Improved Fractionation of Glycinin and *β*-Conglycinin and Partitioning of Phytochemicals

DAVID A. RICKERT,[†] LAWRENCE A. JOHNSON,[‡] AND PATRICIA A. MURPHY*[,†]

Department of Food Science and Human Nutrition and Center for Crops Utilization Research, Iowa State University, Ames, Iowa 50011-1061

Glycinin-rich and *β*-conglycinin-rich products are prepared by soy protein fractionation. Physicochemical characteristics of these proteins affect their unique, important functionality in food systems and industrial applications. Soybean isoflavones and saponins are phytochemicals with potential health benefits. Objectives of this protein fractionation research were to (1) improve protein and phytochemical extraction from defatted soy flakes and recovery in product fractions and (2) evaluate phytochemical partitioning and profile changes during fractionation. Extraction environments (pH, ethanol concentration, temperature, and water-to-flake ratio) were each varied during bench-scale optimization. Optimized conditions of 45 °C and 10:1 water-to-flake ratio were compared with previous conditions of 20 °C and 15:1 water-to-flake ratio and a soy protein isolate process at pilot scale. Optimized conditions yielded more *β*-conglycinin with higher isoflavone and saponin concentrations, but fraction purity was diminished by glycinin contamination. Bench-scale data demonstrated that increased phytochemical extraction did not translate into increased concentrations in product fractions.

KEYWORDS: Glycinin; *β*-conglycinin; soy protein; protein fractionation; pilot plant; isoflavones; saponins

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, including improved cardiovascular health as part of a low-fat diet, antiosteoporotic effects, and anticarcinogenic activity (*3*). Research efforts to define the health impact of each of these soy components continue.

Fractionation of the soluble protein extracted from defatted soy white flakes under mildly alkaline conditions is achieved using ionic strength adjustment and isoelectric precipitation. Bench-scale soy protein fractionation has been utilized to produce enriched glycinin and *β*-conglycinin fractions for protein characterization purposes (*4*−*6*). 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-plant-scale methods for fractionating soy protein from defatted soy white flakes. One method was a scale-up of a modified bench-scale process, termed the modified-Nagano method, yielding an intermediate fraction composed of a mixture of glycinin and *β*-conglycinin,

a glycinin-rich fraction, and a *β*-conglycinin-rich fraction. The other method, referred to as the simplified method, yielded only glycinin-rich and *β*-conglycinin-rich fractions. Compared to the modified-Nagano method, the simplified method produced a larger quantity of *β*-conglycinin-rich fraction, but at the expense of purity.

Both methods of Wu et al. (*10*, *11*) extracted protein at 20 °C, which is a much lower temperature than the temperatures of up to 80 °C that are employed for commercial soy protein isolate production (*12*). The 20 °C temperature was reportedly selected to maximize the amount of native-state storage proteins, which fractionate more effectively in the Wu process. However, the mass yields of protein from these processes are less than those for soy protein isolate. Soy protein isolate is commercially produced by extracting protein from defatted soy flakes under alkaline conditions, followed by isoelectric precipitation (*13*). The dry-basis mass recovery has typically been ∼33% for soy protein isolate produced at the Iowa State University Center for Crops Utilization Research (CCUR) pilot plant. Wu et al. (*10*, *11*) reported dry mass recoveries of only 24 and 29% for the pilot-plant-scale modified-Nagano and simplified processes, respectively.

The isoflavone content and distribution in several soy foods (*14*, *15*) and information about the fate of isoflavones during soy protein isolate processing (*16*, *17*) have been reported, as have the saponin contents of soybeans, soy foods, and soy ingredients (*18*−*21*). However, the partitioning of saponins during soy protein fractionation and soy protein isolate production, as well as mass balance and distribution data, has not been

* Corresponding author [telephone (515) 294-1970; fax (515) 294-8181; e-mail pmurphy@iastate.edu].
[†] Iowa State University.
[‡] Center for Crops Utilization Research.

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reported. Extraction and partitioning differences between these phytochemicals have not been described for these processes.

The primary objective of the current work was to improve the extraction of protein, isoflavones, and saponins from defatted soy white flakes while increasing their recovery in product fractions without substantially decreasing the purities of the glycinin-rich and β-conglycinin-rich fractions. A second objective was to evaluate isoflavone and saponin partitioning and profile changes that occur during processing. Improving protein fraction yields will improve the economics of producing these fractions. Economical production of substantial quantities of glycinin-rich and β-conglycinin-rich fractions will promote their use in clinical trials to understand the potential health benefits of these proteins. In addition, unique functional properties of these protein products, such as emulsifying, gelling, and whipping properties, may then be evaluated directly through functionality testing or indirectly by incorporation into food products, such as comminuted meat products, gravies, or whipped toppings. Understanding the partitioning characteristics of the isoflavones and saponins may provide insight for increasing or reducing the concentrations of these phytochemicals in product fractions or for determining which process streams may be practical sources for further extraction and recovery.

MATERIALS AND METHODS

Materials. Defatted soy white flakes used for bench-scale experiments were obtained from Cargill (Minneapolis, MN), whereas those for the pilot-plant-scale experiments were prepared at the Research Oil Mill, Food Protein R&D Center, Texas A&M University. The Texas A&M flakes were prepared from the 2000 soybean harvest, IA 2042 variety, and had an 84% protein dispersibility index (Woodson-Tenent Labs, Des Moines, IA). Flakes were stored in sealed containers at 4 °C until use.

Soy Protein Processing Methods. To improve the bench-scale process, the following four extraction variables were each evaluated: (1) pH, from 7.5 to 9.0; (2) ethanol concentration, from 0 to 10%; (3) temperature, from 25 to 65 °C; and (4) water-to-flake ratio, from 6:1 to 15:1. Pilot-plant-scale fractionation was used to compare the results from the bench-scale optimized-process conditions with those from process conditions used in a previous work (*10*). Soy protein isolate, produced with the same pilot-plant equipment and from the same lot of starting material, was prepared for comparison purposes.

pH Effects on Bench-Scale Protein Solubilization. Soy slurries were prepared at ambient temperature by combining deionized water and 80.0 g of defatted soy white flakes at a 15:1 water-to-flake ratio and stirring as rapidly as possible with a magnetic stir bar. The slurries were adjusted to pH 7.5, 8.0, 8.5, or 9.0 with 2 N NaOH and maintained at that pH while stirring for 1 h. Large particulate insolubles were then removed using a 60-mesh screen, whereas smaller insolubles were removed by centrifugation at 14000*g* for 30 min at 20 °C. The supernatant protein concentration was determined by using the biuret method (*22*) with fraction V bovine serum albumen as the standard (Sigma Chemical Co., St. Louis, MO).

Ethanol Effects on Bench-Scale Protein Solubilization and Fractionation. Bench-scale fractionation for this study, as well as for the water-to-flake ratio and temperature studies described below, was a modification of the method of Wu et al. (*10*), who used 50 g of flakes, a 120-mesh stainless steel screen to remove large insolubles, and a 30-min protein solubilization period. Soy slurries were prepared at ambient temperature by combining water, containing ethanol concentrations of 0, 0.1, 1.0, or 10%, and 80.0 g of defatted soy white flakes at a 15:1 ratio and stirring as quickly as possible with a magnetic stir bar. The slurries were adjusted to pH 8.5 with 2 N NaOH and maintained at that pH during 1 h of stirring. Insolubles were removed by screening and centrifuging. Sodium bisulfite was added to the supernatant to produce a 10 mM SO_2 concentration, and the pH was adjusted to 6.4 using 2 N HCl. The protein dispersion was chilled

overnight at 4 °C. The glycinin-rich precipitate was recovered by centrifugation at 7500*g* for 20 min at 4 °C. The supernatant was adjusted to 0.25 M NaCl by adding dry NaCl and then adjusted to pH 5.0 with 2 N HCl, stirred for 1 h, and then centrifuged at 14300*g* for 30 min at 4 °C to recover the intermediate protein fraction, which contained glycinin, β-conglycinin, and other proteins. Water was added to the resulting supernatant to dilute by a factor of 3, and the pH was adjusted to 4.8. The β-conglycinin-rich precipitate was removed from the whey by centrifugation at 7500*g* for 20 min at 4 °C. The insoluble fraction and fractionated proteins were weighed and then freeze-dried. Freeze-dried weights were regarded as the dry weights and used in the calculation of solids contents.

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

Temperature Effects on Bench-Scale Protein Extraction and Fractionation. Soy slurries were prepared by combining 80.0 g of defatted soy white flakes at a 10:1 water-to-flake ratio, stirring as quickly as possible with a magnetic stir bar, and heating on a stir plate to 25, 35, 45, 55, or 65 °C within 15 min and then maintaining them at that temperature. Slurries were adjusted to pH 8.5 with 2 N NaOH and maintained at that pH during 1 h of stirring. The storage proteins were then fractionated as described above. The insoluble fraction, whey, and fractionated proteins were weighed and then freeze-dried.

Pilot-Plant-Scale Protein Fractionation. Soy proteins were extracted from defatted soy white flakes (20.0 kg) with a 15:1 water-to-flake ratio and an extraction temperature of 20 °C, as was used by Wu et al. (*10*) for previous pilot-plant-scale fractionation, or a 10:1 water-to-flake ratio and an extraction temperature of 45 °C (optimized conditions). Flakes and temperature-adjusted water were combined and stirred at 22 rpm in a jacketed 800-L tank (Walker Stainless Equipment Co., New Lisbon, WI). The slurry was adjusted to pH 8.5 with 2 N NaOH and maintained at that pH for 1 h while stirring at 13 rpm. The slurry was fed at ~2 L/min with a Moyno transfer pump (electric pump, model IFFCA SSE SAA, Des Moines, IA) to a continuous BTPX disc-stack centrifuge (Alfa Laval Separation Inc., Warminster, PA) set at a 9800 rpm bowl speed (~12000*g*). This jacketed centrifuge was cooled with ~13 °C water.

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 2 N HCl, and then cooled overnight to ~7 °C. The glycinin-rich precipitate was recovered by using the Alfa Laval centrifuge at a 9800 rpm bowl speed. The centrifuge was fed at a rate of ~4 L/min by the transfer pump. The glycinin-rich precipitate was ~8 °C after exiting the centrifuge. The resulting supernatant was adjusted to 0.25 M NaCl by adding dry NaCl and then adjusted to pH 5.0 with 2 N HCl, stirred at 13 rpm for 1 h, and then centrifuged at 9800 rpm bowl speed. The resulting intermediate fraction precipitate comprised a mixture of glycinin, β-conglycinin, and other proteins. The supernatant was diluted by a factor of 3 with water, adjusted to pH 4.8, and chilled overnight to ~7 °C. The β-conglycinin-rich precipitate was recovered by centrifugation at a 9800 rpm bowl speed. The whey fraction was sampled and then discarded. Recovered protein fractions were adjusted to pH 7.5 with 2 N NaOH and then desalted with a model SRT-50 feed and bleed membrane filtration system (North Carolina SRT Inc., Cary, NC) and a 30-kDa regenerated cellulose membrane (North Carolina SRT Inc.). Diafiltration was continued until permeate equaling 5 times 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 an air outlet temperature of 85 °C. In-process supernatants, the whey, the insoluble fraction, and the protein products after neutralization and after diafiltration were collected and freeze-dried.

Soy Protein Isolate Production. Extraction was accomplished using a 10:1 water-to-flake ratio and an extraction temperature of 60 °C.

Twenty kilograms of flakes and temperature-adjusted water were combined and stirred at 22 rpm in a jacketed 800-L tank. The slurry was adjusted to pH 8.5 with 2 N NaOH and then stirred for 30 min at 13 rpm. The slurry was then fed at ∼2 L/min with the Moyno transfer pump to the continuous BTPX disc-stack centrifuge set at a 9800 rpm bowl speed. The insoluble fraction was sampled and then discarded. The supernatant was adjusted to pH 4.5 using 2 N HCl and then chilled overnight to ∼7 °C. The soy protein isolate slurry was fed at ∼4 L/min to the Alfa Laval centrifuge, which was set at a 9800 rpm bowl speed. The recovered isolate was adjusted to pH 6.8 and then spray-dried at the settings described for drying the fractionated proteins. In-process supernatant, insoluble fraction, whey, and neutralized soy protein isolate samples were collected and freeze-dried.

Proximate Analysis. Nitrogen contents of bench-scale samples were determined by micro-Kjeldahl (*23*), whereas the nitrogen contents from the pilot-plant-scale samples were determined by 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 values using a conversion formula (*25*). A correction factor of 6.25 was used to convert from percentage nitrogen to protein content. Moisture and ash were determined (*26*, *27*).

Isoflavone and Group B Saponin Extraction and Analysis. Extractions and HPLC analyses were conducted as reported by Hu et al. (*18*) for saponins and by Murphy et al. (*14*) for isoflavones, with modifications to extraction solvents and/or extraction times that permitted a single extraction to recover isoflavones and saponins simultaneously without a significant loss ($P < 0.05$) in recovery efficiency (for ground, defatted soy flakes). About 2.5 g of freeze-dried slurry, insoluble fraction, or isolate, or 4.0 g of freeze-dried whey fraction was accurately weighed into 125-mL screw-capped Erlenmeyer flasks, and then 14 mL of water and 20 mL of acetonitrile were added to the flasks. For pilot-plant-scale samples, this mixture was neutralized to pH 7.0 with 2 N HCl or 2 N 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.

For quality control purposes, an isoflavone standard solution (genistin, genistein, and daidzein) and a saponin standard solution (saponin I) were analyzed prior to the analysis of experimental samples. Preparation of isoflavone and saponin standards and calibration curves has been described elsewhere (*14*, *18*). Isoflavone recoveries from ground soy flakes and freeze-dried soy protein isolate were evaluated by using a concentrated isoflavone extract produced from soy flakes. The concentrated isoflavone extract contained 7.5 μmol/mL total isoflavones in 80% methanol. Saponin recoveries from these matrices employed a mixture of saponins 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.

Urea SDS-PAGE. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was performed using the method described by Wu et al. (*10*) as a means to describe the composition of process or product fractions in terms of lipoxygenase, glycinin, and β-conglycinin. Densitometry was accomplished by using the Kodak 1D Image Analysis version 3.5 (Kodak, Rochester, NY) on scanned images produced by a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). SDS-PAGE results were calculated as follows: % composition = [(band or sum of subunit bands)/(all bands measured)] × 100.

Surface Hydrophobicity. The surface hydrophobicity of pH 7 soluble proteins from the spray-dried products was determined using a modification of the method used by Wu et al. (*10*). A 1% (w/w) dry-basis sample dispersion was adjusted to pH 7, stirred and maintained at pH 7 for 1 h, and then centrifuged at 10000*g* for 10 min at 20 °C. The supernatant protein content was determined according to the biuret method. Soluble protein was diluted with 0.01 M phosphate buffer (pH 7.0) to obtain concentrations ranging from 6.25 to 1000 μg/mL. 8-Anilino-1-naphthalenesulfonic acid magnesium salt monohydrate (ANS; ICN Biomedicals, Inc., Aurora, OH) solution (8.0 mM ANS in 0.01 M phosphate buffer) was added to samples and standards. Diluted samples were adjusted to 0.1 mM ANS. The fluorescence zero point was standardized with 0.1 mM ANS in phosphate buffer, whereas 40 μM ANS in methanol was assigned a value of 80.0 fluorescence

intensity units. Fluorescence was measured with a Turner Quantech spectrophotometer (Barnstead Thermolyne, Dubuque, IA) using 360 nm (excitation) and 515 nm (emission) filters. Fluorescence was plotted versus percentage protein concentration. The slope of the regression line was considered to be the surface hydrophobicity. Samples were evaluated in triplicate.

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

Statistical Analysis. Least-squares means, least significant differences, and ANOVA results were calculated using the SAS system (version 8.2, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Bench-Scale Extraction: pH Effects. The total protein extracted from 80.0 g (6.6% moisture) of defatted soy flakes increased significantly from 27.4 to 33.3 g as the extraction pH was increased from 7.5 to 9.0, although the differences in protein content of the pH 8.5 and 9.0 extracts were not significant. 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*). Glycinin solubility began to decrease below pH 8 and decreased rapidly below pH 7.0, whereas β-conglycinin solubility did not decrease substantially until pH 7. At pH 7.5, glycinin solubility may have been reduced, accounting for the lower protein extraction observed for the pH 7.5 treatment. However, this hypothesis was not supported by SDS-PAGE data, which showed that glycinin-to-β-conglycinin ratios in the extracts did not differ from pH 7.5 to 9.0. Extractions at pH >9.0 were not evaluated. Although lysinoalanine formation is more extensive as temperature is increased (*29*), increasing the extraction pH above 9.0 increases the risk of lysinoalanine formation. Additionally, isoflavones ionize above pH 9.0 (*30*; J. Krol, Waters Corp., personal communication), possibly increasing their affinity for the soy matrix. Therefore, an extraction pH of 8.5 was selected as optimum.

Bench-Scale Fractionation: Ethanol Effects. Solvents, such as acetonitrile, ethanol, and methanol, have been used to extract soy phytochemicals from food and soy samples (*31*). We hypothesized that a small amount of alcoholic solvent might improve phytochemical extraction from the soy flakes and translate into increased concentrations in product fractions.

Ethanol in the extraction medium had little impact on phytochemical recovery from soy flakes. Although isoflavone concentration in the insoluble fraction, a process waste stream, decreased significantly from 4.53 to 4.08 μmol/g as the ethanol concentration of the extraction medium increased from 0 to 10%, total isoflavone contents of the insoluble fractions were not different. This was due to an increase in insoluble fraction weights as ethanol concentrations increased. Saponin concentration did not change significantly, ranging from 6.19 to 6.36 μmol/g. The isoflavone and saponin concentrations in the soy flakes were 8.68 and 6.51 μmol/g, respectively. This limited effect on extraction was not surprising because 59% acetonitrile and 70% ethanol solutions (*14*, *18*) are commonly used for soy isoflavone and saponin extraction, respectively. These phytochemicals are poorly soluble or insoluble in water. Their transfer into the extract fraction from the starting material was likely either from mass action effect or due to their associations with solubilized proteins.

The dry-basis weight of the insoluble fraction increased significantly from 29 to 34 g per 80 g of flakes as the ethanol

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Table 1. Water-to-Flake Ratio Effects on Fraction Weight and Protein Recovery in Process Fractions Produced during Bench-Scale Fractionation of Soy Storage Proteins[a]

ratio	insoluble	glycinin	intermediate	β-conglycinin	whey	total
		Process Fraction Weights (Grams, Dry Basis)				
6:1	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	3.19	1.22	0.76	ND	1.82	4.39
		Total Protein (Grams, 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	ND	0.40	ND

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

Table 2. Water-to-Flake Ratio Effects on Isoflavone and Saponin Concentrations and Mass during Bench-Scale Fractionation of Soy Storage Proteins[a]

ratio	insoluble	glycinin	intermediate	β-conglycinin	whey	total
		Isoflavone Concentration (Micromoles per Gram, Dry Basis)				
6:1	7.02 a	10.17 a	15.3 b	4.79 a	4.17	
8:1	6.29 b	9.74 b	15.8 a	3.81 b	4.90	
10:1	5.49 c	9.40 c	15.1 b	3.27 bc	4.81	
15:1	4.63 d	8.34 d	13.8 c	2.85 c	4.50	
LSD	0.39	0.15	0.5	0.61	ND	
		Isoflavone Mass (Micromoles, Dry Basis)				
6:1	290 a	55.4	84 c	32.6 a	63 b	525
8:1	240 b	68.7	93 bc	27.1 b	103 ab	532
10:1	185 c	74.0	105 a	21.7 bc	121 a	507
15:1	143 d	66.4	101 ab	18.6 c	146 a	476
LSD	30	ND	9	5.4	43	ND
		Saponin Concentration (Micromoles per Gram, Dry Basis)				
6:1	7.05	2.67 a	16.5	1.51	nd	
8:1	7.16	2.02 b	18.2	1.73	nd	
10:1	7.05	1.62 c	15.8	1.97	nd	
15:1	6.80	1.27 c	14.7	1.74	nd	
LSD	ND	0.39	ND	ND		
		Saponin Mass (Micromoles, Dry Basis)				
6:1	291 a	14.5	90	10.2	nd	406 a
8:1	273 b	14.2	106	12.3	nd	406 a
10:1	238 c	12.8	110	13.0	nd	374 b
15:1	211 d	10.1	108	11.3	nd	340 c
LSD	15	ND	ND	ND		12

[a] Starting material was 80.0 g ("as is" basis, 6.6% moisture) of defatted soy flakes. Letters within a column indicate significant difference at $P < 0.05$. $n = 2$. LSD, least significant difference; nd, none detected; ND, not different.

concentration increased, indicating reduced extraction of soluble components, whereas the weight of the β-conglycinin fraction decreased significantly from 7 to 2 g. SDS-PAGE analysis of the extract did not indicate that β-conglycinin was extracted 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. The intermediate fraction weight did increase significantly between 1 and 10% ethanol. This increase apparently occurred due to diminished β-conglycinin solubility. It is possible that β-conglycinin was more readily denatured by the ethanol than was glycinin, causing differences in partitioning. Roberts and Briggs (*32*) used sedimentation patterns to demonstrate that β-conglycinin (7S fraction) is more readily denatured by ethanol than glycinin (11S fraction). It should be noted, however, that these experiments were based on the ability of the proteins to resolubilize at pH 7.6 after precipitation at pH 5.0 and exposure to ethanol concentrations of 10−100%. The most dramatic differences in solubility were observed after exposure to 40% ethanol for 8 h.

Bench-Scale Fractionation: Water-to-Flake Ratio Effects. Higher water-to-flake ratios should improve extraction of soluble components; however, higher ratios lead to reduced process capacity on a protein basis. 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. For soy flakes, a 6:1 water-to-flake ratio was the lowest ratio that could be used and still maintain the ability to effectively stir the suspension. Water-to-flake ratios of 6:1, 8:1, 10:1, and 15:1 were selected for evaluation.

The dry weight of the insoluble fraction decreased significantly, 24%, between the 6:1 to 15:1 water-to-flake ratios, respectively (**Table 1**), indicating higher water-to-flake ratios were more efficient at extracting solubles from the soy flakes. The glycinin and intermediate fraction weights increased significantly as the water-to-flake ratios increased from 6:1 to 15:1, but differences between the 10:1 and 15:1 water-to-flake ratios were not significant. There were no significant differences among β-conglycinin fraction weights. The total protein contents for all fractions were not different, indicating excellent accounting of the protein mass balance. SDS-PAGE revealed that there were no differences among treatments for glycinin fraction purity. However, the β-conglycinin fraction purity increased from 59 to 66% as the water-to-flake ratio increased from 8:1 to 10:1.

The isoflavone concentration (dry basis) of the insoluble, glycinin, intermediate, and β-conglycinin fractions decreased with increasing water-to-flake ratio (**Table 2**), but total isoflavones in the intermediate fractions increased due to a corresponding increase in fraction weights. The whey isoflavone concentrations were not different, but total whey isoflavones increased as the water-to-flake ratio increased concomitantly with the dry weight of the whey fraction. These data indicate that improved isoflavone extraction from the soy flakes did not translate into increased isoflavone concentration in the product fractions, but rather increased isoflavones in the whey. Shen and Bryan (*33*) disclose in their patent that excessive process water or washing of precipitated protein results in unwanted loss of aglucons. Because our protein precipitation step that produced the intermediate fraction occurred at pH 5.0 and the step that produced the β-conglycinin and whey fractions occurred at pH 4.8, it is not likely that isoflavone partitioning was affected by ionized forms of isoflavones because isoflavones are not substantially ionized at those low pH values.

Isoflavones were preferentially partitioned among the product fractions, with the distribution being about 2:3:1 on a micromole per gram basis for the glycinin, intermediate, and β-conglycinin precipitate fractions, respectively (**Table 2**). Wu et al. (*10*) indicated that most of the glycinin and β-conglycinin proteins comprising the intermediate fraction were no longer in the native state. It is probable that as the native state was lost and due to protein unfolding, more hydrophobic areas were exposed to interact with the isoflavones. However, the native state does not explain why the isoflavone content of the glycinin fraction was twice that of 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 retained substantial quantities of isoflavones.

Saponin partitioning was different from that observed for the isoflavones, being partitioned 1:8:1 on a micromole per gram basis among the glycinin, intermediate, and β-conglycinin fractions (**Table 2**). Because saponins are more hydrophobic than the isoflavones, they may have been even more attracted to the non-native state proteins of the intermediate fractions. Saponins were not detected in the whey. In contrast to isoflavone concentrations, saponin concentrations were significantly different only for the glycinin fraction, ranging from about 3 to 1 μmol/g for the 6:1 and 15:1 ratios, respectively. Saponin concentrations in the insoluble fractions were uniform for all treatments. However, the total protein content of the insoluble fraction decreased by about one-third as the water-to-flake ratio increased; therefore, the saponins may be associated with the insoluble proteins or other insoluble components.

The starting material contained 648 and 486 μmol of isoflavones and saponins, respectively. The mass balance data (**Table 2**) show that when total isoflavones from all fractions were summed, the sums ranged from 507 to 532 μmol, whereas total saponins ranged from 340 to 406 μmol. These values translate to about 80 and 70–84% of the recovery of isoflavones and saponins observed in the starting material, respectively. Physicochemical differences between the matrices of the soy flakes and the process fractions may have been responsible for differences in extraction efficiencies between these materials, leading to mass balance discrepancies.

An analytical recovery study with ground, defatted soy flakes and freeze-dried soy protein isolate (similar to the high-protein fractionation products) revealed considerable analytical recovery differences between these matrices. Recoveries of saponins I and II were ~99% for the flakes, but only 60–70% for the isolate. Analytical isoflavone recoveries were 75–92% for the flakes, with the more hydrophobic aglucons exhibiting the lowest recoveries. Isoflavone aglucon recovery from the isolate material was 65–75%, whereas recoveries for the β-glucosides and malonylglucosides were 78–93%. Neutralizing samples at the time of phytochemical extraction increased saponin I and II recoveries from the isolate samples by about 15 and 25–45%, respectively. Neutralizing samples increased recovery of the aglucon isoflavones from the isolate by 10–15%. The impact of these characteristics on mass balance data depends on the types of isoflavone forms in the various process fractions. If isoflavones are primarily aglucons, the impact could be substantial. In this work, corrections for recovery were not calculated because recovery information was not known for each product matrix.

Bench-scale process samples were not neutralized prior to freeze-drying or phytochemical extraction. Consequently, the pH values of the extraction media used during phytochemical analysis were approximately 4.8 and 5.0 for the β-conglycinin and intermediate fractions, respectively. A glucuronic acid moiety on the saponin aglucon would be expected to have a pK_a of ~4. The pH effect could lead to ionic bonding of the saponins within the protein matrix of the product fractions and poor recovery during phytochemical extraction, although this type of bonding may be of less importance than hydrophobic bonding.

The 10:1 water-to-flake ratio was selected as the optimum ratio. The process fraction weights and total protein for the 10:1 and 15:1 ratios were significantly higher than or not different from the 6:1 and 8:1 ratios, and there were no differences between fraction weights and total protein for the 10:1 and 15:1 ratios. The purities of the β-conglycinin fractions were higher for the 10:1 and 15:1 ratios. The saponin concentrations of the

Table 3. Extraction Temperature Effects on Fraction Weight and Protein Recovery in Process Fractions Produced during Bench-Scale Soy Protein Fractionation of Soy Storage Proteins[a]

temp (°C)	insoluble	glycinin	intermediate	β-conglycinin	whey	total
Process Fraction Weights (Grams, Dry Basis)						
25	32.17 a	8.41 bc	6.49 d	6.71 ab	23.22	76.99
35	29.22 b	9.31 a	7.58 d	7.39 a	26.00	79.49
45	28.05 bc	8.96 ab	9.53 c	7.55 a	25.29	79.36
55	28.62 bc	8.26 c	12.91 b	6.29 b	23.64	79.70
65	27.31 c	6.02 d	22.73 a	1.68 c	23.09	80.82
LSD	1.46	0.60	1.41	1.07	ND	ND
Total Protein (Grams, Dry Basis)						
25	13.0 a	8.31 a	4.91 e	6.34 ab	4.23 ab	37.1
35	10.8 b	9.03 a	5.51 d	6.99 a	4.67 a	36.9
45	10.0 c	8.67 ab	6.96 c	7.10 a	4.32 ab	37.3
55	9.9 cd	8.05 b	9.62 b	5.88 b	3.85 bc	37.7
65	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) of defatted soy white flakes. Letters within a column indicate significant differences at $P < 0.05$. $n = 2$. LSD, least significant difference. ND, not different.

intermediate and β-conglycinin fractions were not different, although the concentrations in the glycinin fractions for the 10:1 and 15:1 ratios were lower. The 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 acceptable because the differences in phytochemical concentrations, although 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 Fractionation: Effect of Temperature. Berk (*12*) reported that as the extraction temperature increased to 80 °C during the extraction step of soy protein isolate manufacturing, soy protein recovery increased. Wu et al. (*10*) expressed concerns that increased extraction temperatures would alter the protein native state and interfere with desired protein fractionation outcomes. Therefore, extraction temperatures of 25–65 °C were selected to test this hypothesis.

The insoluble fraction weight, protein concentration, and total protein decreased with increasing temperature, indicating improved protein extraction (**Table 3**). The intermediate fraction weight increased as extraction temperature increased, whereas β-conglycinin fraction weight decreased significantly at 55 °C and declined sharply at 65 °C. Denaturation was likely the cause for this decline, with β-conglycinin being partitioned to the intermediate fraction. The onset temperatures for β-conglycinin and glycinin denaturation are approximately 68 and 82 °C, respectively (*34*). Glycinin fraction weight was lower at 65 °C. These data support the hypothesis of Wu et al. (*10*) that the intermediate fraction is primarily composed of glycinin and β-conglycinin proteins that are no longer in their native state. The whey fraction weights were not different, indicating that denatured protein was not being partitioned into whey. The β-conglycinin fraction purities were not different from 25 to 45 °C; however, the glycinin fraction purities were higher at 45 °C (86%) compared to 25 and 35 °C (~82%).

The isoflavone concentrations decreased with increased extraction temperature for all process fractions except the whey fraction (**Table 4**). Saponin concentrations behaved similarly to the isoflavones, except there were no saponins in the whey (**Table 4**). This decrease in phytochemical concentrations for all fractions was accounted for in the phytochemical mass in the intermediate fraction because more protein mass was

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Table 4. Temperature Effects on Isoflavone and Saponin Concentrations and Mass during Bench-Scale Fractionation of Soy Storage Proteins[a]

temp (°C)	insoluble	glycinin	intermediate	β-conglycinin	whey	total
Isoflavone Concentration (Micromoles per Gram, Dry Basis)						
25	5.33 a	9.48 a	15.1 a	3.04 a	5.08	
35	5.26 ab	9.10 a	14.5 ab	3.16 a	5.44	
45	5.04 cd	8.44 b	13.9 b	2.65 ab	5.12	
55	5.07 bc	8.35 b	12.3 c	2.31 b	5.47	
65	4.85 d	7.86 c	10.4 d	IS	5.12	
LSD	0.20	0.47	0.7	0.55	ND	
Isoflavone Mass (Micromoles, Dry Basis)						
25	171 a	79.6 ab	98 e	20.5	118	487
35	153 b	84.7 a	109 d	23.4	141	513
45	141 cd	75.7 bc	132 c	20.0	129	498
55	145 bc	68.9 c	159 b	14.5	130	517
65	132 d	47.3 d	237 a	IS	118	535
LSD	10	7.2	10	ND	ND	ND
Saponin Concentration (Micromoles per Gram, Dry Basis)						
25	6.51 a	1.74 a	18.6 a	1.78	nd	
35	6.30 a	1.83 a	18.0 a	1.84	nd	
45	6.05 a	1.09 c	17.0 b	1.90	nd	
55	5.55 b	0.80 d	13.9 c	1.44	nd	
65	4.70 c	1.44 b	9.0 d	IS	nd	
LSD	0.48	0.28	0.8	ND		
Saponin Mass (Micromoles, Dry Basis)						
25	209 a	14.60 b	121 c	11.9 ab	nd	357
35	184 b	17.05 a	136 c	13.6 a	nd	351
45	170 bc	9.76 c	162 b	14.3 a	nd	356
55	159 c	6.61 d	179 b	9.0 b	nd	353
65	128 d	8.68 c	204 a	IS	nd	341
LSD	16	2.07	22	3.1		ND

[a] Starting material was 80.0 g ("as is" basis, 6.6% moisture) of defatted soy flakes. Letters within a column indicate significant difference at $P < 0.05$. $n = 2$. LSD, least significant difference; nd, none detected; IS, insufficient sample; ND, not different.

Table 5. Mass Balance Comparisons for Pilot-Scale Fractionation Processes[a]

fraction	method	wt (kg, db)	ash (%, db)	protein (%, db)	isoflavones (μmol/g, db)	saponins (μmol/g, db)
flakes	Wu	18.06	6.6	59.1*	6.15	5.26
	opt	18.02	6.8	61.6	5.90	5.16
insoluble	Wu	7.90	6.4	49.9	3.89	5.98
	opt	7.38	6.4	48.0	4.11	7.26
extract	Wu	10.04*	9.0	66.1	4.84**	3.01
	opt	10.68	9.0	67.1	5.93	3.31
GLY P	Wu	2.55	3.6	98.5	6.25	1.17*
	opt	2.34	3.0	99.8	6.37	0.57
GLY SD	Wu	2.25	1.9	104.2	2.30	0.70
	opt	2.04	1.7	105.6	1.83	0.36
1st supt	Wu	7.65**	13.9	52.1**	6.86	3.46
	opt	8.41	12.6	55.8	6.40	3.44
INT P	Wu	1.64	13.9	75.9	8.33	14.74
	opt	1.64	13.7	78.3	8.57	11.09
INT SD	Wu	1.21	2.6	91.5	3.09	14.09
	opt	1.23	2.5	95.5	2.28	11.35
2nd supt	Wu	10.18	50.4**	26.2*	2.19	0.12
	opt	9.81	40.1	33.8	2.29	0.15
BCON P	Wu	1.40	9.2	89.7	1.96*	1.50
	opt	2.45	7.1	91.3	3.33	5.22
BCON SD	Wu	1.29*	2.6	99.4	0.31*	1.13**
	opt	2.10	2.6	98.9	0.67	3.66
whey	Wu	8.77*	59.4**	13.7	3.42	0.24
	opt	7.23	53.4	14.2	4.09	0.32

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

partitioned into the intermediate fraction. The sums of isoflavone and saponin masses from the process fractions, as observed for the water-to-flake study, were low compared to their total masses in soy flakes.

On the basis of fraction and protein weights and fraction purity, a protein extraction temperature of 45 °C was selected as optimum. However, the phytochemical concentrations tended to be lower for this treatment compared to the 25 and 35 °C treatments.

Pilot-Plant-Scale Fractionation: Wu Modified-Nagano versus Optimized Process. Previous pilot-plant-scale work by our research group used the Wu modified-Nagano process (*10*), referred to here as the Wu process. The Wu-process extraction conditions included a 20 °C extraction temperature and a 15:1 water-to-flake ratio. The conditions for our optimized process were 45 °C and a 10:1 water-to-flake ratio. The process described by Wu et al. (*10*) required a major modification due to pilot-plant centrifuge problems. Wu utilized a two-pass system through a Sharples P660 decanting centrifuge to separate the extract from the insoluble fraction. In the present work, a one-pass system employing an Alfa Laval BTPX 205 disc-type centrifuge was substituted for the two-pass system.

Soy protein isolate was produced at the pilot-plant scale so that fraction weight, total protein, and phytochemical recovery could be compared with protein fractionation process results. The extraction temperature and water-to-flake ratio employed for soy isolate production were 60 °C and 10:1, respectively.

One of the most interesting differences between the two processes was the increased fraction weight of the optimized-

method β-conglycinin fraction (**Table 5**). The fraction weight of the optimized-process spray-dried β-conglycinin was 2 kg, whereas that of the Wu process was 1 kg. The total protein relationship for these two fractions was similar to that of the fraction weights. SDS-PAGE analysis of these fractions indicates that the optimized-method β-conglycinin had significantly lower purity, 68%, compared to 79% for the Wu method.

The protein extract of the optimized method contained higher dry basis concentration and mass of isoflavones (**Table 5**). Higher isoflavone concentrations in the optimized-process extract did not necessarily translate into high isoflavone concentrations in the product fractions compared to the Wu-process product fractions. The isoflavone concentrations and total mass were different, however, for the β-conglycinin fractions of the two processes ($P < 0.10$).

The saponin concentrations and total masses for the insoluble and extract fractions were not different between the Wu and optimized processes (**Table 5**). The Wu-method saponin concentrations and totals for the glycinin and intermediate fractions were higher, but lower for the β-conglycinin fraction. High proportions of isoflavones and saponins were partitioned into the intermediate fraction. This partitioning was likely due to the prevalence of denatured protein in the intermediate fraction. DSC results suggested that very little native-state protein remained in the intermediate fractions for both processes (data not shown). The optimized intermediate fraction demonstrated high surface hydrophobicity, 183, compared to 129 and 124 for the glycinin and β-conglycinin fractions, respectively, supporting the hypothesis for the high saponin content of the intermediate fraction. However, hydrophobicity values for the optimized-process glycinin and β-conglycinin did not explain why the saponin concentration was 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

Table 6. Isoflavone Distribution for Selected Pilot-Scale Soy Protein Fractionation and Soy Protein Isolate Process Fractions[a] (Micromoles per Gram, Dry Basis)

process	fraction	MDIN	DIN	AcDIN	DEIN	MGLY	GLY	GLYEIN	MGIN	GIN	AcGIN	GEIN	total
SF	flakes	1.92 a	0.71 bc	0.08 ab	0.09 de	0.22 a	0.17 b	0.02	1.83 c	0.81 cd	0.09 ab	0.07 j	6.01 b
Wu	GLY P	1.36 b	0.08 de	0.07 abcd	0.76 bc	0.15 abc	0.04 cd	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	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	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	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	0.08	0.86 e	0.07 g	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	0.02	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	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	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	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	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	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	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 d	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	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	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	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	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	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	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	0.07	1.40 d	2.24 a	0.08 bcde	0.29 ghij	7.73 a
LSD		0.33	0.23	0.06	0.36	0.14	0.06	ND	0.35	0.41	0.04	0.35	1.15

[a] $n = 2$. Letters within a column indicate significant difference at $P < 0.05$. 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; nd, none detected; LSD, least significant difference; ND, not different.

glycinin, intermediate, and β-conglycinin, respectively. It may be possible that saponins have an affinity for denatured β-conglycinin, explaining the high saponin concentration in the intermediate fractions, which contained little native-state β-conglycinin. The β-conglycinin fraction of the optimized method had less native-state β-conglycinin in the β-conglycinin fraction and, thus, a higher concentration of saponins in the β-conglycinin fraction compared to the Wu method.

Sodium chloride addition during processing was based on volume, so the Wu-process second supernatant and whey fractions contained significantly more ash. Diafiltration was used in our work to reduce the ash concentrations in product fractions to minimize the effect of salts during protein functionality analyses. Dry-basis ash concentrations prior to and after diafiltration ranged from 3.0 to 13.9% and from 1.6 to 2.7%, respectively. Diafiltration of the protein product fractions caused ~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 during diafiltration, 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 kDa, whereas the molecular weights of the isoflavones and saponins are all <1.1 kDa. In contrast to diafiltration, spray-drying did not lead to a measurable loss in isoflavones or saponins.

The mass and protein yields, as well as the total isoflavone and saponin masses, of the combined product fractions were evaluated for differences between the Wu, optimized, and soy protein isolate processes. The comparisons were based on the precipitate fractions because the Wu and optimized processes employed diafiltration prior to spray-drying, which resulted in substantial losses of isoflavones and saponins. Mass yields for the Wu, optimized, and isolate fractions were 31, 36, and 37%, respectively, and protein yields were 47, 53, and 54%, respectively, but these yields were not different. The total phytochemicals recovered in the soy protein isolate were much higher than

for the protein fractions of the Wu and optimized methods, possibly due to the relatively high proportion of denatured protein or more limited exposure to process water.

Processing conditions can affect the prevalence and distribution of the several forms of soybean isoflavones and saponins. Soy isoflavones are composed 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. The malonylglucoside of each aglucon family is the predominant form in soybeans (35) and in the soy flakes used here, with prevalence of combined forms generally being genistein ≈ daidzein ≫ glycitein on a mole basis. When soy is processed under mild conditions, malonylglucosides will gradually convert to the β-glucoside forms (14, 16). Conversion of malonylglucosides to acetylglucosides occurs during more abusive conditions, such as toasting and extrusion (36, 37). In the presence of native soybean β-glucosidases, the glucoside isoflavones will be converted to their respective aglucon forms (16).

For the Wu process, optimized process, and soy protein isolate process, 30−35% of the malonylglucoside isoflavones present in the defatted soy flakes were converted to the β-glucoside and aglucon forms or were lost in the mass balance (**Table 6**). The malonyldaidzin concentration in the soy flakes was 1.92 μmol/g compared to 1.24, 1.36, and 1.28 μmol/g for the isolate precipitate, Wu-process glycinin precipitate, and optimized-process glycinin precipitate, respectively. The glycinin fraction was chosen for this comparison because processing times were similar for glycinin and soy protein isolate precipitates, and these fractions all had similar total isoflavone concentrations.

The data suggest that there were two competing conversions acting on the malonylglucosides. For the isolate, 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 extraction temperature, 60 °C, drove the conversion to the β-glucosides,

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Table 7. Saponin Profiles of Selected Pilot-Scale Soy Protein Fractionation and Soy Protein Isolate Process Fractions[a] (Micromoles per Gram, Dry Basis)

process	fraction	V	I	II	αg	βg	βa	total
SF	flakes	0.01	0.62 fg	0.35 efgh	0.19	2.98 a	1.13 a	5.28 ef
Wu	GLY P	nd	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
LSD		ND	1.52	0.86	ND	0.48	0.16	2.27

[a] Letters within a column indicate significant difference at $P < 0.05$. $n = 2$. Abbreviations: V, I, and II are non-DDMP saponins; αg, βg, and βa are DDMP saponins; Wu, Wu 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; ND, not different.

and this temperature probably limited the glucosidase activity responsible for conversion to the aglucons (*16*). The isoflavone conversion schemes were different between the Wu and optimized processes and also different from what was observed for the soy protein isolate. The data support the hypothesis that protein solubilization temperature affected the conversion process. In the Wu-method glycinin precipitate, malonylglucosides were converted to aglucons. In fact, the β-glucosides present in the flakes were converted to aglucons as well. These data suggest that there was considerable glucosidase activity, time, and low process temperature. For the optimized process, the conversion of malonylglucosides to aglucons was not as substantial. Daidzin concentration decreased only slightly compared to the flakes, and genistin concentrations actually increased slightly. The 45 °C extraction temperature must have limited the action of the glucosidases. The intermediate and β-conglycinin precipitates display the same conversion features described for the glycinin precipitate. The isoflavone mass balance discrepancies were probably due to poor recovery of the aglucons from the glycinin fraction matrix. As reported above, isoflavone aglucon recovery may be as low as 65−75% from soy protein isolate. Spray-drying did not cause a conversion among the isoflavone forms.

The intermediate fraction had the highest concentration of aglucons. If surface hydrophobicity or lack of protein native state has a role in the affinity of relatively hydrophobic phytochemicals, it is predictable that the most hydrophobic isoflavones, the aglucons, would be present in higher concentrations in the intermediate fraction. However, the β-conglycinin fraction had the highest percentage of aglucons, >40%. The time for enzymatic hydrolysis, surface hydrophobicity, and partitioning of more hydrophilic forms into earlier fractions probably all contributed to the high concentration of aglucons in the β-conglycinin fraction.

The group B saponins measured by the Hu method are soyasaponins V, I, II, αg, βg, and βa. The latter three have a 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP)

moiety, the bond linkage of which is labile. Thus, the DDMP forms readily convert to non-DDMP forms V, I, and II, respectively. Group B saponins III, IV, γg, and γa are present in soybeans but only in very low concentrations.

The DDMP forms of the saponins were in relatively high concentrations in the defatted soy flakes (**Table 7**). During processing, the DDMP forms were mostly converted to non-DDMP forms. Surprisingly, this conversion occurred substantially during the diafiltration process, which lasted only 3−4 h at a temperature near ambient. This was most easily observed for the intermediate fraction. Spray-drying did not cause a conversion among the saponin forms.

In conclusion, the optimized process yielded a larger β-conglycinin fraction by weight than did the Wu process, whereas there was no significant difference between the processes for glycinin or intermediate fraction weights. The optimized-process β-conglycinin fraction purity was less than the Wu-process β-conglycinin fraction purity due to glycinin contamination. Functionality evaluations will indicate whether decreased β-conglycinin purity is a disadvantage. The intermediate fractions from both fractionation processes and the soy protein isolate contain substantial quantities of non-native state proteins. These products also had higher concentrations of phytochemicals than did the glycinin and β-conglycinin fractions from both fractionation processes. It will be difficult to further modify the fractionation process to increase phytochemical concentrations in the glycinin and β-conglycinin fractions while maintaining the ability to efficiently and effectively fractionate the proteins because different physicochemical protein properties (native state vs non-native state proteins) are needed to achieve these two goals. The insoluble fractions from both fractionation processes contain substantial quantities of isoflavones and saponins, making these waste stream fractions an attractive source for additional extraction and recovery of these phytochemicals. The same is true for the whey waste streams with regard to isoflavones.

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