higher than most of the other protein fractions. High surface hydrophobicity can lead to lower solubility due to attraction of hydrophobic regions of the protein molecules, causing aggregation. However, surface hydrophobicity is not always a good indicator of solubility. Bovine serum albumen (BSA), as measured here, had a surface hydrophobicity of 2001, compared to 227 for the soy protein isolate. However, the percentage solubility of the BSA was 99% at pH 5, while soy protein isolate solubility was less than 5% at pH 5. The difference between these proteins is that BSA has a high ratio of hydrophilic to hydrophobic groups on its surface, so its solubility is not as influenced by its isoelectric point (Damodaran 1997). BSA also has a molecular weight of about 68 kD, much less than the 180 and 320 kD molecular weights of β-conglycinin and glycinin, respectively.

The optimized-process intermediate fraction displayed higher solubility across the measured pH range compared to the Wu-process intermediate fraction. The second replication of the optimized-process intermediate fraction demonstrated considerably higher solubility across the range of pH values, compared to the first replication, greatly influencing the average pH values of the replications. The second replication had three times as much native-state glycinin, measured by DSC, and had about 10% more glycinin and 50% less lipoxygenase, as measured by SDS-PAGE. These aspects may have altered the solubility profile. Compared to the first replication, more acid was required to adjust the pH to 5.0 to precipitate the intermediate fraction of the second replication of the optimized method. The dry-basis fraction weight of the second replication glycinin fraction was about 11% less compared to the first replication. This suggests that more glycinin was available for precipitation and was precipitated into the intermediate fraction of the second replication of the optimized method.

The intermediate fractions tend to have lower solubilities, but the solubilities of the glycinin, intermediate, and β-conglycinin fractions were not so substantially different to be recommended for different applications. The largest solubility differences were due to the isoelectric points of the proteins, (e.g. glycinin compared to β-conglycinin) and exploiting these differences may lead to varied applications.

Bian and others (2003) evaluated solubility of Wu-process (called Nagano process in their paper) glycinin, β-conglycinin, and intermediate fractions from material prepared by Wu and others (1999). Results at pH 2 to 10 were nearly identical to those reported here.

Emulsification capacity

Emulsification capacity is a critical functionality in food systems such as sausages, batters, and salad dressings. The role of proteins in emulsion formation and stability is to decrease the interfacial tension between water and oil fractions in the food system. Ability of the protein to move rapidly to the interface and remain at the interface, preventing coalescence, determines the effectiveness of an emulsifier. Other factors affecting emulsification are protein concentration, surface charge, and molecular flexibility capable of revealing hydrophobic regions that can better interact with the oil phase.

The emulsification capacity assay employed here involved rapid mixing of oil into a protein dispersion. Mixing continued until phase inversion occurred. This method measured how much oil the protein can stabilize, but it did not provide a measure of the texture of the emulsion and the stability of the emulsion. As discovered through mixer calibration, energy input can easily have a more dramatic effect on the results than the nature of the protein. Effect of mixing and shearing on emulsification results has been documented (Tornberg 1978, Waniksa and others 1981). Emulsification capacity was evaluated at pH: 7, 5, and 3. These values were selected because they fall within the range of most food systems and to determine whether pH might influence protein fraction choice for maximum emulsification capacity.

At pH 7, the β-conglycinin fractions of the Wu and optimized processes had the highest emulsification capacities (Table 5), each over 600 g oil/g protein. This suggests that the surface hydrophobicity of the β-conglycinin was rapidly altered under emulsification conditions or actually had higher surface hydrophobicity than reported above. The surface hydrophobicities of BSA, soy isolate, and Supro 670, a commercial isolate with no remaining native-state storage proteins, were 2002, 227, and 94, respectively, while their emulsification capacities were 1026, 431, and 578 g oil/g sample, respectively. The low emulsification capacity of the Supro 670 soy protein isolate compared to our soy protein isolate was surprising, because the surface hydrophobicity of the Supro 670 was actually higher. High surface hydrophobicity should translate to high emulsification capacity. Perhaps the lower solubility of the Supro 670, about half that of soy protein isolate at pH 7, affected emulsification capacity by altering its ability to move to the emulsification interface and stabilize the interface.

The emulsification capacities of the glycinin fractions were significantly lower than those observed for β-conglycinin. Aoki and others (1980) showed that a β-conglycinin-rich fraction generally had better emulsifying capacity than did a glycinin-rich fraction. It has been suggested that because β-conglycinin is a smaller molecule than glycinin, β-conglycinin can move to the interface more quickly (Utsumi and others 1997). In addition, the intra- and intersubunit disulfide bonds in glycinin cannot undergo rapid rearrangement at the interface and expose hydrophobic regions that interact with the oil phase, making it less effective than β-conglycinin for emulsification. Bian and others (2003) demonstrated that at pH 7, the Wu-type glycinin, intermediate, and β-conglycinin fractions had emulsification capacities of about 190, 220, and 275 g oil/g sample, respectively. The emulsification capacities of our Wu-process fractions were 371, 208, and 621 g oil/g sample for glycinin, intermediate, and β-conglycinin fractions, respectively. It was not surprising that the emulsification capacities reported by Bian were different from our Wu-process fractions even though the fractionation process was essentially the same. The soy flakes were from different crop years, probably from different soybean varieties, and prepared at

Table 5 - Emulsification capacity of soy protein isolate and fractionated soy proteins (g oil/g sample, dry basis)

	pH 7[a,b]	pH 5	pH 3
Soy protein isolate[c]	431_{ghij}	272_{k}	477_{efgh}
Wu-process glycinin	371_{ij}	583_{abc}	530_{cdef}
Optimized-process glycinin	173_{l}	579_{abc}	623_{ab}
Wu -process intermediate	208_{kl}	591_{abc}	244_{kl}
Optimized-process intermediate	424_{ghij}	363_{j}	484_{defg}
Wu-process β-conglycinin	621_{ab}	453_{fghi}	554_{bcde}
Optimized-process β-conglycinin	651_{a}	394_{hij}	571_{abcd}

[a]Subscript, lowercase letters within the table indicate significant differences at $P<0.05$.
[b]Least significant difference = 88.
[c]n=2.

different facilities. Thus, the fractionated proteins may have had different protein characteristics. Another important difference was the mixing devices used during emulsification. Even though the mixing units were similar, we have observed that a relatively small difference in mixing speeds can cause a dramatic difference in emulsification capacity. Slower speeds and less power produce a higher emulsification capacity.

Changing the protein dispersion pH prior to emulsification had a dramatic effect on some fractions. The emulsification capacities of the soy protein isolate and the β-conglycinin fraction were 40% less at pH 5 compared to emulsification capacities at pH 7, while the glycinin and intermediate fraction capacities increased by 14 to 70%. Emulsification capacities at pH 3 generally returned close to those at pH 7 for the isolate, intermediate, and β-conglycinin fractions. However, the emulsification capacities of the glycinin fractions remained near the pH 5 capacities. Bian and others (2003) showed that the glycinin fraction at pH 2 had an emulsification capacity of about 320 g oil/g protein compared to about 200 g oil/g protein at pH 7, while differences for the β-conglycinin were not significant between these treatments. Franzen and Kinsella (1976) showed that emulsifying capacity of soy protein isolate follows the pH solubility profile. That trend was not observed in the present work for the glycinin fractions and the Wu-intermediate fraction; however, the solubility profile of glycinin does begin to decrease around pH 7.

Commercial soy isolate at pH 5.3 to 5.6 has been shown to provide poor emulsifying properties for frankfurter and sausage meats (Hutton and Campbell 1977). The emulsification capacities reported in the present work suggest that the glycinin or intermediate fractions are better suited for meat applications because at pH 5 they exhibited about twice the emulsification capacity of our soy protein isolate.

Emulsification activity and stability

Emulsification activity and stability are additional means of evaluating emulsifying properties of proteins. The emulsification capacity assay demonstrates the maximum amount of oil that can be emulsified per unit protein. Driving an emulsion to phase inversion, from

oil-in-water to water-in-oil, probably does not accurately reflect what occurs in a food system, but it is commonly used to evaluate emulsifying properties of proteins. Emulsions for the emulsification activity assay are prepared well below the inversion point. Dilution of the emulsion permits turbidimetric evaluation. The amount of turbidity immediately after emulsification is an indication of how much protein is adsorbed to the surface of the oil fraction, which is disrupted into fine particles during blending.

Emulsification stability is a measure of how long the emulsion from the emulsification activity assay persists, resisting creaming, flocculation, and coalescence. Others have sampled and diluted the emulsion at one or more specific intervals after the initial emulsion was evaluated for turbidity. Decreased turbidity was taken to indicate a loss of stability of the emulsion. Here, only one dilution of the emulsion was made. The turbidity was measured immediately after dilution and then at 15 min. This change was implemented to eliminate problems with subsequent sampling of the emulsification.

The soy protein isolate, optimized-process intermediate fraction, and optimized-process β-conglycinin exhibited significantly higher emulsification activities compared to the other process fractions (Table 6). The same was true for emulsification stability. Process fractions demonstrating high emulsification activity had low or diminished protein native state and had high surface hydrophobicity. An advantage of the optimized process was improved emulsifying properties, at least for the intermediate and β-conglycinin fractions. Bian and others (2003) used an emulsification activity index to express emulsification activity. Their method demonstrated roughly the same relationship for glycinin, intermediate, and β-conglycinin as observed here (absorbance values of 0.088, 0.150, and 0.158, respectively). Their method gave values of about 105, 530, and 430 m^2/g, respectively. Both methods used similar emulsification and dilution steps, employing spectrophotometric measurement of turbidity to evaluate emulsification characteristics. As noted for the emulsification capacity assay, mixing speed and power characteristics of the blender used to create the emulsion, as well as blending time, will influence assay results.

Table 6 - Emulsification activity and stability of soy protein isolate and fractionated soy proteins

	Emulsification activity[a] (absorbance at 500 nm)	Emulsification stability (min)
Soy protein isolate[b]	0.242_a	150_b
Wu-process glycinin	0.088_c	49_c
Optimized-process glycinin	0.074_c	60_c
Wu-process intermediate	0.150_b	72_c
Optimized-process intermediate	0.273_a	313_a
Wu-process β-conglycinin	0.158_b	86_c
Optimized-process β-conglycinin	0.228_a	132_b
LSD[c]	0.053	40

[a]Subscript, lowercase letters in the same column indicate significant difference at $P<0.05$.
[b]n=2.
[c]LSD, least significant difference.

Khatib and others (2002) evaluated the glycinin and β-conglycinin fractions from four soybean lines for emulsification activity index and emulsification stability. Averaged values across the four lines showed that the emulsification activity was higher (386 vs. 324 m^2/g protein) for the glycinin fraction, but there was no statistical difference between the glycinin and β-conglycinin fractions.

Foaming properties

The same physicochemical properties necessary for emulsification are important for foaming. For foaming, protein stabilizes the interface between the water and air phases. The protein must move rapidly to the interface and provide sufficient viscosity and elasticity to prevent membrane rupture. To promote foam stability, the protein must reduce interfacial tension and form continuous films around the air bubbles. Proteins in the films are held together by electrostatic, van der Waals, and hydrophobic interactions (Kinsella and others 1985).

There were statistical differences in FC for the protein products, with FCs ranging from 0.83 to 1.34 (Table 7). The foaming capacities of the optimized-process fractions were higher for all three fractionation products compared to those from the Wu process. Some evidence provided here suggests that there is less native-state protein in the optimized-process fractions compared to the Wu-process fractions. Limited heat treatment of soy protein dispersions, with temperatures of 75-80 °C being optimal (Eldridge and others), reportedly increases foaming properties. Heating may cause dissociation of protein subunits and diminished β-conglycinin native state, producing proteins that more readily unfold at the air-water interface and stabilize the film. However, in the current work β-conglycinin gave the lowest FCs. The FC of the soy protein isolate was similar to the FCs of glycinin and intermediate fractions.

Due to the considerable variability in time for one-half of the foam to coalesce into liquid ($t_{1/2}$), there were no significant differences between K values. The β-conglycinin fractions for both processes were significantly slower for liquid-to-foam conversion. Bian and others

Table 7 - Foaming properties of soy protein isolate and fractionated soy proteins

	FC[a,b]	K $(mL\ min)^{-1}$	v_i (mL/min)
Soy-protein isolate[c]	1.13_b	0.0072	19.5_a
Wu-process glycinin	1.19_b	0.0131	14.6_{ab}
Optimized-process glycinin	1.34_a	0.0149	15.6_a
Wu-process intermediate	1.16_b	0.0057	19.5_a
Optimized-process intermediate	1.24_{ab}	0.0158	16.6_a
Wu-process β-conglycinin	0.83_d	0.0140	7.3_c
Optimized-process β-conglycinin	0.98_c	0.0142	10.1_{bc}
LSD[d]	0.14	Not different	5.4

[a]Subscript, lowercase letters within a column indicated significant difference at $P<0.05$.

[b]Abbreviations: FC, foaming capacity; K, foam stability rate constant; v_i, rate of liquid incorporation into foam.

[c]n=2.

[d]LSD, least significant difference.

(2003) were not able to show a difference in FCs for the glycinin, intermediate, and β-conglycinin fractions they evaluated. Their glycinin demonstrated a significantly higher K value than the intermediate and β-conglycinin fractions, indicating that the glycinin foams were less stable. However, in contrast to the liquid-to-foam conversion (v_i) observed here, their β-conglycinin fraction demonstrated a significantly higher rate of liquid-to-foam conversion compared to the intermediate and glycinin fractions. Their fractionation process conditions were nearly identical to the Wu process used here.

Apparent viscosity

Viscosity, a measure of the resistance to flow, is an important aspect of protein dispersions for formation and stability of emulsions and foams. Dispersions of 10% (w/w) for the fractionated proteins and the soy protein isolate were evaluated across a range of shear rates (Table 8). For all fractions, as shear rate increased, apparent viscosity decreased. This trend was also observed by Circle and others (1964) for sodium soy proteinates. At low shear rates protein molecules are not all oriented with the direction of flow, causing higher apparent viscosity. At higher shear rates, the molecules become oriented with the direction of flow and apparent viscosity decreases.

At low shear rates, there were no significant differences in apparent viscosity among the storage protein fractions and the soy protein isolate (Table 8). However, as shear rate increased apparent viscosities significantly increased for the intermediate fractions relative to the other fractions and the soy isolate. Particle size and shape are important factors affecting viscosity. Shen (1976) showed that soy protein in native state at pH 7 had lower intrinsic viscosity than soy protein denatured with guanidine hydrochloride or alkali treatment at pH 12. Unfolded or non-native state proteins, such as those of the intermediate fractions, would have a larger particle size and may more readily participate in protein interactions, such as hydrogen bonding, electrostatic interactions, and hydrophobic interactions. Though not significant, there were differences in apparent viscosities between the Wu and optimized processes for the intermediate and β-conglycinin fractions, with the optimized-process

Table 8 - Apparent viscosities of soy protein isolate and fractionated soy proteins

	Apparent viscosity (Pa·s) at stated shear rate (s^{-1})			
	10	100	200[a]	500
Soy protein isolate[b]	0.028	0.020	0.018_{bc}	0.016_{c}
Wu-process glycinin	0.011	0.007	0.006_{c}	0.005_{c}
Optimized-process glycinin	0.013	0.006	0.005_{c}	0.004_{c}
Wu-process intermediate	0.360	0.142	0.108_{ab}	0.075_{ab}
Optimized-process intermediate	0.479	0.185	0.140_{a}	0.096_{a}
Wu-process β-conglycinin	0.026	0.021	0.019_{bc}	0.018_{bc}
Optimized-process β-conglycinin	0.051	0.034	0.030_{bc}	0.026_{bc}
LSD[c]	Not different	Not different	0.090	0.058

[a]Subscript, lowercase letters within a column indicate significant difference at $P<0.05$.
[b]n=2.
[c]LSD, least significant difference.

fractions having higher viscosities. If non-native state proteins have molecular size, shape, and interactions favoring increased viscosity, then these differences would be expected based on DSC results. There was no correlation between apparent viscosity and emulsification capacity, emulsification activity, or foaming capacity. Recall that viscosity affects the rate at which proteins can reach the interface and stabilize a foam or emulsion.

Gelling

Gelling is an extremely important food system functionality. Sausages, yogurt, tofu, cheese, and pudding are examples of food systems using protein gelation. For gelation to occur, a protein must be at least partially denatured, and then the protein molecules must associate or aggregate into a structure, either highly ordered or random. Gel formation is affected by protein concentration, pH, ionic strength, ionic species, and heating considerations, such as temperature and time (Circle and others 1964).

Native-state analysis by DSC was used to determine appropriate gelation temperatures. The minimum temperature for protein gel production is the onset temperature for denaturation. The hardest gels are obtained between the peak denaturation temperature and the endpoint of denaturation (Matsumura and Mori 1996). The average peak and endpoint temperatures for the β-conglycinin and glycinin from the isolate and fractions evaluated in the current work were 73 and 79 °C for β-conglycinin and 90 and 96 °C for glycinin, respectively. To ensure that this temperature range was met for the interior of the samples, the 10% protein dispersions were heated in a water bath at 80 or 99 °C for 30 min. Gelling was evaluated at three pH values, 7, 5, and 3, to suggest what applications might be best suited for the different fractions.

Although peak force (firmness) and total force (work) values for glycinin fractions are shown in Table 9, they did not gel at pH 7, 80 °C. The lack of gelling was likely due to insufficient change in native state or denaturation from the heat treatment and did not permit the second step in gel formation, the association of denatured proteins into a network. Glycinin heated at 99 °C did form opaque gels. Utsumi and others (1997) commented in

Table 9 - Gel strength characteristics of soy protein isolate and fractionated soy proteins

	Dispersion pH and gelling temperature					
	pH 7 80 °C[a]	pH 7 99 °C	pH 5 80 °C	pH 5 99 °C	pH 3 80 °C	pH 3 99 °C
Peak force or firmness (N)						
Soy protein isolate	0.8_{jkl}	5.3_{ef}	NGF[b]	2.0_{ghijkl}	3.5_{fghi}	GNP
Wu-process glycinin	0.2_{kl}*	4.5_{efg}	NGF	8.5_{cd}	GNP	NGF
Optimized-process glycinin	0.2_{kl}*	3.9_{fgh}	NGF	13.7_{a}	GNP	NGF
Wu-process intermediate	0.5_{kl}	1.0_{ijkl}	NGF	3.2_{fghij}	NGF	1.3_{hijkl}
Opt.-process intermediate	3.5_{fghi}	5.5_{ef}	NGF	1.9_{ghijkl}	2.8_{fghijk}	GNP
Wu-process β-conglycinin	14.0_{a}	12.3_{ab}	UTM[c]	GNP[d]	14.4_{a}	GNP
Opt.-process β-conglycinin	8.7_{cd}	10.1_{bc}	7.0_{de}	GNP	10.1_{bc}	GNP
LSD[e] for peak force = 2.65						
Total force or work (Nmm)						
Soy protein isolate	4.8_{ij}	39.2_{d}	NGF	12.7_{fghi}	24.7_{defgh}	GNP
Wu-process glycinin	1.7_{j}*	36.1_{d}	NGF	25.7_{defg}	GNP	NGF
Optimized-process glycinin	1.7_{j}*	31.9_{de}	NGF	40.4_{d}	GNP	NGF
Wu-process intermediate	3.1_{ij}	7.8_{ij}	NGF	16.9_{efghi}	NGF	9.3_{hij}
Opt.-process intermediate	28.5_{def}	35.5_{d}	NGF	11.9_{ghij}	18.1_{efghi}	GNP
Wu Process β-Conglycinin	90.5_{b}	73.3_{c}	UTM	GNP	142.8_{a}	GNP
Opt.-process β-conglycinin	58.2_{c}	63.3_{c}	32.6_{de}	GNP	98.8_{b}	GNP
LSD for total force = 15.95						

[a]Subscript, lowercase letters within a table indicate significant difference at $P<0.05$.
[b]NGF, no gel formed.
[c]UTM, unable to measure.
[d]GNP, gel not prepared.
[e]LSD, least significant difference.
*Although values were given, samples had not gelled.

their review of this topic that glycinin gels have been formed at 80 °C, but the forces maintaining the network structure were different than gels formed at 100 °C. Hydrogen bonding may have been more prevalent in the gel structure formed at 80 °C because there was less unfolding of the glycinin. At 100 °C, unfolding would have been more extensive, leading primarily to hydrophobic interactions.

Like the glycinin fractions, soy protein isolate heated at 80 °C at pH 7 did not gel. This was probably due to the approximately equal portions of glycinin and β-conglycinin, as shown by SDS-PAGE analysis (Table 1), with the glycinin disrupting the gelling properties of the β-conglycinin at this temperature. The β-conglycinin fractions did produce firm gels at 80 °C and pH 7. Again, this functionality was because the heat treatment was sufficient to cause the protein to denature, the first step in the gelling process. The firmness of the β-conglycinin gels was not significantly different for gelling treatments of 80 °C and 99 °C at pH 7, indicating that the increased temperature did not have a detrimental effect on the gel-forming properties.

The Wu-process intermediate fraction behaved very differently compared to that of the optimized process at both 80 and 99 °C at pH 7. The firmness of the optimized-process intermediate gels was 3.53 and 5.45 N, respectively, compared to 0.47 and 0.96 N for the Wu-process gels. There was no significant difference in glycinin content between these process fractions, and during the 99 °C treatment any differences in protein unfolding should have been eliminated. The DSC data also does not assist in explaining this difference. For the β-conglycinin fractions, firmness of the Wu-process material was higher at both temperatures than the optimized-process material. The smaller amount of native-state β-conglycinin in the optimized-process β-conglycinin fraction may have caused reduced firmness compared to the Wu-process β-conglycinin fraction gels, but this contradicts what was observed for the intermediate fractions. The Wu-process intermediate fraction had more native-state β-conglycinin, but had a lower firmness. These firmness values were difficult to explain with the available data.

The gel characteristics at pH 5 were quite different compared to gels formed at pH 7. Gels, if they formed, were white, opaque, and tofu-like. For the glycinin and intermediate dispersions heated at 80 °C, the protein precipitated, but did not form a network. The Wu-process β-conglycinin sample heated at 80 °C did form a hard, tofu-like gel, but the height of the gel was so limiting that the texture could not be measured. The firmness of the optimized-process β-conglycinin gel at pH 5, 80 °C was not significantly different than the pH 7 gel heated at 80 °C, but the pH 5 optimized-process β-conglycinin gel looked tofu-like. Matsumura and Mori (1996) cited several studies suggesting that highly ordered "string of beads" type gels tend to be translucent or transparent, while randomly aggregated protein gels tend to be opaque. It is possible that because the proteins were near their isoelectric point, the protein structures could not associate in an ordered manner. Glycinin gels prepared at pH 5, 99 °C were significantly firmer than gels from the soy isolate or intermediate fractions formed under these conditions. β-Conglycinin gels were not prepared at pH 5 and 99 °C because gels could be formed at 80 °C.

Gels that formed at pH 3 were generally translucent, like the gels formed at pH 7. The glycinin fractions would not gel at either 80 or 99 °C at pH 3. However, β-conglycinin formed gels that were similar in firmness to gels formed at pH 7. Soy protein isolate gels formed at pH 3, 80 °C were not significantly different in firmness from those formed at pH 7, 99 °C. Soy isolate gels were not prepared for analysis at pH 3, 99 °C, but gels were observed to form under these conditions. The Wu-process intermediate fraction would not form a gel at pH 3 unless it was heated at 99 °C. Even under those conditions it was still a very weak gel, as was the gel produced for the optimized-process intermediate fraction at pH 3, 80 °C.

Probably the most interesting result of the gelling study was the strength of the β-conglycinin gels that could be formed at pH 3. This protein fraction may perform well in some high acid food systems or in jellied products containing acidic fruit juice or flavorings. The β-conglycinin fraction would be the preferred protein among those studied here for use at pH 7 if firm gelling properties were desired.

Conclusions

There were functionality differences among the unique, fractionated products, and with respect to our soy protein isolate. This was especially true for emulsification and gelling. Functionality differences between the glycinin and β-conglycinin fractions may potentially be exploited and utilized. Further modification of these fractions may lead to additional products with unique functional properties. However, fractionated proteins may not be as economical as soy protein isolate, or even a modified isolate, due to increased processing requirements. Future work should include a more exhaustive evaluation of the functional properties of the process fractions, for example at additional pH values and for different ionic strengths. The performance of these fractions in actual food systems, when compared to commercial isolates, would be interesting as well.

Acknowledgment

This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project N. 6571, was supported in part by USDA NRI grant No. 2001-35503-10814, NASA Food Technology Commercial Space Center, and by Hatch Act and State of Iowa funds.

Amalia Tziotis provided training on the TA-XT2 Analyzer. Igor Kovalenko provided rheology expertise. Ojash Raval assisted with the emulsification capacity analyses.

References

AACC. Approved Methods of the American Association of Cereal Chemistry, 8th ed.; AACC: St. Paul, MN, 1983a; Method 44-15A.

AACC. Approved Methods of the American Association of Cereal Chemistry, 8th ed.; AACC: St. Paul, MN, 1983b; Method 08-03.

Anonymous. 2003. Soy protein reference guide. http://soyasa.org/documents/0/SPI_RefGuide.htm.

AOAC. Official Methods of Analysis of Association of Official Analytical Chemists, 11th ed.; AOAC: Washington, DC, 1970; Method 2.066.

AOAC. Official Methods of Analysis of Association of Official Analytical Chemists, 15th ed.; AOAC: Arlington, VA, 1990; Method 990.03.

Aoki H, Taneyama O, Inami M. 1980. Emulsifying properties of soy protein: characteristics of 7S and 11S proteins. J Food Sci 45:534-538.

Berk Z. 1992. Soybean protein concentrates. In: Berk Z, editor, Technology of Production of Edible Flours and Protein Products from Soybeans. 1st ed. Rome, FAO. p 73-82.

Bian Y, Myers DJ, Dias K, Lihono MA, Wu S, Murphy PA. 2003. Functional properties of soy protein fractions produced using a pilot-scale process. J. Am. Oil Chem. Soc. 80:551-555.

Bringe NA, inventor; Monsanto Company, assignee. 2001 Jan 9. High beta-conglycinin products and their use. U.S. patent 6,171,640.

Chove BE, Grandison AS, Lewis MJ. 2001. Emulsifying properties of soy protein isolate fractions obtained by isoelectric precipitation. J Food Sci 81:759-763.

Circle SJ, Meyer EW, Whitney RW. 1964. Rheology of soy protein dispersions. Effect of heat and other factors on gelation. Cereal Chem 41:157-172.

Damodaran S. 1997. Food proteins: an overview. In: Damodaran S, Paraf A, editors. Food Proteins and Their Applications. 1st ed. New York: Marcel Dekker, Inc. p 1-24.

Davidson RM, Sand RE, Johnson RE, inventors; Anderson, Clayton & Co., assignee. 1979 Oct 30. Method for processing soy protein and composition of matter. U.S. patent 4,172,828.

Eldridge AC, Hall PK, Wolf WJ. 1963. Stable foams from unhydrolyzed soybean protein. Food Technology 17:1592-1595.

Fehr WR, Hoeck JA, Johnson SL, Murphy PA, Nott JD, Padilla GI, Welke GA. 2003. Genotype and environment influence on protein components of soybean. Crop Sci 43:511-514.

Fisher MS, Marshall WE, Marshall, Jr. HF. 1990. Carbon-13 NMR studies of the effects of gelation and heat and chemical denaturation at neutral pH on soy glycinin and β-conglycinin. J Agric Food Chem 38:957-966.

Franzen KL, Kinsella JE. 1976. Functional properties of succinylated and acetylated soy protein. J Agric Food Chem 24:788-795.

Hayakawa S, Nakai S. 1985. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. J Food Sci 50:486-491.

Hutton CS, Campbell AM. 1977. Functional properties of a soy concentrate and a soy isolate in simple systems and in a food system. J Food Sci 42:457-460.

Ishino K, Okamoto S. 1975. Molecular interaction in alkali denatured soybean proteins. Cereal Chem 52:9-21.

Jung S, Rickert DA, Deak NA, Aldin ED, Recknor J, Johnson LA, Murphy PA. 2003. Comparison of Kjeldahl and Dumas nitrogen methods for protein content determination in soy products. J Am Oil Chem Soc In press.

Khatib KA, Herald TJ, Aramouni FM, MacRitchie F, Schapaugh WT. 2002. Characterization and functional properties of soy β-conglycinin and glycinin of selected genotypes. J Food Sci 67:2923-2929.

Kinsella JE. 1979. Functional properties of soy proteins. J. Am. Oil Chem. Soc. 56:242-258.

Kinsella JE, Damodaran S, German B. 1985. Physicochemistry and functional properties of oilseed proteins with emphasis on soy proteins. In: Altschul AM, Wilcke HL, editors. New Protein Foods. Vol 5. New York:Academic Press p 108-180.

Lakemond CMM, de Jongh HHJ, Hessing M, Gruppen H, Voragen AGJ. 2000. Soy glycinin: influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. J Agric Food Chem 48:1985-1990.

Lehnhardt WF, Gibson PW, Orthoefer FT, inventors; A. E. Staley Manufacturing Company, assignee. 1983 Jan 25. Fractionation and isolation of 7S and 11S protein from isoelectrically precipitated vegetable protein mixtures. U.S. Patent 4,370,267.

Liu M, Lee DS, Damodaran S. 1999. Emulsifying properties of acidic subunits of soy 11S globulin. J Agric Food Chem 47:4970-4975.

Maruyama N, Sato R, Wada Y, Matsumura Y, Goto H, Okuda E, Nakagawa S, Utsumi S. 1999. Structure-physicochemical function relationships of soybean β-conglycinin constituent subunits. J Agric Food Chem 47:5278-5284.

Matsumura Y, Mori T. 1996. Gelation. In: Hall, GM, editor, Methods of Testing Protein Functionality. 1st ed. New York, Blackie Academic & Professional. p 76-109.

Messina MJ. 1997. Soyfoods: their role in disease prevention and treatment. In: Liu K, editor, Soybeans: Chemistry, Technology, and Utilization. 1st ed. New York, Chapman & Hall. p 442-477.

Murphy PA, Resurreccion AP. 1984. Varietal and environmental differences in soybean glycinin and β-conglycinin content. J Agric Food Chem 32:911-915.

Nagano T, Hirotsuka M, Mori H, Kohyama K, Nishinari K. 1992. Dynamic viscoelastic study on the gelation of 7S globulins from soybeans. J Agric Food Chem 40:941-944.

Nagano T, Fukuda Y, Akasaka T. 1996. Dynamic viscoelastic study on the gelation properties of β-conglycinin-rich and glycinin-rich soybean protein isolates. J Agric Food Chem 44:3484-3488.

Nielsen NS. 1985. Structure of soy proteins. In: Altschul AM, Wilcke, HL, editors. New Protein Foods. Vol. 5 New York, Academic. p 27-64.

Nielsen PM. 1997. Functionality of protein hydrolysates. In: Damodaran S, Paraf A, editors. Food Proteins and Their Applications. 1st ed. New York: Marcel Dekker, Inc. p 443-472.

Riblett AL, Herald TJ, Schmidt KA, Tilley KA. 2001. Characterization of β-conglycinin and glycinin soy protein fractions from four selected soybean genotypes. J Agric Food Chem 49:4983-4989.

Schwenke KD. 1997. Enzyme and chemical modification of proteins. In: Damodaran S, Paraf A, editors. Food Proteins and Their Applications. 1st ed. New York: Marcel Dekker, Inc. p 393-424.

Shen JL. 1976.Solubility profile, intrinsic viscosity, and optical rotation studies of acid precipitated soy protein and of commercial soy isolate. J Agric Food Chem 24:784-788.

Staswick PE, Hermodson MA, Nielsen NC. 1984. Identification of the cysteines which link the acidic and basic components of the glycinin subunits. J Biol Chem 259:13431-13435.

Thanh VH, Okubo K, Shibasaki K. 1976. Major proteins of soybean seeds. A straightforward fractionation and their characterization. J Agric Food Chem 24:1117-1121.

Tornberg E. 1978. Functional characterization of protein stabilized emulsions: creaming stability. J Food Sci 43:1559 - 1562.

Utsumi S, Damodaran S, Kinsella JE. 1984. Heat-induced interactions between soybean proteins: preferential association of 11S basic subunits and β subunits of 7S. J Agric Food

Chem 32:1406-1412.

Utsumi S, Matsumura Y, Mori T. 1997. Structure-function relationships of soy proteins. In: Damodaran S, Paraf A, editors. Food Proteins and Their Applications. 1st ed. New York: Marcel Dekker, Inc. p 257-292.

Wagner JR, Guéguen J. 1995. Effects of dissociation, deamidation, and reducing treatment on structural and surface active properties of soy glycinin. J Agric Food Chem 43:1993-2000.

Wagner JR, Sorgentini DA, Añón MC. 1996. Thermal and electrophoretic behavior, hydrophobicity, and some functional properties of acid-treated soy isolates. J Agric Food Chem 44:1881-1889.

Wagner JR, Guéguen J. 1999a. Surface functional properties of native, acid-treated, and reduced glycinin. 1. Foaming properties. J Agric Food Chem 47:2173-2180.

Wagner JR, Guéguen J. 1999b. Surface functional properties of native, acid-treated, and reduced glycinin. 2. Emulsifying properties. J Agric Food Chem 47:2181-2187.

Waniksa R, Shetty JK, Kinsella JE. 1981. Protein-stabilized emulsions: effects of modification on the emulsifying activity of bovine serum albumin in a model system. J Agric Food Chem 29:826-831.

Wu S, Murphy PA, Johnson LA, Fratzke AR, Reuber MA. 1999. Pilot-plant fractionation of soybean glycinin and β-conglycinin. J. Am. Oil Chem. Soc. 76:285-293.

CHAPTER 6. CONCLUSIONS

This body of work sought to answer questions about the effects of soy protein fractionation process scale up, protein and phytochemical partitioning during processing, accurate accounting of the fate of phytochemicals during processing, and how different fractionation process conditions alter the functionality of glycinin, intermediate, and β-conglycinin fractions. Explanation of the observations with respect to the physicochemical aspects of the proteins and phytochemicals was desired.

Scale up of the fractionation process from 15 to 50 kg of starting material increased total protein recovery about 2.4-fold. However, about 12% less protein was recovered in the product fractions. This was primarily due to poor protein extraction from the defatted soy flakes. It is difficult to determine from available information whether this difference in extraction efficiency was due to functionality characteristics of the different soy flake starting material or equipment efficiency. It is possible that the flakes used in the scaled-up process had a lower protein dispersibility index compared to the white flakes used by Wu and others (1999) for the 15-kg process. Varietal, environmental, and storage differences may have also affected results. It would be interesting to perform a scale-up study using identical material in the same time frame to get a more accurate picture of this issue. The efficiency of both processes was poor with respect to protein recovery during soy protein isolate production. For research purposes, the current efficiency may be acceptable. However, the fractionated proteins would need to have very unique functional properties, or require minimal additional processing to yield unique properties, in order to be competitive in the marketplace. The fractionation process is long and capital intensive.

The process improvement study provided interesting results. The water-to-flake study demonstrated that improved isoflavone extraction from soy flakes could be achieved at increased water-to-flake ratios, but unfortunately this did not translate into increased isoflavone concentrations in the protein products. Increased isoflavone extraction did lead to increased isoflavones in the whey waste stream, so the whey waste stream may be a viable

source for isoflavone recovery. Recovered isoflavones could then be added back to the protein products prior to spray drying.

The physicochemical characteristics of the isoflavones and saponins were observed to be quite different with regard to the ability to move into and remain in the aqueous fraction. The whey contained a significant quantity of isoflavones but almost no saponins, probably due to hydrophobicity characteristics of the saponins and their apparent affinity for the soy protein. This affinity was observed again during diafiltration. The β-conglycinin precipitate lost 80-85% of its isoflavones to diafiltration but only 10-20% of its saponins. This finding presents a useful application for nutritionists wishing to evaluate the impact of isoflavones, saponins, and soy protein on serum cholesterol and lipid levels. Isoflavone concentration of the protein fractions, or isoflavone to saponin ratio, could be manipulated using diafiltration. Diafiltration could prevent the need to ethanol extract isoflavones from protein fractions for such studies. Ethanol extraction, as well as the heating process to remove the ethanol, denatures the proteins and may diminish the impact that β-conglycinin or other protein peptides, recovered through the intestine after digestion, may have on serum cholesterol and lipid levels.

Protein functionality testing did show differences between glycinin, intermediate, and β-conglycinin fractions. Some differences were observed between a given protein fraction produced by the optimized and the control pilot-scale fractionation processes. The effect of native state protein on solubility and emulsification was demonstrated. Probably the most useful information was provided by the emulsification capacity and gelling studies. Based on pH of the system, it may be possible to match a given fractionated protein for a specific application. The gelling characteristics of β-conglycinin at pH 3 were especially interesting. Future research with the remaining material should include solubility, emulsification, and gelling analyses at varied ionic strengths. If more product was available, evaluation of the proteins in actual food systems, such as a whipped product or in a meat product, would be ideal.

Finally, the importance of recovery studies became very clear. It is not useful to be frustrated by inaccurate mass balances, such as experienced with the isoflavones and saponins, without attempting to understand why those mass balances are inaccurate. The recovery study, described in Chapter 3, was very enlightening, demonstrating how very different soy matrices can be in their ability to retain phytochemicals. The soy protein isolate, and presumably the fractionated proteins that remain to be evaluated, displayed a much stronger affinity for the phytochemicals, especially the aglucon isoflavones and saponins, than the soy flake starting material. It also was discovered that pH can have a dramatic effect on isoflavone and saponin phytochemical extraction from soy products. This may be helpful for increasing accurate quantification of these phytochemicals, although laboratories may not choose to take the time and absorb the expense to evaluate this effect on the samples they analyze.

APPENDIX A. ANOVA TABLES FOR CHAPTER 3 TOTAL ISOFLAVONE AND SAPONIN CONCENTRATION DATA

Soy Protein Isolate Experiment – ANOVA for total isoflavone concentrations for the insoluble fraction.

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr>F
Temperature	1	0.627	0.627	22.88	0.0014
pH	2	4.022	2.011	73.36	<0.0001
Temp*pH					
Neutralization	1	0.546	0.546	19.90	0.0021
Temp*Neut	1	0.036	0.037	1.33	0.2821
pH*Neut	2	0.292	0.146	5.33	0.0338
Temp*pH*Neut					
Error	8	0.219	0.027		

Soy Protein Isolate Experiment – ANOVA for total isoflavone concentrations for the isolate fraction.

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr>F
Temperature	1	0.009	0.009	0.15	0.7063
pH	2	3.579	1.789	29.96	0.0002
Temp*pH					
Neutralization	1	1.296	1.296	21.70	0.0016
Temp*Neut	1	0.349	0.349	5.84	0.0421
pH*Neut	2	1.003	0.502	8.39	0.0109
Temp*pH*Neut					
Error	8	0.478	0.060		

Soy Protein Isolate Experiment – ANOVA for total isoflavone concentrations for the whey fraction.

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr>F
Temperature	1	1.911	1.911	3.18	0.112
pH	2	4.069	2.035	3.39	0.086
Temp*pH					
Neutralization	1	0.029	0.029	0.05	0.832
Temp*Neut	1	0.285	0.285	0.47	0.510
pH*Neut	2	0.558	0.279	0.46	0.644
Temp*pH*Neut					
Error	8	4.802	0.600		

Soy Protein Isolate Experiment – ANOVA for total saponin concentrations for the insoluble fraction.

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr>F
Temperature	1	0.138	0.138	0.98	0.352
pH	2	1.598	0.800	5.68	0.029
Temp*pH					
Neutralization	1	0.403	0.403	2.86	0.129
Temp*Neut	1	0.086	0.086	0.61	0.457
pH*Neut	2	0.091	0.046	0.32	0.733
Temp*pH*Neut					
Error	8	1.127	0.141		

Soy Protein Isolate Experiment – ANOVA for total saponin concentrations for the isolate fraction.

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr>F
Temperature	1	0.769	0.769	0.730	0.419
pH	2	32.908	16.454	15.570	0.002
Temp*pH					
Neutralization	1	45.794	45.794	43.330	0.0002
Temp*Neut	1	0.218	0.218	0.210	0.662
pH*Neut	2	0.504	0.252	0.240	0.793
Temp*pH*Neut					
Error	8	8.455	1.057		

Soy Protein Slurry Experiment – ANOVA for total isoflavone concentrations

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr>F
Temperature	1	2.627	2.627	20.99	0.0006
pH	2	4.675	2.337	18.68	0.0002
Temp*pH	2	1.085	0.542	4.33	0.0383
Neutralization	1	1.804	1.804	14.42	0.0025
Temp*Neut	1	0.260	0.260	2.08	0.1747
pH*Neut	2	1.121	0.561	4.48	0.0352
Temp*pH*Neut	2	0.570	0.285	2.28	0.1449
Error	12	1.501	0.125		

Soy Protein Slurry Experiment – ANOVA for total saponin concentrations

Source	Degrees of freedom	Sum of squares	Mean square	F value	Pr>F
Temperature	1	1.445	1.445	4.41	0.0577
pH	2	0.053	0.026	0.08	0.9232
Temp*pH	2	0.889	0.444	1.35	0.2948
Neutralization	1	2.178	2.178	6.64	0.0243
Temp*Neut	1	0.200	0.200	0.61	0.4503
pH*Neut	2	0.051	0.026	0.08	0.9250
Temp*pH*Neut	2	0.953	0.476	1.45	0.2725
Error	12	3.938	0.328		

APPENDIX B. MICROBIAL LOAD OF DEFATTED SOYBEAN FLAKES AND PILOT-SCALE PROCESS FRACTIONS

Colony forming units on trypticase soy agar aerobic plates for materials from protein fractionation and soy protein isolate processes.

	Isolate R1[a]	Isolate R2	Wu R1	Wu R2	Optimized R1	Optimized R2
Soy Flakes	2.6×10^3/g	2.5×10^2/g	2.7×10^3/g	6.2×10^2/g	5.9×10^3/g	3.1×10^3/g
Slurry 1[b]	1.5×10^2/mL	5.8×10^2/mL	7.7×10^2/mL	1.9×10^3/mL	1.7×10^3/mL	6.1×10^3/mL
Slurry 2	2.0×10^3/m L	5.3×10^2/mL	1.4×10^4/mL	1.1×10^4/mL	5.9×10^3/mL	2.2×10^4/mL
Glycinin SD			$>3.0 \times 10^6$/g	4.6×10^5/g	2.2×10^7/g	8.6×10^6/g
Intermediate SD			8.9×10^5/g	8.0×10^4/g	9.3×10^5/g	1.3×10^5/g
B-Conglycinin SD			7.5×10^5/g	1.6×10^5/g	2.5×10^5/g	3.3×10^5/g
Isolate SD	3.5×10^4/g	7.8×10^4/g				

[a]Abbreviations: R, replication; SD, spray dried.

[b]Slurry 1 was the soy slurry just prior to centrifuging to remove the insoluble fraction. Slurry 2 was the precipitated protein fraction that had chilled overnight that, when centrifuged, yielded the soy protein isolate or glycinin fraction.

Coliform colony forming units on violet red bile agar for materials from protein fractionation and soy protein isolate processes.

	Isolate R1[a]	Isolate R2	Wu R1	Wu R2	Optimized R1	Optimized R2
Soy Flakes	<10/g	<10/g	<10/g	<10/g	<10/g	<10/g
Slurry 1[b]	<1/mL	<1/mL	10/mL	1.8×10^2/mL	5/mL	4/mL
Slurry 2	<1/mL	16/mL	>250/mL	1.7×10^2/mL	1.3×10^1/mL	$>3.0 \times 10^2$/mL
Glycinin SD			2.2×10^3/g	4.0×10^1/g	<10/g	10/g
Intermediate SD			7.1×10^1/g	<10/g	<10/g	<10/g
B-Conglycinin SD			4.2×10^1/g	<10/g	<10/g	<10/g
Isolate SD	<10/g	<10/g				

[a]Abbreviations: R, replication; SD, spray dried.

[b]Slurry 1 was the soy slurry just prior to centrifuging to remove the insoluble fraction. Slurry 2 was the precipitated protein fraction that had chilled overnight that, when centrifuged, yielded the soy protein isolate or glycinin fraction.