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Refunctionalization of extruded-expelled soybean meal by hydrothermal cooking

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GENERAL INTRODUCTION

Project Significance

Extruding-expelling (EE) is an alternative low-cost technology to extract oil from soybean. The soybean meal that is produced has limited applications due to heat denaturation of protein. If the functional properties of soy protein in EE meal can be improved, EE meal and its enriched protein products will have more markets in the food industry. EE is uniquely suitable for identity-preserved soybean processing due to its small scale and flexibility. The success of EE technology will encourage producing specialty soybeans, which usually have higher returns for the farmers. More producer income and job opportunities will result and be retained in agricultural communities where such a technology is utilized.

Characteristics and Significance of Extruding-Expelling Process

In 1969, a Des Moines, IA, company, Triple "F" Inc., patented a dry soybean extruder to produce animal feeds (1). Unlike alternative extrusion technologies, dry extrusion does not involve steam generation and injection into the extruder, hence the invention was termed "dry extrusion." The extruder was designed to be powered by a farm tractor so that livestock feed could be produced on the farm where used. Extrusion cooking was important to inactivate trypsin inhibitor and improve feed conversion. Because of the rapid acceptance of dry extrusion, another company, Insta-Pro International, was chartered to manufacture and market the dry extruders (2).

Nelson *et al.*(3) were the first to use dry extrusion to replace steam-heated driers to prepare soybeans for screw pressing and a new concept of combining extrusion with expelling was developed to

extract oil from soybeans. This continuous process involved pressing the hot semi-fluid extrudate from dry extrusion. Over 70% oil yield was achieved in a single pass, leaving cake containing 50% protein and 6% residual oil, while achieving 90% inactivation of trypsin inhibitors. Insta-Pro International added screw presses (expellers) to their product line under the trademark name of ExPress™ to commercialize this concept.

In the first step of the process, the friction, shear, and pressure generated during dry extrusion disrupted the soybean cell structure, denatured proteins, and released the oil from the spherosomes. In the second step, the released oil was removed from the solids by mechanically pressing before the solids cooled. This technology couples dry-extrusion with screw pressing or expelling, hence the name extruding-expelling (EE) became accepted.

A major advantage of the EE process compared with the modern solvent-extraction process using hexane, which dominates the oil seed processing today, is that EE is purely a mechanical process and no organic solvent is involved, which avoids hazardous emissions and even explosions. Due to the relatively simple machinery used, EE processing requires low capital investment and is ideal for de-centralized soybean processing in rural areas close to where livestock are fed as well as for identity-preserved (IP) processing of specialty soybeans.

Properties and Applications of EE Products

Most EE processing is used to produce high-energy, high-protein livestock feed because EE meal has significant amounts of protein and residual oil, and the anti-nutritional factors, such as trypsin inhibitors,

are inactivated. Feeding experiments using chickens have shown that higher extrusion temperatures improved the digestibility of EE meals (4). Extruding at 138-154˚C produced protein with quality close to that of toasted, hexane-defatted soybean meal when used as poultry feed. Studies on the digestibilities of EE meals with or without hulls indicated that EE meals had higher apparent ideal digestibility of amino acids, higher digestible and metabolizable energy than white flakes (5).

Additional studies have shown that the body-weight-gain/feed ratio of growing pigs increased when fed EE meals exposed to higher extrusion temperatures, with the most significant body-weight-gain/feed ratio increase at an extrusion temperature of 149°C (6). Pigs fed EE meal had high body-weight-gain/feed ratios compared with those fed toasted, solvent-extracted meal when the pigs grew from 25 to 61 Kg. For the overall growing period, the average daily gain (ADG) was not affected but average daily feed intake (ADFI) declined, indicating feed efficiency improved when the pigs were fed EE meal with or without added fat (6).

EE soybean oil, having a pleasant nutty flavor, can be used for food applications with minimal refining. Compared with solvent-extracted soybean oil, EE oil has lower levels of free fatty acids and dramatically lower levels of phospholipids. EE oil typically has higher peroxide values indicating more oxidation than solvent-extracted soybean oil, which was attributed to prolonged heating during EE processing and poor oil storage conditions at EE plants (7). A natural oil refining technique, in which the EE oil was subjected to settling at refrigeration temperature, followed by water-degumming, free fatty acid adsorption, and mild deodorization (lower temperature than typical), was shown to be effective in producing stable and high quality soybean oil with more tocopherols and carotenoids retained than

conventionally refined soybean oil (8). Commercial trials have shown physically refined EE oil to be a promising frying oil. Compared with solvent-extracted, partially hydrogenated oil, the minimally refined EE oil had longer frying life, did not contain trans-fatty acids, and the fried products tasted better, although its Oxidation Stability Index (OSI) value tended to be lower (9). The mechanism underlying the longer frying life despite low OSI is yet to be understood.

The partially defatted EE meals made from dehulled soybeans can be further processed into foods or food ingredients (10). By changing EE processing parameters, EE meals with different residual oil contents and protein dispersibility indices (PDI, an indirect measurement of soluble and native protein) can be readily produced for use in different applications (11). Pressing twice reduced the residual oil while maintaining PDI of the EE meal compared with single-pressing. As expected, PDI and residual oil have shown a strong correlation. The temperature in the extruder, which could be manipulated by adjusting the feeding and extruder configurations, negatively correlated with both PDI and residual oil content. High temperature also inactivated trypsin inhibitor and lipoxgenase. EE processing can be tailored to produce EE meals with suitable characteristics for different applications. For example, lower extrusion temperature can result in EE meal with higher PDI and moderate lipoxgenase activity, which may be desirable in baking applications (11). Lipoxygenase activity can improve mixing properties of bread dough and bleach carotenoids.

The relationships between PDI/residual oil content and major functionalities, such as solubility, water-holding capacity, fat-binding capacity, emulsification capacity, etc, were investigated by Heywood *et al.* (12). They found that EE meals with lower PDI had lower emulsification capacities than those with

higher PDI. PDI and residual oil were negatively correlated with water-holding capacity, but positively correlated to fat-binding capacity. Similar relationships between residual oil and water-holding capacity were observed in texturized EE meal (13). No significant difference, however, was found in water-holding capacities of texturized proteins over a range of PDI values. High residual oil content resulted in lower hardness values for the texturized proteins, which was attributed to oil inhibition of protein interactions during texturization (13). EE meal-wheat flour blend had comparable water-holding capacity to defatted soy flour-wheat flour blend although EE meal had significant amount of oil (14).

Sensory evaluation of texturized EE-meal-extended ground-beef patties with controls of non-extended beef and commercial patties has been conducted (13). The cooking loss of texturized EE meal-extended ground-beef patties was similar to that of the pure-beef patties or commercial controls. Trained panelists detected soy flavor in patties prepared with texturized proteins from EE meal having moderate PDI but they found the overall taste was similar to that of the control (13). This study demonstrated that EE meal can be further extruded to produce texturized low-fat soy protein products for food applications.

The low processing capacity and flexibility make the EE technique ideal for identity-preserved processing to produce specialty soybean ingredients, such as soy oil and soy proteins with claims of "organic," "all natural," or "non-GMO," depending on the beans processed. The volumes and market shares of food products with such claims are steadily increasing, being driven by the demands of health-conscious customers. For example, natural products sales in the United States enjoyed a 9.1% growth rate in 2005 with $51.39 billion in total sales (15).

Soy Protein Structure

Proteins constitute about 40% of dry mass of the soybean seed (the rest being 34% carbohydrates, 21% oil, and 5% ash) (16). Glycinin and β-conglycinin account for 51 and 19% on average, respectively, of the seed protein (17). They are the two major storage proteins that act as nitrogen reservoirs for the geminating embryo. The molecular weights of glycinin and β-conglycinin are about 350 and 180 kDa (18), when each exists as its typical structure, i.e. hexamer and trimer, respectively. Glycinin contains more sulfur than β-conglycinin.

Glycinin has five different subunits, A1aB1b, A1bB2, A2B1a, A3B4, and A5A4B3, which are composed of two groups of components, acidic (A and a) and basic (B and b) polypeptides, and are covalently bridged by disulfide bonds (except A4) (18-20). The molecular sizes of the acidic and basic components are 38 and 20 kDa (21), respectively. Each pair of acidic and basic components is encoded by one gene (22). The final polypeptides are the products of complex proteolytic cleavage during translation (22-23). The five types of glycinin subunits can be separated into two groups (group I: A1aB1b, A1bB2, and A2B1a; group II: A3B4, and A5A4B3). Group I subunits have molecular weights of 58 kDa and 5-8 methionine groups, while those in group II have molecular weights of 62-69 kDa and about 3 methionine groups. The sequence homology between subunits is greater than 80% within the same group but only 40-50% between subunits across different groups (18).

The association and dissociation of the subunits are influenced by various factors. Glycinin exists as hexamers at pH 7.6 and 0.5 ionic strength, and trimers at pH 3.8 and 0.03 ionic strength. Between two

extreme conditions glycinin exists as an intermediate mixture of both hexamers and trimers (24). Proteolytic tests verified that the acidic polypeptides are located in the exterior of the glycinin complex (24).

β-Conglycinin is a trimeric glycoprotein containing about 5% carbohydrates, and consists of three subunit types, α, α', and β with molecular sizes of about 67, 71, and 50 kDa (106), and having 2, 2, and 1 N-glycosylation consensus sequences, respectively (25). Glycosylation occurs during translation and the polypeptides were modified after translation in a slow and complex process (23). The α and α' subunits have extension regions of 125 and 141 residues, respectively, and a core region of 418 residues, whereas the β subunit has a core region of only 416 residues (25). The core structure is responsible for thermal stability and surface hydrophobicity while the extension regions render high solubility and emulsifying properties to the subunits (26). The carbohydrate moieties tend to prevent the formation of heat-induced aggregates (26). For the β-conglycinin heterotrimers, hydrophobicity and solubility were mainly decided by the α or α′ subunits (27). Comparisons of circular dichroism spectra between naturally occurring α, α′, and β homotrimers with their recombinant counterparts (without N-linked glycans) indicated that the carbohydrate moieties do not have a big influence on the secondary and probably the 3-D structures. The recombinant homotrimers had similar thermal stability as the native ones. However, the existence of glycan chains increased the solubility of each subunits (28). Contrary to glycinin, the subunits of β-conglycinin are associated by hydrophobic and hydrogen bindings and no covalent disulfide bonds are involved, which gives β-conglycinin several heterogeneities (29). β-Conglycinin has an isoelectric point of 4.8-4.9 (30).

The structure of β-conglycinin can undergo dramatic changes during storage at extreme conditions. For example, the extraction yield of extractable β-conglycinin dropped from 12.6 to 0.2% after soybean stored at 84% relative humidity and 30°C for 9 mo. The decrease of β-conglycinin extraction was attributed to the formation of intramolecular disulfide bonds based on the detection of disulfide bonds and unchanged molecular mass after storage (31). Similar observations were found in glycinin (32).

Soy Protein Denaturation

The compositional and structural differences of the two proteins give them different physiochemical properties, such as different isoelectric points, different denaturation temperature and functionalities. Soy proteins are thermodynamically most stable in their native state as in the seeds. When their secondary, tertiary, and quaternary structures change while the primary structure or linear peptide bonds remain intact, it is considered "denaturation". Many factors can cause protein to denature, such as physical agents (heat, pressure, shear, etc.) and chemical agents (acid, alkali, organic solvents, surface active chemicals, salts, etc.) (33). The denaturation or the loss of native ordered structure of the protein usually causes the loss of bioactivity. The most common denaturation agents for soy proteins are heat and pH. Heat treatment significantly reduced the yield of soy protein extraction by decreasing its solubility and more heat treatment usually results in greater solubility loss (34-35). The insolubility of denatured protein in neutral or mild alkaline conditions makes soy protein difficult to be separated from insoluble fractions such as fiber, which results in low SPI yield.

Wolf *et al.* (34) studied the behavior of soybean 11S protein (glycinin) heated at 100ºC in an aqueous system at different ionic strengths. They found that heating breaks the quaternary structure of protein into two major groups of subunits. One group consisted of soluble subunits even after prolonged heating, the other interacted with each other to form soluble aggregates within a short time but they continued to grow into larger aggregates until insoluble precipitates formed. The denaturation product of glycinin heated at temperature 100ºC and 0.5 ionic strength also exhibited two peaks on gel filtration chromatography (36). SDS-gel electrophoresis showed that one peak had highly polymerized subunit complexes, while the other contained a basic subunit monomer and seven oligomers formed by different proportions of basic subunits to an acidic subunit (36). Heating glycinin alone produced soluble aggregates that were composed exclusively of the basic glycinin subunits and their association was through hydrophobic interactions (37-38). Heating at 80ºC caused dissociation of both glycinin and β-conglycinin and the dissociated subunits formed soluble aggregates with molecular weights of over 1 M (39). The aggregates consisted of mainly the basic glycinin subunit with β-conglycinin β subunit (39). Apparently, heat denaturation was a progressive process involving dissociation and re-association of various polypeptides.

Studies by Lakemond *et al.* (40) showed that the disulfide bonds between acidic and basic subunits were broken when heated at pH 7.6, where the glycinin was present as hexamers. Glycinin partly dissociated into the 7S complex at pH 3.8 but at pH 5.2, near the isoelectric point of glycinin, no disruption was found. Koshiyama *et al.* (41) found that at pH 7.6 as ionic strength increased from 0.1 to 0.5, the glycinin denaturation temperature shifted from 78 to 90ºC. Glycinin showed endothermic

denaturation when heated in salt solution, whereas exothermic denaturation was observed when heated with the presence of alcohols, such as 2-mercaptoethanol, ethanol, or 2-propanol. If 2-mercaptoethanol concentration was increased from 0.01 to 10%, the exothermic denaturation temperature dropped from 95 to 46°C (42). These observations demonstrated that disulfide bonds and hydrophobic interactions affected the thermal behavior of soy glycinin (42). The surface hydrophobicity and viscosity of glycinin increased by four and two times, respectively, after treatment with 5 mM dithiothreitol, which reduces the disulfide bonds (43). Cleavage of the intermolecular and intramolecular disulfide bonds greatly improved the proteolysis of glycinin (44) due to the exposure of more sites of enzymatic attack. Studies on the binding of Cresol Red and Acid Orange 10 to soy protein after different extents of heating suggested that more heat treatment caused progressive exposure of hydrophobic residues, which bonded with dyes (45).

Heating duration dramatically affected the functionalities of denatured soy protein. Soy protein solubility was reduced after prolonged heating (30 min) and aggregates formed and water-holding capacity and viscosity increased; but if heated for a short time (5 min), the soy protein had high solubility and surface hydrophobicity (46-47). Heat-denatured soy protein has low solubility but high water-holding capacity because the matrix formed by denatured protein entraps a considerable amount of water (48). Similar results were found by Arrese *et al.* (49). Water-imbibing capacity increases with more denaturation. Unfolded soy protein peptides are necessary to form a matrix system that retains more water (49). Emulsification capacity increases after heating soy protein for a short period, while treated with reducing agents (i.e. sodium sulfide) and urea. The treated protein had high surface hydrophobicity and solubility (50-52).

In summary, heating dissociates the subunits in native soy protein structure and the subunits re-associate into large but still soluble aggregates. Further heat treatment can cause disulfide bonds to cleave and the polypeptides to unfold, exposing more hydrophobic groups and resulting in larger insoluble aggregates. The newly formed complexes have different functional properties with the native soy protein.

Acid treatment (except pH around the isoelectric point) tended to increase soy protein functionality. For example, when a soy protein solution was heated at 95°C in 0.05 N HCl for 30 min, solubility, emulsifying and foaming properties improved (53). Deamidation occurred at the same time. The improved functionalities are believed to be the results of increased surface hydrophobicity caused by acid denaturation (53). Soy protein extracted after being solublized at pH 1.5-3.5 was more soluble than that made after alkali solublization and then isoelectric precipitation (54).

Isoelectric precipitation during conventional soy protein extraction, however, seemed to damage soy protein fucntionalities. Nash *et al.* (55) compared the solubilities of water-extracted proteins acidified to pH 4.5 and then equilibrated in pH 7.6 buffer with proteins having the same treatment except acidification. They found that longer treatment time and higher acidity caused more denaturation. Protein solubility decreased by 12% when acidified to pH 4.5 for 2 h (55).

Alkali is widely used to solublize soy proteins during SPI preparation to increase protein yield. When a soy protein suspension was treated with alkali to pH $\geq$ 11, the disulfide bonds were cleaved, subunits dissociated and unfolded, whereas only aggregation and hydration were observed at pH $\leq$ 11 (56). When the pH was about 12.8, the hydrophobic core was disrupted (56). Apparently, alkali solublizes soy

protein by breaking disulfide bonds and hydrogen and hydrophobic interactions, making the native rigidly packed soy protein more dispersible in aqueous systems (56).

In addition to changing structure, high pH can produce undesirable chemical modifications to soy protein. These undesirable changes include the destruction of amino acids and production of amino acid derivatives by racemization and crosslinking of amino acids (57), which may reduce digestibility and nutritional value of the proteins (57). For example, cystine started to be destroyed at pH above 11 and the loss increased with increasing pH (56). Heating soy protein solutions at pH 12.5 and 65ºC for 2 h produced about 2% lysinoalanine and the total sulfur amino acid bioavailability was reduced by 71% (58). Similar results were found by Wu *et al.* (59), although SPI treated at pH 12 and 100ºC for 1 h improved most functional properties (such as solubility and emulsification), significant amounts of lysinoalanine were produced. Lysinoalanine production increased with temperature until about 70ºC, after which there was no significant change in lysinoalanine content (60). Studies by Friedman (61) showed that high pH and temperature and long exposure time favored lysinoalanine formation; however, the formation of lysinoalanine could be suppressed by adding cysteine, copper salts, dimethyl sulfoxide, and glucose (62). The toxicity of lysinoalanine in humans is unresolved (63).

As discussed before, soy protein undergoes a progression of unfolding, association and dissociation of polypeptides during denaturation. When the concentration of glycinin or β-conglycinin solution is right, the protein can aggregate to form a vast interlinked structure called gel. This is an important characteristic of soy protein denaturation. For example, when heated at 100ºC for 1 min, both 0.5 and 5% glycinin solutions form soluble aggregates. After prolonged heating, only the 5% glycinin

solution formed gel. Many believe that the soluble aggregates were intermediates during gel formation (64). The molecular weight of the aggregates formed in 5% glycinin solution heated at 100ºC increased with heating time until gelation (65). Transmission electron microscopic observations suggested that the soluble glycinin aggregates formed strand-like structures, which interacted with each other to form a three-dimensional matrix (65). Gels with similar structures were formed when 10% glycinin solution was heated at 100°C for both 1 and 20 min (66). Nevertheless, the hardness of the gel produced after 1 min was only 1/4 that produced after 20 min of heating. The former was totally dissolved in buffer with denaturation agents, such as 2-mercaptoethanol or urea, whereas the latter had only a fraction dissolved with the same treatment. This demonstrated that both disulfide bonds and non-covalent interactions formed during glycinin gelation. Prolonged heating produced more and/or stronger non-covalent forces, which may include hydrophobic interactions or hydrogen bonds (66). Renkema *et al.* (67) observed similar phenomena. Their study has shown that the stiffness of the gel formed at gelation temperature increased with the concentration of denatured proteins and the stiffness of gel formed at gel onset temperature increased with prolonged time. These increases may be due to the rearrangement of denatured protein in the gel network or more proteins incorporated into the gel structures (67).

pH had profound effects on soy protein gelation. The gel formed at pH ≤ 6 had higher stiffness values than those formed at pH ≥ 6 (68). This observation suggested that fewer protein components participated in the gel network at pH ≥ 5 than that at pH 3-5. At pH 7.6 with prolonged heating, extensive rearrangement was observed in the gel matrix. Similar rearrangement was not found at pH 3.8 (68).

Hermansson (69) also showed that glycinin aggregates formed strand-like structures in gel. The gel structure formed by conglycinin, however, looked more irregular or having more branches.

Since the denaturation properties of glycinin and β-conglycinin are slightly different, it was not surprising that the gelation properties of glycinin/β-conglycinin mixture were different from those of glycinin (70). The proportion of the two proteins influenced gel hardness, gel turbidity and gelation time. The two proteins formed soluble aggregates by non-covalent interactions in the process of gelation (70).

In summary, the denaturation pattern of soy protein is the result of a combination of various factors, such as heat, pH, ionic strength, and the presence of solvent or reducing agent. The functionalities of protein depend on the specific conditions of a denaturation process, even the same factor at different values may produce protein with totally different properties.

Soybean Protease Inhibitors

There are two major soybean protease inhibitors in soybeans, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI). They are the soybean's defense weapons again insect attacks (71). Their structures are unique compared with storage proteins such as glycinin and β-conglycinin. KTI is a sphere-shaped protein with dimensions of 45Å×42 Å×40 Å. It has 12 antiparallel β-strands connected by loops and one 310-helix. It consists of three similar subdomains each composed of four β-strands (about 60 amino acid residues each). The three subdomains are arranged in a way that gives KTI a nearly 3-fold symmetry. Two disulfide bonds stabilize such a structure by interlinking the loops between two ends (72). BBI has two reactive subdomains, one binds chymotrypsin, the other binds trypsin, all through insertion

loop motifs. The two subdomains are composed of two β-hairpin structures, stabilized by disulfide bonds and each has three antiparallel β-strands. A unique conformation feature of BBI is that its two hydrophobic patches expose to the environment, and an electronically charged cluster and five water molecules are buried in the interior. The existence of seven disulfide bonds, salt bridge, and probably hydrogen bonds are the major forces in maintaining such unusual structure (73). Studies on the crystal structure of BBI and bovine trypsin complex showed that one subdomain specifically bonded trypsin through interactions of polar residues, hydrophilic links and weak hydrophobic forces. While another subdomain interacted with both trypsin and chymotrypsin through close hydrophobic contacts across the interface. A buried salt-bridge responsible for trypsin binding was stabilized in a polar environment (74).

Due to their protease-inhibiting activity, KTI and BBI have carcinogenesis suppression functions. Their potentials as chemopreventive agents were extensively studied (75). For example, KTI induced programmed cell death to human leukemia Jurkat cell (76), suppressed the UV-induced up-regulation of cytokine expression in human skin to prevent further damage (77). KTI prevented ovarian cancer cell invasion by blocking urokinase up-regulation (78). BBI suppressed prostate carcinogenesis in rats (79), suppressed breast cancer cells by inhibiting proteasome (80), inhibited tumor growth by inducing the expression of gene Connexin 43 (81), reduced lung cancer cells in mice (82).

Major Allergens in Soybeans

Components of soybeans that can cause allergic reaction in some consumers also draw considerable attentions from the scientific community. The major allergic soy protein is Gly m Bd 30 K, an N-linked

glycoprotein (83) that exists in the protein body as a glycoprotein precursor during soybean seed maturation (84). This protein can bind with α and α ′subunits of β-conglycinin through disulfide bonds in soy milk (85). Another major allergic soy protein, Gly m Bd 28 K, also a glycoprotein, was also identified (86). Gly m 1.0101 and Gly m 1.0102, designed to transfer lipids from liposomes to mitochondria, are two significant allergic proteins. Both proteins are highly hydrophobic (87).

Immunological analysis using pig plasma showed that α subunit of β-conglycinin was a potential allergen in pigs (88, 89). This subunit was resistant to pepsin digestion. Deglycosylation did not eliminate the immunoreactivity. The immunogenic epitopes were identified as two β-strands and a loop linking them. These epitopes are located on the surface of the protein (88). Similar results were found using E. coli produced recombinant αα′-subunit without glycosylation (90). Its reaction with antibodies implied glycosylation is not necessary for the immunogenicity. The immunoreactive domains are highly hydrophilic and located in the extension region (90).

Although other subunits of glycinin may also have allergenicity, the strongest allergenicity lies in its acidic subunits (91). Soybean Kunitz trypsin inhibitor is also a potent allergen whose allergenicity may come from its stable anti-parallel β-sheet structure (92).

Major Functional Properties of Soy Proteins in Foods

Commercial soy protein products are mixtures of proteins and other components. Their functional properties important in food products are determined by their concentration and their collective physicochemical properties of each individual component. Major functional properties of soy proteins

include interaction with water (solubility, water-binding, water-holding capacities, viscosity, etc.), with oils and fats (emulsification properties and fat-binding), with air and water (foaming). Gelation is the interaction among protein molecules to form a three-dimensional structure in an aqueous system. There is no single standard method to quantify each functional property and a wide variety of measurement conditions are used by different workers (14, 35). Nevertheless, the major soy protein functional properties are summarized in the following paragraphs.

Solubility is probably the most important characteristic of soy protein. In aqueous systems, high solubility is usually desirable because soluble proteins have sufficient interactions with other components. Less processed soy protein products generally have better solubility. Heat, isoelectric pH, and alcohol reduce solubility. However, some solubility-reducing processing is necessary, such as heating to inactivate the anti-nutritional factors and to reduce the microorganism counts for adequate food safety (35). The reduced solubility/dispersibility can be compensated by either further processing (such as increasing pH) or by addition of other ingredients (i.e., lecithin).

Water-binding is the amount of water bound by the proteins that cannot be removed by centrifugal force. The hydration of soy protein concentrate has been studied by nuclear magnetic resonance (NMR) and sorption isotherm methods (93). It found that about 0.065 g water/g solids is tightly bound (this water may bind with ionic or polar sites of the protein), and about 0.25 g water/g solids is loosely bound, above which the water is considered bulk water. The hydration of soy protein concentrate by vapor starts with random sorption of water on the protein particle surfaces, followed by redistribution to highly polar sites, and then binding at lower affinity sites as “loosely bound” water (94). In general, soy protein products

with higher protein contents bind more water but the relationship is not linear (95). The water-binding has no relationship with particle size of the protein product, because the tightly-bound water is associated with the polar amino acid residues instead of being adsorbed at the particle surface. Heat denaturation can improve the water-binding capacity of soy protein, while highly soluble protein has low water-binding properties (35). Contrary to solubility, pH had little effect on water-binding capacity of the soy protein, indicating that there is no direct relationship between water-binding capacity and solubility (96).

Water-holding capacity is the ability of soy protein to trap water in the food system. Water-holding capacity is important in meat products because it influences juiciness and texture of the final products (97). Protein products with higher soy protein concentration exhibited higher water-holding capacity and alkali treatment improved the water-holding capacity of soy protein (98). When wheat flour blended with soy flours, its water-holding capacity increased (99). Both pH and temperature significantly affected the water-holding capacity of SPI and there was interaction between pH and temperature (100). At pH 7, the maximum water-holding capacity was achieved around temperature of 40ºC. Increasing the pH from 5 to 7 dramatically increased water-holding capacities. Residual fat in the soy protein reduces the water-holding capacity (13, 101).

The stability of oils/fats in an aqueous system is critical to many food systems. Soy protein can maintain emulsion stability because it lowers interfacial tension between hydrophilic and hydrophobic phases, i.e., at the boundary between water and lipids. Soy protein with higher solubility usually has higher emulsification capacity (35, 102), whereas insoluble soy proteins are poor emulsifiers (52). pH affects emulsification properties. Data showed that both emulsification capacity and stability corresponded

positively to the solubility profile, i.e. decreasing to lowest level in the isoelectric region (103). Emulsification capacity is influenced by temperature and other components in the system (104). Soy protein treated by heat and urea had higher emulsification capacity and stability, as well as hydrophobicity (105), which was attributed to the cleavage of disulfide bonds and exposure of more hydrophobic surfaces. Similar observations that short-time heating, alkali or Na_2SO_3 and urea increased the emulsification properties of soy protein isolate were found by Petruccelli *et al.* (52). The high emulsification activities from these treatments were attributed to the change of association-dissociation degrees. Desirable emulsification properties usually correspond to high surface hydrophobicity and solubility.

Due to the heterogeneity of soy protein, protein fractions with better emulsification properties can be extracted by tailoring the preparation conditions. For example, soy protein fractions recovered at isoelectric point range of 5.1-5.6 had superior emulsification stability and activity than those recovered at the isoelectric point of around 4.5 (106). Acidic glycinin subunits have better emulsification properties than whole glycinin and the difference was contributed to the likelihood that the hydrophobic residues buried in the whole glycinin molecules are released after being fractionated into subunits (107).

Emulsification properties can be modified by many approaches, such as partial hydrolysis (103), succinylation (108), acylation (109), lipohilization (110), lecithin complexing (111-112), and ethanol treatment (113). Tryptic digestion of heat-denatured soy protein yielded three fractions: precipitate fraction, high molecular weight fraction, and low molecular weight fraction (with molecular weights of 30, 20, and 10 kDa, respectively) (103). The precipitate fraction had poorer emulsification capacities than

unhydrolyzed soy protein, while the high molecular weight fraction had better emulsification and stability than native soy protein if the digestion was done in short time (103).

Succinylation improved the emulsification capacity by three-fold (108). Acetylation of glycinin significantly increased the emulsifying activity of the protein at pH above isoelectric point and more acetylation resulted in larger increase (109). When palmitic acyl residue was incorporated into soybean glycinin, the emulsifying activity of the protein increased to 2.5 times of that of the original glycinin, probably due to the introduction of palmitic acyl group (110). The palmitoyl proteins remained soluble, which was believed that the aggregation of the proteins was perhaps hindered by the hydrophobic tails. Lecithin-soy protein complex had better emulsification capacity because lecithin is a potent emulsifier and interacts with the soy protein both hydrophobically and hydrophilically modifying the surface charge of soy protein, and making it more negatively charged and thus more dispersible (113).

Phosphatidylcholine-SPI had higher emulsifying activity than SPI without phospholipid and the emulsion made from denatured SPI-phospholipid complex was more stable than that formed by native SPI-phospholipid complex (112). The emulsification properties of lecithin-soy protein complex can be dramatically improved by ethanol treatment (113). Lecithin associated with soy protein during the aggregation process caused by ethanol denaturation. The emulsification activity of the ethanol-treated lecithin-soy protein complex was improved compared with the original lecithin-soy protein complex. Such improvement was thought to be the result of increased hydrophobic areas. Heat caused the aggregation of soy protein but the aggregation only increased the emulsification activity of glycinin not the β-conglycinin protein. The emulsification activity of the ethanol-treated lecithin-soy protein complex was not affected by

NaCl concentration; however, the emulsification activity of the heat-treated lecithin-soy protein complex declined with increasing NaCl concentration (113).

Foaming is also an important property of proteins in aerated foods such as cakes, whipped toppings, frozen desserts, etc. Foaming is the ability of protein to form gas droplets entrapped inside thin liquid films consisting of soy protein solution. Foam stability correlated well with the amount of surface hydrophobic region (114). After alkali-treatment and papain-modification, the foaming capacity of soy protein increased to rival that of egg white (114). When treated with mild acid (0.05 N HCl at 95°C for 30 min), soy protein was deamidated without major peptide cleavage (53) and the foaming properties, along with solubility and emulsification properties, were improved. The improvement corresponded to the increasing surface hydrophobicity. Enzymatically deamidated soy protein showed improved foaming properties (115). Similar observations that foaming capacity increased with the increasing hydrophobicity were made by others (116). Heat or enzyme hydrolysis increased foaming stability, because unfolded subunits had more hydrophobic area enabling soy protein to have stronger interactions at the foam surface (117-118), however, thermal treatment decreased the foaming capacity due to the loss of protein solubility (52). As well as mild heat, addition of calcium chloride increased foam stability (119). Succinylation improved the foam stability and capacity of soy protein by 50 and 20%, respectively, but acetylation had little impact (109).

Soy protein forms gels when heated above certain temperature or when coagulating agents, like calcium salts, are added. A protein gel is a three-dimensional matrix formed by protein molecules in which the water is entrapped. Tofu is probably the best known soy protein gel. Calcium increases the sizes of the

aggregates and the pores inside the calcium-induced soy protein gel, and both protein and calcium concentration improved the elastic modulus of the gel (120).

Producing a heat-set soy protein gel is a denaturation process, which can be affected by many factors such as protein concentration, pH, heating temperature and duration, salt concentration, etc. Heat denaturation is a prerequisite of soy protein gelation because it disrupts the native structures of the protein, exposes the functional groups that re-associate to form the three-dimensional structure (121). The re-association involves hydrogen bonds and disulfide bonds (122). Other results have shown that noncovalent protein-protein interactions rather than covalent disulfide bonds are formed during soy protein gelation (67). This reconciles with the findings by Babajimopoulos *et al.* that hydrogen bonds and van der Waals interactions are the major forces in the gelation of soy protein, whereas the hydrophobic and electrostatic forces are marginal (123). Another study has shown that the interaction may be between β-sheets (121).

Gel hardness increased with increasing protein concentration (121, 124-125). NaCl stabilizes the quaternary structure of glycinin and the denaturation temperature increases with the increasing NaCl concentration (68-69), but the effect was pH-dependent. The salt concentration effect was less pronounced in the pH range of 5-8 (68). High temperature favored the formation of more ordered strand structure by dissociation of the quaternary structure and re-association into a new ordered structure of gel. β-Conclycinin formed a denser gel than glycinin at lower temperature due to its lower denaturation temperature (69). β-Conclycinin was more responsible for gel elasticity, whereas glycinin was responsible for hardness and fracturability of gels (126).

pH influences the denaturation temperature of soy protein and thus affects the onset temperature of gelation. For example, the denaturation temperature is the highest at the isoelectric point (68), while at the extreme pH values like pH 2 or 10, soy protein is partially denatured (69). Gels formed at acidic pH (<6) had higher stiffness than those formed at higher pH (>6) (68). Gels formed at pH 3.8 had coarse, granular, and white appearance, with high storage modulus values; whereas the gels formed at pH 7.6 showed finer, smoother, and opaque appearance with lower storage modulus values (127). The difference was due to cleavage of disulfide bonds in glycinin when heated at higher pH.

Denaturation extent and preparation method significantly affected the gelation properties of soy protein isolate (SPI). Highly denatured SPI formed a gel composed of the swelling particles (69). Completely denatured SPI has low gelation capacity, although some have high solubility (49).

Other treatments can also alter the gelation properties of soy protein. For example, autoclaving SPI with reducing sugar dramatically increased the rheological properties of soy protein gels. The change was attributed to reduced pH from neutral to 5.5 and additional covalent bonds produced during Maillard reactions between soy protein and reducing sugars (128). Heating at 90°C before or after pressurization at 300–700 MPa caused soy protein to lose gelation properties (129). Oxidation of SPI increased gel elasticity by modifying amino acid side chains (130) and the presence of oil droplets increased the storage and loss moduli of the soy protein gel (131).

Some enzymes can cause soy protein gelation, such as the modification of covalent and noncovalent interactions of the soy protein polypeptides by pepsin, trypsin, papain, bromelin, ficin (132, 133, 134) or polymerization of soy protein subunits by transglutaminase (135).

Applications for Soy Proteins

Soybeans are a vital crop in many cultures, probably the most important protein source for the sustenance of human society, either in the converted forms of animal proteins (muscle meat and dairy products) from livestock feed by domestic animals, or in the form of numerous traditional soy products (including soy milk, tofu, etc) that can be consumed directly by humans, or as protein ingredients used in endless processed foodstuffs that some people may never realize. The usage of soy protein ingredients in food is steadily increasing due to its versatility, health benefits, and acceptance by mainstream customers.

Soy protein concentrate (SPC) and SPI are two important commercial soybean protein products. SPC is an edible protein product with a protein content of at least 65% on dry weight basis (136), whereas SPI is a product with at least 90% protein on dry weight basis (137). Livestock feed is the largest usage of soy protein. Another less-mentioned major application is in pet foods where soy protein is a substitute for meat due to its high nutritional value and low cost.

Depending on how much oil is removed from the beans, soy protein products can be classified as full-fat, partially defatted, and defatted products. Based on the protein content, these products can be categorized into soy flours (about 50% protein or less), SPC, and SPI. Soy flour, the least processed soy protein product, can be full-fat or defatted, and enzyme-active or heat-treated. Both full-fat and defatted enzyme-active soy flour are used in baking industry, primarily for the activity of lipoxygenase and β-amylase, which can give the baked products desired mixing properties, color and texture (138). Soy β-amylase is an ethanol-soluble enzyme that can tolerate extensive ethanol treatment at room temperature

(139) and can be used to hydrolyze starch from various sources (140). Heat-treated soy flours are mainly used in baked products giving good water retention and fat absorption capacities.

Both SPC and SPI are made from defatted white flakes. SPC preparation from white flakes involves washing away the soluble sugars while insolubilizing the protein. There are three commercial processes: aqueous alcohol washing (20-80% alcohol), acid washing (at the isoelectric pH 4.5), and hot water leaching (141). In order to prepare SPI, both insoluble fiber and soluble sugars have to be removed. Many techniques were developed but the conventional procedure starts with solublizing the protein at elevated temperature and pH (such as 60ºC and pH 9-11), followed by filtration or centrifuging to remove the fiber, then precipitating the protein at the isoelectric point, decanting or centrifuging to recover the purified precipitate, and lastly neutralizing and spray-drying (141). Their functionalities can be easily modified to meet different requirements in food processing.

SPC is widely used in breakfast cereals, nutritional bars for its protein nutrition and reasonable price (141-143); in meat products as inexpensive functional fillers; in bakery products for nutritional benefits and functionality. SPI is extensively used in processed meat products including emulsified, ground, and injected products, mainly for its adhesion, water-holding, fat-emulsifying and stabilizing capacities, and gelling properties (141, 143-144). It is also used in bakery products to fortify protein, to lower the carbohydrate level, and to improve the texture. SPI is also widely used in liquid, paste, or frozen dessert products as a protein fortifier, emulsifier, thickener, stabilizer, or texture/mouth-feel enhancer.

Soy flour, SPC, and SPI can be extruded with or without other ingredients to make texturized protein products, which have another broad range of applications in many foodstuffs, such as breakfast cereals, energy bars, etc.

Although soy protein products are traditionally used in foods more for their economic and functionality reasons, their nutritional benefits are being increasingly recognized by food processors and the general public, e.g. as fat and carbohydrate dilution agents. The approval of soy protein's "heart-healthy" claim by FDA in 1999 was one of the cornerstones in food applications for soy protein (145). Soy protein will find more uses in food products due to the increasing public awareness and the availability of new soy-protein-based ingredients.

The amount of soy protein used in non-feed, non-food, industrial or biobased products is small (146), nevertheless, its industrial application potential cannot be underestimated (147). In fact, early soy protein production was driven by its suitability in industrial applications, such as in paper coatings, adhesives, water-based paint, etc. Paper coating is still one of the major industrial uses for soy protein today. With the anticipated depletion of fossil resources and environmental pollution associated with the petrochemical industry, interest in industrial applications of soy protein has rekindled (148). Compared with its synthetic counterparts, soy protein is a renewable material and its products are biodegradable with less negative impacts on the environment. One of the promising uses for soy protein is in adhesives for wood composite materials. Soy protein adhesives can reduce the emissions of volatile organic compounds commonly found in traditional wood composite products and in the work environment where those products are manufactured. Soy protein can be co-spun with polyvinyl alcohol to produce high quality

fibers. Apparel made of fabrics weaved from this kind of fibers are available on the market today (149-150). Plastics made from soy protein, starch, natural fiber and other biodegradable materials have also been developed (148). The prevalence of these novel industrial applications depends on the cost of massive production and the properties of the new materials compared with the existing counterparts.

Soy Protein Products Preparation

Two most commonly available and widely used concentrated forms of soy protein are SPC (>65% protein) and SPI (>90% protein). These ingredients are typically made from white flakes (flash-desolventized, solvent-extracted soybean meal, with protein content about 50%) by removing non-protein components, such as soluble saccharides for SPC, or both soluble sugars and insoluble fiber for SPI (151-152).

Soy protein extraction started in the 1930's. One of the earliest extraction methods was patented by Cone *et al.* (153) to produce soy proteins for paper coating. In the early days, pH manipulation was the most frequently used method. The protein was usually solublized in alkali (pH 8.0 and above) at ambient or elevated temperatures (153, 154), precipitated at around the isoelectric point of pH 4.5 (50, 154, 155), the insoluble fraction was separated by adding lime (153), centrifuging or filtering (54, 154-156).

Soy protein can be solublized at acidic conditions. For example, when solublized at pH 1.5-3.5, the extracted protein was found to be more soluble than that made by isoelectric precipitation (54). Soy protein can be extracted with other chemicals in addition to pH manipulation, such as with salt (ionic strength) (157) and calcium chloride (158), which significantly improved the purity of SPI (158).

Other methods used in soy protein preparation include density fractionation (159) and ultrafiltration (160-162). Similar to conventional protein extraction (158), adding calcium hydroxide instead of sodium hydroxide to solublize soy proteins prior to ultrafiltration increased protein purity of SPI (163). This may indicate that the binding of calcium ion prevented soy protein from binding to other impurities. Compared with conventional isoelectric precipitation processes, the ultrafiltration process gave higher protein yield, because the protein normally lost in the whey was recovered (163). The efficiency of ultrafiltration was improved after treating the soy flour with commercial pectinases (164-165).

SPC and SPI are mixtures of the two major storage proteins, glycinin and β-conglycinin, among other non-protein components. One of the major interests in soy protein processing during the past decade is the fractionation of these two soy proteins. The early efforts were done at bench-scale with main objectives of collecting enough purified samples for the basic research to understand the structural and physicochemical properties of the individual proteins. The fractionation was based on the slightly different pH solubility profiles in the presence of buffer and reducing agent (166-167). Larger scale fractionation has been tried in recent years (59, 168-171). All of these methods involved the manipulation of pH which produced additional salt. A desalting step is usually needed to remove this salt.

A recent novel technique avoided this problem. Instead of using mineral acids, such as hydrochloric acid, a volatile acid, CO_2 was used to lower the pH. The pH of the solution was precisely controlled by manipulating the CO_2 pressure (172). This approach resulted in higher fraction purity and yield than the conventional fractionation method (172). This may be because the accuracy in pH control avoided localized excessive acid, which damages the protein. Another characteristic of this pressurized

CO_2 fractionation method was that the precipitated protein particles had nearly perfect spherical shapes, unlike the irregular shapes in conventional mineral acid precipitation (173). Under optimum conditions, the spherical particles could grow to as large as 500 μm. It was also possible to co-aggregate with a second protein (173). This method could be used to make coatings or carrier agents for the pharmaceutical industry (174).

Hydrothermal Cooking (HTC)

Due to the heat-denaturation of soy proteins during the EE process, functional properties of EE meal are altered. Most properties are lower than those of less denatured soy proteins, such as white flakes. Protein denaturation also reduces SPI yield. Improving the functional properties and/or increasing extractability of protein can lead to more applications for EE meal and add more values and application potentials to EE technology. So far very little research in this area has been reported.

One promising technology is hydrothermal cooking (HTC). HTC is a term used to describe "any heterogeneous reaction in the presence of aqueous solvents or mineralizers under high pressure and temperature conditions to dissolve and re-crystallize (recover) materials that are relatively insoluble under ordinary conditions" (175). The HTC technology in the food industry is generally called jet-cooking, which involves injecting steam into slurries of various food materials through a small orifice to modify their physiochemical properties. Its applications in food industry include starch modification, oil/starch or oil/protein emulsion formation, dietary fiber modification, pasteurization, etc (176-178).

One of earliest applications of HTC in food processing involved treating the gluten fraction in corn wet milling. HTC at 138-193˚C was found to be effective on improving the filterability and protein content of the gluten fraction (179). The higher the temperature, the shorter the time needed. HTC treatment at 141-160˚C removed the typical beany flavor and other detrimental odors in isolated soy protein, making it more suitable for food applications (180). Another patent described HTC treatment of soy protein at 121-177˚C, pH 6.8-7.2, for less than 6 min (181). After HTC, the slurry was flashed and cooled under reduced pressure in a vented chamber. The inventors claimed that HTC had the benefits of microorganism destruction, trypsin inhibitors inactivation, and deodorization (181). After HTC treatment at 110-140˚C, pH 6.5-9, for 2-180 s, the Nitrogen Solubility Index (NSI), flavor and color of the denatured defatted soy flakes from alcohol extraction or desolventization was effectively improved (99).

When treating soy protein materials at 107-204˚C for 3-180 s with the pH of the slurry around the isoelectric point (pH 3.5-5.5) instead of neutral or mild alkaline conditions, then neutralizing the treated slurry immediately without cooling, soluble protein was increased from 60 to 90% compared with conventional extraction (182).

Studies by Johnson *et al.* (183-185) showed that when treated with HTC at 154˚C, the solids and protein yields of soy milk increased from 62 to 86% and from 73 to 90%, respectively. The trypsin inhibitor activity was reduced to <10% in about 40 s of heat treatment. HTC has been shown to be effective on improving the major functionalities of alcohol-washed SPC, in which protein was denatured by ethanol (186). The nearly flat solubility profile curve of denatured protein was restored to a typical U shape of non-denatured soy protein. HTC also improved the emulsification and foaming properties (186).

Although it is believed that the high shear and temperature are the major forces during HTC, the exact mechanism of protein refunctionalization is not fully understood. Wang *et al*. (187) showed that HTC disrupted the large particles into smaller, more dispersible aggregates. The spray-dried HTC-treated soy protein samples had slightly darker color than the untreated control, probably because of the Maillard reactions during HTC (186).

A recent study demonstrated that the functional properties of the wheat gluten were altered when treated by HTC in presence of corn syrup (188). Polymers of large molecular size were formed during this process.

Other similar techniques include the employment of shear force, heat and mild alkali to modify the soy protein. For example, treating soy flakes through a shearing orifice at around the isoelectric point (pH 3.5-5.5) resulted in a soy protein concentrate with higher viscosity than conventional method (189). Soy protein products with high NSI were produced after subjecting the less-soluble soy protein materials (such as alcohol-washed soy flour) to successive shear force and cavitation cycling in a homogenizer at temperature of 50-150˚C, pH 6.5-9.0 (190). All these imply that HTC has the potential to modify plant proteins.

Hypothesis and Objectives of the Dissertation

There is little understanding on whether or how HTC can improve the functional properties of heat-denatured soy proteins especially those from the EE process. Based on the aforementioned findings

that HTC increased soy milk yield (183) and refunctionalized alcohol-denatured soy protein (186, 191), we hypothesized that HTC can restore the lost functionality of heat-denatured EE proteins.

The overall objective of this project was to improve the functional properties of EE meals using HTC and to extract value-added soy protein products from HTC-treated EE meal. Our research was divided into four parts corresponding to four manuscripts in the following chapters. In Chapter 1, the potential of HTC to improve the functionalities of EE meal was determined under different HTC operating conditions. In Chapter 2, the feasibility of preparing SPC and SPI from EE meals was evaluated. In Chapter 3, the potential of HTC with alkali to enhance functionality and the preparation of SPI from EE meal was explored. In Chapter 4, a model was developed to explain the mechanism of HTC in restoring functionality of EE meal.

AN EXPLANATION OF THE DISSERTATION ORGANIZATION

This dissertation is organized in the following order: four individual papers published on the Journal of the American Oil Chemists' Society, which are in the designated format for that journal, followed by the general summary chapter and recommendations for future work.

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Chapter 1. Refunctionalization of Extruded-Expelled Soybean Meals

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Running title: Hydrothermal cooking of EE meals

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ABSTRACT: Soybean meals produced by extruding-expelling (EE) have poor functional properties due to heat denaturation of the proteins, which limits their utilization in foods. Hydrothermal cooking (HTC), a treatment in which steam (150°C) and high shear are applied to a slurry of soybean meal, was used to refunctionalize EE protein meals. Two EE samples with protein dispersibility indexes (PDI) of 35 and 60 were used, along with solvent-extracted white flakes and full-fat whole soy meal as controls. Two HTC methods were explored: one method used treatment temperature of 154°C and seven different residence times, controlled by varying the holding tube length; the other involved flashing the treated slurry directly into atmosphere without any back-pressure regulation or holding. Effects of residence time on functional properties of the samples were investigated. The maximum effect of HTC conducted with the use of holding tubes (with-holding-tube HTC) was also compared with that of flash-out HTC. Solids dispersibility, protein dispersibility, and emulsification capacity of both EE meals were significantly improved by both types of HTC treatments. The flash-out HTC showed more benefits than the with-holding-tube HTC in refunctionalizing heat-denatured EE protein. For example, the solids dispersibility, protein dispersibility and emulsification capacity of EE meal with PDI of 35 were improved 2.0, 4.4, and 2.1 times, respectively, by flash-out HTC treatment. Therefore, the HTC treatment partially restored the native functional properties of soy protein in heat-denatured samples.

KEY WORDS: Dispersibility, emulsification capacity, extruded-expelled soybean meal, foaming property, functional properties, hydrothermal cooking, refunctionalization.

Modern solvent extraction of soybean oil has replaced the traditional mechanical oil extraction, because of its processing scale and efficiency, its high oil recovery, and its yield of fully functional defatted flakes. But recently, interest in mechanical processing has been rekindled, especially after the introduction the extruding-expelling (EE) concept. In 1987, Nelson *et al.* (1) investigated coupling dry extrusion with the mechanical expelling process. When soybeans passed through the extruder, heat was generated, plant tissues and cell structures were disrupted, and oil was released from seed matrix. The discharged extrudate was conveyed to an expeller where the oil was pressed out. A typical oil recovery of 70% and a press cake with 50% protein, 6% oil, and 90% inactivation of trypsin inhibitors can be obtained from dehulled soybean (1). Compared with solvent extraction, EE has several advantages, such as low capital cost, relatively simple machinery, no solvent use, and a small production scale. It is ideal for identity-preserved (IP) processing.

With high stability, low phospholipid and FFA contents, and a pleasant nutty flavor, EE oil may be consumed after minimal refining or even without refining. Studies by Wang and Johnson (2, 3) have shown that with minimal refining, a unique oil product can be obtained. Utilization of EE proteins as a food ingredient has also been studied (4, 5). Traditionally, EE meals are used as livestock feed (6, 7) because their poor functional properties, caused by protein heat-denaturation, limit food use. If the heat-denatured protein can be refunctionalized, production of various new protein products will be possible and more benefits can be realized by EE technology.

A promising method of protein refunctionalization is hydrothermal cooking (HTC), which involves a system commonly referred to as jet-cooker, where high-temperature steam and a protein slurry

are infused into a holding tube through a restriction orifice. Johnson *et al.* (8) showed that for soymilk preparation, HTC treatment increased soybean protein recovery from conventional 70% to about 90%. Wang and Johnson (9) employed HTC to refunctionalize ethanol-denatured soy protein concentrate, and their results showed significant increases in the major functional properties, including dispersibility, emulsification, and foaming. There has been little effort to refunctionalize heat-denatured proteins, especially those from EE process. We hypothesize that HTC can improve the functional properties of heat-denatured EE protein. The objective of this study was to examine the effects of HTC on the functional properties of EE protein meals having different degrees of heat denaturation.

EXPERIMENTAL PROCEDURES

Soybean meal samples. Two EE flours (EE35 and EE60) were made from cracked and dehulled soybeans (Stine Seed Co., Adel, IA), and their protein denaturation and oil content were measured as protein dispersibility index (PDI) of 35.3 and 62.0 and oil content of 7.6 and 13.6%, respectively. The EE system consisted of an Insta-Pro International extruder Model 2500 and screw press Model 1500. The following extrusion parameters were used to produce the extruded protein meals: 11-11-6-6 shear lock configuration, double flight screws, and a restriction die setting at 3/8 in. (0.94 cm). The temperature in the last segment of the extruder barrel was 132-143°C, and the total residence time was about 20-25 s. One sample of solvent-defatted white flakes (ADM Nutrisoy® defatted flakes, PDI of 90; ADM, Decatur, IL) and Iowa-produced soybeans were selected as controls. All samples were ground by FITZ®MILL (Model DAS06; The Fitzpatrick Company, Elmhurst, IL) with a 40-mesh screen. To avoid any further protein

denaturation, caution was taken to prevent any overheating of the mill. All samples were made into a 20% slurry stirred with a Biomixer™ handheld mixer (ESGE Ltd., Mettlen, Switzerland), then pumped through a Stephan mill (Type MC15; A. Stephan u. Söhne GmbH & Co., Hameln, Germany) to achieve thorough dispersion before HTC treatment.

HTC (jet-cooker) treatment. A Moyno pump (2MI type SSQ; Robin and Myers, Inc., Springfield, OH) was connected to a hydroheater (size 300, type B; Hydrothermal Co., Milwaukee, WI) where culinary-grade steam (~90 psi pressure, 6.5 kg/cm^2) was infused to give the heat and shear treatment (Fig. 1). Two types of HTC treatments were used. One used holding tubes (and was termed "with-holding-tube HTC") and involved combinations of holding tubes (2.54 cm i.d. and 2.66 cm o.d.) of various lengths that gave seven holding times for each sample. All the holding tubes were insulated. The lengths of the seven holding tubes were: 2.58, 4.48, 6.31, 8.22, 10.66, 13.17, and 16.28 m. Due to viscosity differences among the samples, the residence times of different samples were different even when using the same tube length. The residence time was measured by injecting Brilliant Blue R-250 into the slurry that was about to go into the stabilized or equilibrated HTC system and observing the time needed for the colored material to emerge from the outlet. A back-pressure valve, after which the sample was released into the flash tank, was used to adjust the temperature. Cooking temperature was maintained at 154±0.6°C and monitored using a thermocouple. The second type of HTC treatment was flashing-out HTC. After the slurry had been infused with steam in the hydroheater, the product was discharged directly into atmosphere without any holding tube or back-pressure control. There was still a short, open tube from hydroheater to outlet, but the temperature was about 104°C, much lower than 154°C.

The feeding speed for all samples was maintained at a constant rate, 1.5 kg/min, as standardized with water. The released slurry was cooled to below 40°C in a jacketed ice bath. Treated samples were stored in a cold room at 5°C for further analyses.

Characterization of functional properties. All concentrations used were based on dry-matter basis (measured after drying at 130°C for 3 h). Protein content was measured by Kjeldahl method (with a conversion factor of 6.25). Solids dispersibility and protein dispersibility were measures of the total dry matter and protein matter, respectively, in the supernatant of a 10% suspension after centrifuging at 1050 × g and 5°C for 5 min, which was a modification to a method of Johnson (10).

There are many methods to measure the solubility (or dispersibility) of soybean proteins. Some methods are recommended by industry associations and thus are more popular such as protein dispersibility index (PDI) or nitrogen solubility index (NSI). Others are used in feed situations (such as KOH solubility). The key steps for measuring PDI are blending 20 g of protein in 300 ml water using a standardized blender at 8,500 rpm for 10 min at 25°C, centrifuging at 1,400 x g for 10 min, and then measuring protein content in the supernatant (14). For NSI, the key steps are mixing 5 g protein with 20 ml water using a blender at 120 rpm for 2 h at 30°C, centrifuging at 1,500 rpm for 10 min, filtering the supernatant through a plug of glass fiber, and then measuring protein content in the filtrate (15). For KOH solubility the key steps are stirring 1.5 g protein in 75 ml 0.2% KOH for 20 min on a magnetic stir plate, centrifuging at 2,700 rpm for 15 min and then measuring protein content in the supernatant (16). Despite differences, their rationales are fundamentally the same, i.e. the sample is blended in water or diluted alkali solution, centrifuged or filtered to remove insoluble (or indispersible) and the protein remaining in the

supernatant is quantified as the soluble (or dispersible) fraction.

Emulsification capacity was measured based on a method of Swift *et al.* (11). Twenty-five mL of a 2% dispersion was put into a 400-mL plastic beaker, and a handheld Biomixer™ (BioSpec Prodcts, Bartlesville, OK) was used at high speed (1,200 rpm) to emulsify the protein suspension with a commercially refined soybean oil (Bakers & Chefs™ vegetable oil; North Arkansas Wholesale Co., Bentonville, AR), which was introduced at a rate of about 0.5 g/s. Emulsification capacity was determined as the amount of oil that had been used when an inversion was observed. The inversion point was visually determined as the breakdown of the emulsion, i.e., the creamlike emulsion suddenly broke into separate oil and water phases and viscosity dropped suddenly. A fat-soluble dye, Red Fat 7B, was added at about 4 ppm to the oil to make the end point easy to observe.

To quantify foaming properties, a foaming device, made by fusing a fritted ceramic disk to a graduated glass column, was used (12). Nitrogen gas was purged at 16.7 mL/s to produce 300 mL of foam from 100 mL of 1% sample suspension. Three measurements were made: time of foaming ($t_{f,}$ in s), volume of sample suspension consumed at the end of foaming ($V_{max,}$ in mL), and time used for one-half of the incorporated suspension to drain back ($t_{1/2,}$ in s). From these measurements, three foaming parameters were calculated: foaming capacity (FC) = $300/(16.7 \cdot t_f)$, which was the number of milliliters of foam formed per milliliter of N_2 purged; K value = $1/(V_{max} \cdot t_{1/2})$, in units of 1/(mL·s), which was used to describe foam stability (a higher value indicates lower stability); and $V_i = V_{max}/t_f$, in unit of mL/s, which was foaming speed, i.e., the speed of liquid being incorporated into foams.

Experimental design and data analysis. HTC treatments were conducted in duplicate. All

functional property measurements were in triplicate except for Kjeldahl, which was done in duplicate. Statistical analysis was performed using the General Linear Model procedures of SAS 8.02 (13). For all the functional properties of each sample, the differences among the seven residence times were examined. Comparisons were also made among samples of control, maximum value of the seven with-holding-tube HTC, and flashing-out HTC treatments.

RESULTS AND DISCUSSION

Effect of HTC holding time on EE meal refunctionalization. HTC significantly improved the solids dispersibility of EE35 and whole soy meal (with short residence time), but it reduced that of white flakes. No statistically significant effect was found on EE60 (Fig. 2A). The LSD values used to compare these differences are presented in Table 1. The two EE meals followed a similar trend. They reached the treatment maximum and then remained relatively unchanged despite the increase in residence time. Whole soy meal achieved the maximum solids dispersibility, from 53 to 70%, at a residence time of 33 s, which then decreased. This result agreed with that of Johnson *et al.* (6), in which solids dispersibility increased from 65 to 86% at 34 s. The different maximal values may be due to different shear forces between two systems. After 33 s the solids dispersibility of the whole soy meal decreased steadily until it became stable at 50% after 60 s. EE35 achieved the highest value of 52% at 42 s; after that, the values remained at nearly 50% except at 100 s (unexplainable drop). The solids dispersibility of white flakes decreased from 61 to 40% at about 16 s, after which it increased to 51% and then decreased to about 40%, which indicates protein denaturation by HTC treatment. All data show that the two EE meals and the whole soy meal

benefited from HTC, whereas the white flakes were damaged by such treatment.

Protein dispersibility followed a trend similar to that of solids dispersibility, but the changes with the increase of residence time were more notable (Fig. 2B) than that of solids dispersibility. EE60 performed more like whole soy meal than EE35. The increases for EE60 and whole soy meal were from 47 to 57% and 57 to 66%, respectively. The maximum protein dispersibility for EE35 was 43%, an increase of more than three times from the untreated value of 13%. After HTC, the protein dispersibility of white flakes was reduced from 70 to 36%, after which it was increased to 47% then decreased to about 23% with an increase of residence time. It is interesting to observe that after a residence time 120 s, EE35, EE60, and whole soy meals had almost the same protein dispersibility of 42%, although the protein denaturation in the starting materials were different (their PDI were 35, 60 and 90, respectively). The white flakes from the solvent extraction process had less than 1% of oil, whereas whole soy meal was full-fat. Their different performance during HTC treatment suggests that the oil in the samples may have played protective roles in preventing soybean protein from forming big, non-dispersible aggregates, or in other words, oil on the surface of the particles might have helped smaller aggregates or protein molecules (though mostly denatured) stay dispersible. EE60 had an oil content of about 12% and relatively high PDI, so it is understandable that it showed a response similar to that of the whole soy meal during HTC treatment. After HTC, EE35 had a greater quality improvement than EE60. In the EE process, soybean protein experienced high shear, pressure, and heat, and more and larger denatured protein aggregates were probably formed in EE35 than in EE60. During HTC treatment, the soybean slurry was subjected to several forces and conditions: One was the shear force, generated mainly at the point of steam infusion;

another was the high-temperature treatment (154°C). Supposedly, shear forces act to break down the big aggregates into smaller ones, whereas high temperature facilitates the aggregation of native proteins. The outcomes of HTC are believed to be the mixed actions of all these major forces.

The emulsification capacities (EC) of untreated materials were very different.Samples having the most native proteins had much higher value (128 and 148 g oil/0.5 g sample for whole soy meal and white flakes, respectively), whereas values for the two EE meals were below 60 g oil/0.5 g sample (Fig. 3). After HTC, both EE meals quickly gained EC to about 100 g oil/0.5 g sample at the shortest residence times of 24 and 26 s, respectively. They remained almost the same even with longer residence time. EC values of whole soy meal and white flakes dropped abruptly to 90 and 100, respectively. There were some similarities between EC and protein dispersibility, e.g., for untreated samples, high solids or protein dispersabilities always corresponded to high EC. But the EC curves of HTC-treated samples were quite smooth, with no apparent peaks and fluctuations as shown in protein dispersabilities. This might be because that EC test itself was not as sensitive to aggregate size as protein dispersibility was.

HTC did not significantly affect the foaming capacity of EE60, white flakes, and whole soy meal, but it significantly increased that of EE35 (Fig. 4). HTC improved the foaming speed of EE35, white flakes, and whole soy meal. For EE35, the foaming speed increased steadily as residence time increased. For EE60, the value decreased at the beginning but increased slowly with the increasing residence time. Whole soy meal and white flakes showed considerably different curves, and in general, their values slightly increased with time. The two EE samples had very similar performances with longer residence times. *K* value is an indicator of foaming stability, and the lower the value, the higher the stability of the

foam. HTC lowered the *K* value of EE35, which indicates significant improvement in its foaming stability. For the other three samples, the values fluctuated, but the overall stability did not change considerably during the various lengths of HTC treatments. In general, HTC improved the foaming properties of EE35, but its effects on EE60, white flakes, and whole soy meal were not significant.

Effect of flashing-out HTC treatment on EE meal refunctionalization. When testing the effect of residence time, we observed that most of the functional property (such as dispersibility and EC) improvements happened at short residence times (Figs. 2-4). Longer residence time had limited (if any) effects on further refunctionalization of EE meals. Therefore, a flash-out HTC experiment was carried out in which all the conditions were the same as with-holding-tube HTC except the treated slurry was flashed out without a valve to maintain temperature at 154°C. Because there was a short tube from the point of steam infusion to flash outlet, a 6-s "residence time" was actually applied. Temperature at the point of infusion was about 160°C, and at the outlet, about 104°C.

For comparison, the maxima of all quality measurements were selected for each sample from with-holding-tube HTC experiment. Since not all the maxima were achieved at the same residence time (or at the same tube length), these data represent actual achievable maxima of with-holding-tube HTC. Untreated samples were also included in the comparison.

Flashing-out HTC resulted in two-fold increase in solids dispersibility of EE35, which was also significantly higher than the maximal value in the with-holding-tube HTC treatment (58.4 vs 52.3%) (Table 2). It also improved the solids dispersibility of whole soy meal, and the increase was the same as the maximal value of with-holding-tube HTC treatment. There was no treatment difference for EE60. For

white flakes, flashing-out HTC did not change its solids dispersibility, but with-holding-tube HTC reduced it from 62 to 51%. Similar to solids dispersibility, flashing-out HTC increased protein dispersibility more than the with-holding-tube HTC treatment, expect for EE60. For EE35, flashing-out HTC increased the protein dispersibility by more than four times, compared to two times for solids dispersibility. A similar trend was observed for whole soy meal and white flakes. It indicates that increase in protein dispersibility was directly related to the improvement in solids dispersibility. Flashing-out HTC resulted in solids and protein dispersibilities that are as good as or better than those of with-holding-tube HTC. This may be explained by less heat treatment in flashing-out HTC (shorter time and lower temperature after steam infusion), resulting in less denaturation or production of smaller protein aggregates.

As with-holding-tube HTC, flashing-out HTC significantly improved the ECs of EE35 and EE60 but reduced that of white flakes and whole soy meal (Table 3). Flashing-out HTC improved EC to lesser degree than with-holding-tube HTC did for the two EE samples. Flashing-out HTC reduced EC to a greater degree than with-holding-tube HTC did for the whole soy meal and white flakes. For soy proteins, EC is believed to relate to amphiphilicity, which is the overall ability to interact with both polar molecule and nonpolar molecules to produce a stable emulsion. More heat treatment (higher temperature or longer time) changed soybean protein surface properties in such a way that more amphiphilic ability was produced in with-holding-tube HTC than in flashing-out HTC. Despite not all the functional property maxima could be achieved under the same conditions in with-holding-tube HTC and that the operations were not as simple as flashing-out HTC, flashing-out HTC was a very useful HTC method to refunctionalize EE soybean meals.

Flashing-out HTC increased the foam stability of EE35, but its stability was lower than the maximum in with-holding-tube HTC. For EE60, there was no significant difference among the treatments, and no significant difference was observed between the two HTC treatments for white flakes (Table 3). Flashing-out HTC significantly reduced the foaming stability of whole soy meal. No significant difference was observed between the stability of untreated whole soy meal and that of maximal value of with-holding-tube HTC. Foaming capacity and foaming speed showed similar mixed results (data not shown).

This study demonstrated that HTC can effectively improve most functional properties of heat-denatured soy protein. Most of the maximal treatment effects were achieved in short residence time at temperature <154°C. Longer residence time usually decreased the functional properties. The results suggested that solids and protein dispersibilities were improved much more in flashing-out HTC than in with-holding-tube HTC. Emulsification capacities of the two EE samples were also increased by flashing-out HTC treatment, but the values were lower than the maximal increases achieved by with-holding-tube HTC. Foaming properties were somewhat mixed by the two HTC treatments. Flashing-out HTC showed a certain promise, not only for the effective refunctionalization but also for the ease of operation. For both HTC treatments, the more heat-denatured the EE protein is, the more refunctionalization can be achieved. This observation suggests that oil recovery in EE process need not to be sacrificed if HTC treatment is to be used for protein refunctionalization. Since EE meals with PDI of 30 are typical EE processing products, they can be readily hydrothermally cooked to produce highly functional value-added food ingredients. This will further enhance the value and application of

extruding-expelling processing.

ACKNOWLEDGMENTS

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Figure captions:

FIG. 1. Hydrothermal cooking system (jet-cooker).

FIG. 2. Effect of residence time during hydrothermal cooking on (A) solids and (B) protein dispersibilities of various soy protein samples.

FIG. 3. Effect of residence time during hydrothermal cooking on the emulsification capacities of various soy protein samples.

FIG. 4. Effect of residence time during hydrothermal cooking on foaming properties of various protein samples (A) foaming capacity; (B) *K* value, or foam stability; (C) foaming speed.

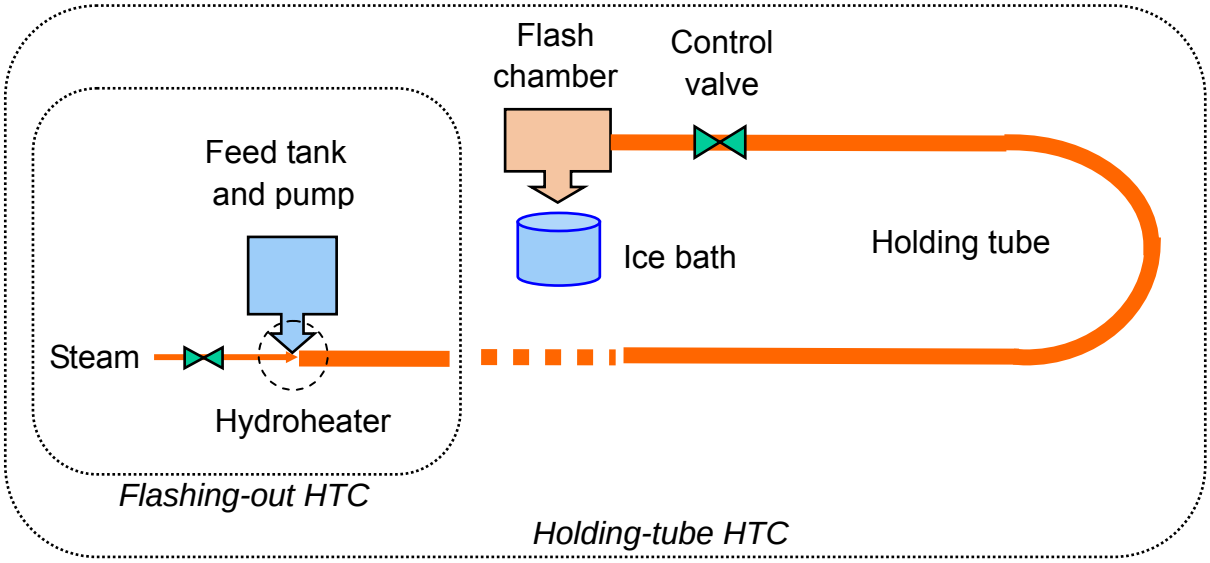

FIG. 1. Hydrothermal cooking system (jet-cooker).

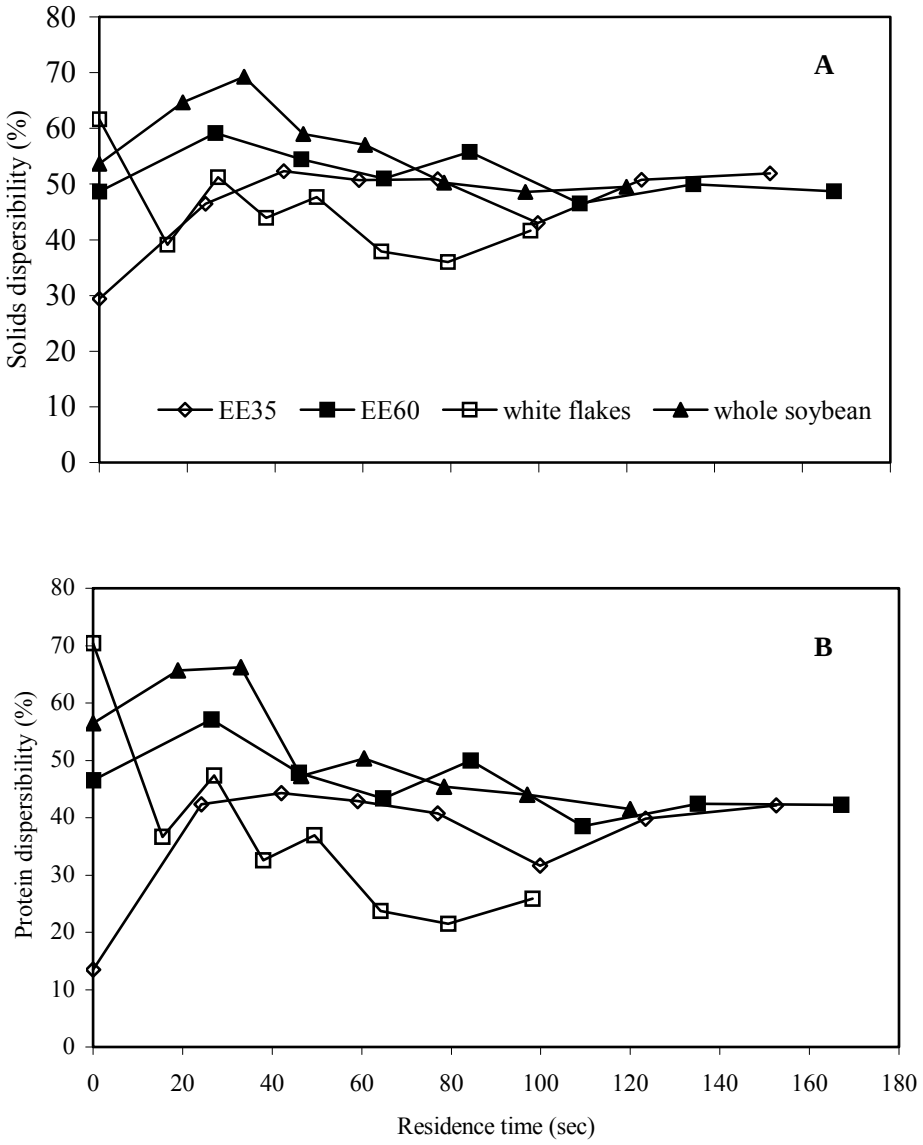

FIG. 2. Effect of residence time during hydrothermal cooking on (A) solids and (B) protein dispersibilities of various soy protein samples.

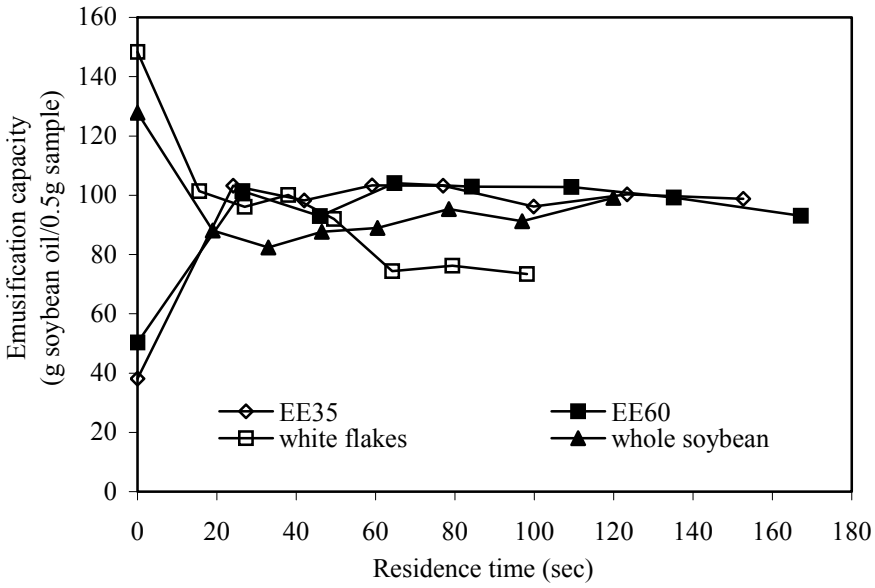

FIG. 3. Effect of residence time during hydrothermal cooking on the emulsification capacities of various soy protein samples.

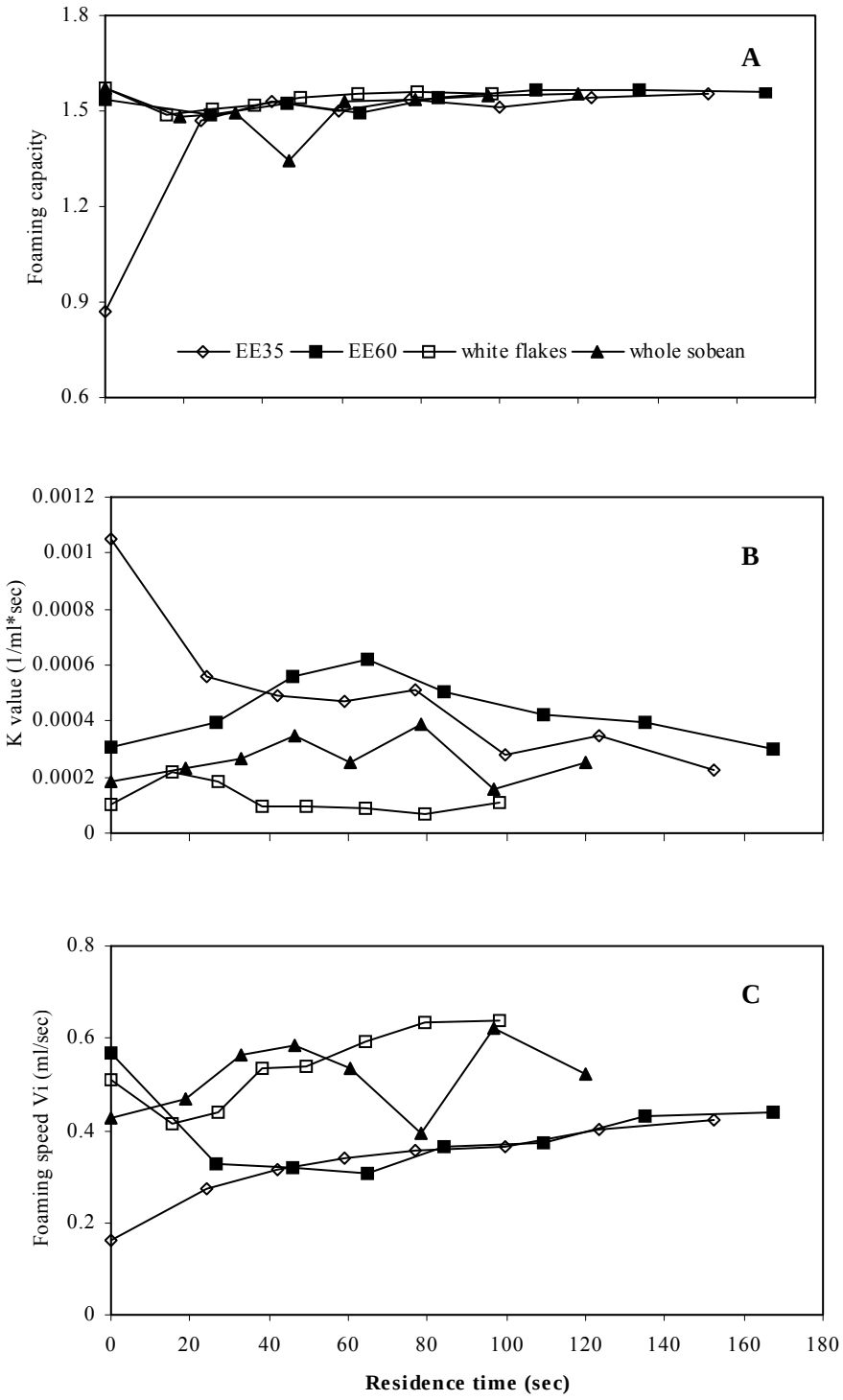

FIG. 4. Effect of residence time during hydrothermal cooking on foaming properties of various protein samples (A) foaming capacity; (B) *K* value, or foam stability; (C) foaming speed.

TABLE 1

$LSD_{0.05}$ Values for the Functional Properties of Soy Protein Meals Subjected to Hydrothermal Cooking Treatments[a]

Property	EE35	EE60	White flakes	Whole soybeans
Solids dispersibility	2.50*	6.74	7.46*	7.99*
Protein dispersibility	3.30*	9.20	11.02*	15.96*
Emulsification capacity	6.65	6.77*	16.58*	4.73*
Foaming speed	0.051*	0.054*	0.106*	0.381
Foaming capacity	0.048*	0.039*	0.043*	0.206
Foaming K value	0.0002	0.0002	4.7E-5*	0.0003

[a] *, LSD values with significant treatment (residence time) effect ($P<0.05$).

Units: solids and protein dispersibilities, %; emulsification capacity, g soybean oil/0.5g sample; foaming speed, mL/s; capacity, mL/mL; and *K*, 1/(mL·s).

TABLE 2

Comparison of Solids and Protein Dispersibility between Flashing-out Hydrothermal Cooking (HTC) and With-holding-tube HTC Treatments [a]

Property/Treatment	EE35	EE60	White flakes	Whole soybeans
Solids dispersibility, %				
Untreated	29.4[c]	48.7[a]	61.6[a]	53.6[b]
Flashing-out HTC	58.4[a]	61.0[a]	62.5[a]	69.8[a]
With-holding-tube HTC	52.3[b]	59.2[a]	51.3[b]	69.3[a]
Protein dispersibility, %				
Untreated	13.5[c]	46.5[a]	70.4[a]	56.5[c]
Flashing-out HTC	58.6[a]	64.2[a]	74.4[a]	78.7[a]
With-holding-tube HTC	44.3[b]	50.0[a]	47.3[b]	66.2[b]

[a]The with-holding-tube HTC treatment values are the highest values obtained with various residence times; means with same superscript are not significantly different (P=0.05) within the same column and under the same quality parameter.

TABLE 3

Comparison of Emulsification Capacity and Foaming *K* Value between Flashing-out HTC and With-holding-tube HTC Treatments [a]

Property/Treatment	EE35	EE60	White flakes	Whole soybeans
Emulsification capacity				
Untreated	38.1[c]	50.3[c]	148.3[a]	127.8[a]
Flashing-out HTC	80.0[b]	91.8[b]	96.3[b]	51.9[c]
With-holding-tube HTC	103.2[a]	104.1[a]	101.4[b]	99.1[b]
Foaming K value				
Untreated	0.0011[a]	0.0003[a]	0.0001[a]	0.0002[b]
Flashing-out HTC	0.0004[b]	0.0003[b]	0.0001[a]	0.0004[a]
With-holding-tube HTC	0.0002[c]	0.0003[a]	0.0001[a]	0.0002[b]

[a]The with-holding-tube HTC treatment values are the highest values obtained with various residence times; means with same superscript are not significantly different (P=0.05) within the same column and under the same quality parameter. Emulsification capacity is expressed as g soybean oil/0.5g sample; foaming *K* value is in 1/(mL·s).

Chapter 2. Preparation of Soy Protein Concentrate and Isolate from Extruded-Expelled Soybean Meals

A paper published in the Journal of American Oil Chemists' Society[1]

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ABSTRACT: Soy protein concentrates (SPC) and soy protein isolates (SPI) were produced from hexane-defatted soybean white flakes and from two extruded-expelled (EE) soybean meals with different degrees of protein denaturation. Processing characteristics, such as yield and protein content, and the key protein functional properties of the products were investigated. Both acid- and alcohol-washed SPC from the two EE meals had higher yields but lower protein contents than SPC prepared from white flakes. Generally, acid–washed SPC had much better functional properties than those from alcohol washing. The SPI yield was highly proportional to protein dispersibility index (PDI) of the starting material, so the EE meal with lower PDI had lower SPI recovery. The protein content in SPI prepared from EE meals was about 80%, which was lower than that from white flakes. Nevertheless, SPI from EE meals had functional properties similar to or better than those from white flakes. The low protein contents in SPC and SPI prepared from EE meals were mainly due to the presence of residual oil in the final products. SPI made from EE meals had higher concentration of glycinin relative to β-conglycinin than that from white flakes.

KEY WORDS: Extruding-expelling, functional property, soy protein concentrate, soy protein isolate.

Soy protein products have become increasingly popular because of their low price, high nutritional quality, and versatile functional properties. Two important soybean protein products are soy protein concentrate (SPC) and soy protein isolate (SPI). SPC is defined as an edible protein product with a protein content of at least 65% on dry weight basis (1), whereas SPI is a product with at least 90% protein on dry weight basis (2). Currently, flash-desolventized solvent-extracted white flakes (typically containing 50% protein) are generally the starting materials for SPC and SPI preparation. Other soybean meals or flours in addition to white flakes may also be used as starting materials provided that the final products meet protein content specifications and demonstrate desired functional properties.

Soybean meals produced from the extruding-expelling (EE) processing of soybeans may be used as starting materials for SPC and SPI preparation. EE is a mechanical processing technology that allows small-scale production of protein meals having a high oil content and partial recovery of oil. Extrusion, the first step of the processing, provides a heat treatment that reduces trypsin inhibitors, permitting the use of the full-fat or defatted protein meals as livestock feed. The extrudate can be pressed by an expeller to partially recover the oil. The protein in the meal typically is extensively heat-denatured by extrusion. Depending on the processing conditions, EE meals with different oil contents and protein denaturation can be achieved (3). Advantages of EE technology include process simplicity, low capital investment, no need for organic solvents, and applicability to identity-preserved (IP) processing due to its flexibility and efficiency in processing small lots of soybeans.

SPC preparation involves insolubilization of the protein to remove soluble sugars. In SPI preparation, proteins are solublized first to remove the insoluble fiber, then they are precipitated to remove

soluble sugars. How the yield and functionality of the SPC and SPI are affected when the proteins in the starting material are heat denatured are unknown. We hypothesized that EE meals would be good starting materials for SPC and SPI products because of they are partially defatted and have reasonably high protein solubility. The objectives were to determine the feasibility of preparing SPC and SPI from EE meals and to evaluate the functional properties of these SPC and SPI products in comparison with those produced from defatted, or white, soy flakes.

EXPERIMENTAL PROCEDURES

EE meals and defatted white flakes. An Insta-Pro International Model 2500 extruder and Model 1500 screw press were used to process the dehulled and cracked soybeans (Stine Seed Co., Adel, IA) into EE meals. The following extruder processing parameters were used: 11-11-6-6 shear lock configuration, double flight screws, and a restriction die opening setting of 3/8 in. (0.94 cm). The temperature in the last segment of the extruder barrel was 132-143°C, and the total residence time was about 20-25 s. EE processing was conducted in the commercial facility of Nutriant (Vinton, IA). Two EE flours (ground meals), EE35 and EE60, with oil contents of 7.6 and 13.6% and PDI of 35.3 and 62.0, respectively, were prepared. Defatted white flakes (Nutrisoy® , 90 PDI) were purchased from Archer Daniels Midland (Decatur, IL). EE meals and defatted soy flakes were ground into flour using a Fitz MILL® (Model DAS06, The Fitzpatrick Company, Elmhurst, IL) with a 40-mesh screen. To avoid any further heat denaturation of the proteins, care was taken to minimize heat generation during milling. All flours were stored in sealed plastic bags at -20°C before use.

SPC and SPI preparation. Acid-washed SPC, alcohol-washed SPC, and isoelectric SPI were prepared in the pilot plants of the Center for Crops Utilization Research (CCUR) employing modified protocols of standard methods (4) (Figs. 1-3). For acid washing, the standard method uses a ratio of 10:1 to 20:1 of water/soybean meals, but 10:1 ratio was used in this study. The centrifugation force was 14,000 × g, at 15°C to reduce the protein solubility in the whey. For alcohol washing, 60% aqueous alcohol was used, compared with 20-80% required in conventional method. According to Berk (5), on either side of 60%, soy protein solubility tends to increase. For the SPI procedure, pH 8.5 was used to solublize the soy protein, compared with pH 7.5-9 in conventional procedure. According to Berk (5) cystine tends to be destroyed at >9 pH with the formation of dehydroalanine, which can further react with free ε-amino groups of lysine to produce lysinoalanine, whose toxicological aspect is not fully understood. The supernatant was refrigerated at 4°C after adjusting pH to 4.5, to facilitate the formation of larger and stronger curds. Centrifugation conditions were the same as those used in SPC preparations.

Analytical methods. All concentrations and final data were expressed as dry weight basis (measured after drying at 130°C for 3 h). All protein contents were measured by Kjeldahl method (6), and a 6.25 conversion factor was used to calculate protein content. Solids dispersibility and protein dispersibility were measured based on the method of Johnson *et al.* (7). Briefly, a 10% w/w (protein product) dispersion was prepared by stirring for 20 min and cooling for 1 h at 5°C. After centrifugation at 1,050 × g, for 5 min at 5°C, the supernatant fraction was quantified. The dispersible solids was measured by drying and weighing of the total solids in supernatant, and the dispersible protein was measured by using the Kjeldahl method as just discussed to quantify proteins in the supernatant fraction. This protein

dispersibility is different from the standard PDI by the AOCS official method (8) in that the measurement conditions are different.

Emulsification capacity was measured based on a method introduced by Swift et al. (9). A 25-mL aliquot of a 2% (w/w) dispersion of protein product was placed in a 400-mL plastic beaker. Fully refined soybean oil was added at about 0.5 g/s and mixed with a hand-held mixer. Emulsification capacity was defined as the amount of oil that could be emulsified until the inversion point was observed (9). A fat-soluble dye, Red Fat 7B (Sigma-Aldrich Co., St. Louis, MO) was added in oil at about 2 ppm to enhance the detection of the inversion point.

Measuring foaming properties involved using a foaming device, which consisted of a graduated glass cylinder with a ceramic frit fused at the bottom. Nitrogen gas was purged at 16.7 mL/s to make a final 300 mL foam from 100 mL of 1% protein sample suspension. Three measurements were made (10): time to reach the final volume (t_f, in s), volume of liquid sample converted to foam at the very end of foaming (V_{max}, in mL), and time required for half of the liquid incorporated into foam to drain back into the liquid fraction ($t_{1/2}$, in s). From these measurements, three foaming parameters were calculated: (i) foaming capacity (FC), an indication of the milliliters of foam formed per milliliter of N_2 purged, and calculated as $FC = 60 \times 300/(16.7 \times t_f)$ in mL/mL unit; (ii) *K* value, which describes foam stability (a higher value indicating lower stability), calculated as $K = 1/(V_{max} \times t_{1/2})$ in $ml^{-1} \times s^{-1}$ unit; and (iii) foaming speed (FS), V_i, which describes the rate of liquid incorporated into foam and is calculated using $V_i = V_{max}/t_f$ in units mL/s.

Composition of alcohol-washed SPC and SPI. The total lipid content as determined by acid

hydrolysis and crude fiber were determined by Woodson-Tenent Laboratories, Inc. (Des Moines, IA), according to standard AOAC methods (11, 12). The total carbohydrate was quantified using the phenol/sulfuric acid method (13). The ratio of β-conglycinin to glycinin in the protein products was evaluated by scanning densitometry following the method of Rickert *et al.* (17). The SDS-PAGE gels were prepared according to Jung *et al.* (18) with glycinin and β-conglycinin standards provided by P. Murphy at Iowa State University.

Experimental design and data analysis. All analyses were repeated three times except for Kjeldahl measurement, which was duplicated. SPC preparation was performed following a 3×2 factorial design, with three protein samples and two washing methods (alcohol and acid). For SPI, three protein samples and only one preparation method were used. Statistical analysis was performed using General Linear Model procedures of SAS 8.02 (14).

RESULTS AND DISCUSSION

Preparation and functional properties of SPC. The method of SPC preparation, i.e., acid or alcohol wash, and the type of soybean material significantly influenced yield and protein content of SPC, as shown in Table 1. Wash method and sample type had significant interactions with all quality and functional parameters except for protein content. Alcohol washing resulted in significantly higher SPC and protein yields than acid washing, especially when using white flakes, however, protein contents of SPC from acid washing were statistically higher than those from alcohol washing. These data suggest that the acid washing removed more soluble sugars and recovered relatively more proteins than alcohol washing. SPC

prepared from the two EE meals had significantly lower protein content than prepared from white flakes. The difference was caused mostly by residual oil content in the SPC (Table 2). For example, total lipid contents of alcohol-washed SPC were 3.4, 22.2, and 12.0%, whereas the protein contents were 67.5, 52.2, and 58.8% for white flakes, EE60, and EE35, respectively. Oil in initial EE meals could not be removed by either alcohol or acid washing, resulting in lower protein content in the final SPC products. It is noteworthy that the oil contents measured by the acid hydrolysis method were always higher than those measured by standard total lipid quantification methods, such as the Goldfish or Soxhlet method. For example, oil contents of EE35 and EE60 meals were 7.6 and 13.6% by Soxhlet extraction, but 9.8 and 16.7% by the acid hydrolysis procedure (Table 2).

In contrast to alcohol washing, protein yields from both EE meals by acid washing were higher, by about 5%, than those from white flakes as a result of the heat denaturation of protein during EE processing, which made protein less soluble in acid. Such differences disappeared in alcohol-washed samples as a result of the strong denaturation power of alcohol.

The solids dispersibility for acid-washed SPC directly related to PDI of the starting material. Alcohol-washed SPC had significantly lower solids dispersibility than acid-washed SPC, and they did not correlate with the initial PDI as a consequence of protein denaturation by alcohol. The same was true for protein dispersibility. SPC from acid washing had much higher emulsification capacity than that from the alcohol washing, because the alcohol-denatured protein did not disperse well in either water or oil phases. The emulsification capacity of acid-washed SPC from EE35 was similar to that of the white flakes despite its significantly lower protein content, and its emulsification capacity was significantly higher than that of

EE60. It is possible that with the higher degree of protein denaturation, the more hydrophobic regions were exposed, which might have contributed to a higher emulsification capacity. Alcohol-washed SPC showed an opposite trend, indicating that alcohol denaturation of protein is different from heat denaturation. The higher residual oil content in SPC from EE60 may also contribute in its lower emulsification capacity.

The foaming properties of acid-washed and alcohol-washed SPC showed quite different patterns also. Under the same wash method, SPC from white flakes had a higher foaming speed, foaming capacity, and foam stability values than the SPC from EE samples. Acid-washed SPC from EE35 had significantly higher foaming speed, capacity, and stability values than that from EE60. However, alcohol-washed SPC from white flakes, EE60, and EE35 showed different trends compared with acid-washed products. For alcohol-washed SPC, product from EE35 had the lowest foaming speed, capacity, and foam stability. This also implies that alcohol and heat denature protein in different manners.

SPI Preparation and functional properties of SPI. SPI yield, protein yield, and protein content were significantly different among SPI prepared from different materials (Table 3). SPI from samples with higher PDI values had higher yields and protein contents than those from lower-PDI materials. Since the total carbohydrates in SPI from white flakes and EE60 were similar (Table 3), the differences in protein contents were apparently partially due to the residual oil content in the SPI. For EE35, the higher total carbohydrates and total oil together contributed to the lower protein content in SPI compared with that from white flakes. Both SPI yield and protein yield showed a strong linear relationship with PDI of the raw materials: SPI yield (%) = 10.3 × PDI + 14.1, $R^2 = 0.99$; protein yield (%) = 16.4 × PDI + 25.4,

R^2=0.98. This shows that denaturation of protein strongly affects the amount of proteins that can be extracted into SPI. Although the protein content differences among SPI from white flakes and two EE meals were significant, the difference in SPI between the two EE meals was much smaller than that between white flakes and EE meals (Table 2).

SPI from both EE60 and EE35 meals had solids and protein dispersibilities of 100%. The SPI from white flakes had slightly lower solids and protein dispersibilities, about 96 and 94% respectively. The difference might be due to a small portion of unstable proteins in white flakes that was recovered into SPI but became insoluble during SPI handling and testing, whereas the corresponding proteins in EE meals never had this chance because they endured a much harsher treatment earlier in EE process and went with the insoluble fractions during SPI preparation.

Contrary to yield, SPI from two EE meals had significantly higher emulsification capacities than those from the white flakes. To explain this observation, the ratio of β-conglycinin to glycinin was determined by SDS-PAGE and densitometry analysis (Table 2). The ratio for SPI from white flakes was 0.72, but the ratios were 0.57 for EE60 and 0.47 for EE35. Apparently, EE processing denatured relatively more β-conglycinin than glycinin, resulting in decreased β-conglycinin-to-glycinin ratio. The lower the PDI value (thus the harsher the EE processing), the lower was the ratio. This is reasonable since the denaturation temperature of β-conglycinin was lower than that of glycinin. For instance, in water solutions, denaturation temperatures were about 70 and 90°C for β-conglycinin and glycinin, as measured by DSC in our own research (Wang, H., L.A. Johnson, and T. Wang, unpublished data). Thus, the EE process resulted in a partial fractionation of β-conglycinin and glycinin during SPI preparation and increased the glycinin

fraction in the final SPI. The emulsification capacity of β-conglycinin was 1.5- to 4.0-fold higher than that of glycinin, as reported by Bian *et al.* (15), and 1.7- or 3.8-fold higher as reported by Rickert *et al.* (16); therefore, we expected to have a lower emulsification capacity for the SPI from EE meals. However, the emulsification capacity of SPI from EE35 was significantly higher than that from white flakes, which was contrary to the expected outcome based on the β-conglycinin and glycinin ratio change. One possible explanation may be that the soybean proteins in SPI recovered from heat-denatured materials experienced special conformational changes during EE processing such that the emulsification performance was altered.

For the foaming properties, i.e., foaming speed and foaming capacity (Table 3), the differences were not significant. The differences for foam stability (*K* value) were minor, although they were statistically significant.

Overall, although the SPI and acid- and alcohol-washed SPC produced from EE meals had lower protein content than their counterparts from white flakes, certain functional properties, such as emulsification capacity and dispersibility of acid-washed SPC, and emulsification capacity of SPI made from EE meals, were similar to, or higher than, those from white flakes. This indicates that certain soy protein products with good functional properties can be made from protein meals processed by EE.

ACKNOWLEDGMENTS

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Figure Captions:

FIG. 1. Procedure for producing soy protein concentrate using the acid-wash method.

FIG. 2. Procedure for producing soy protein concentrate using the alcohol-wash method.

FIG. 3. Procedure for producing soy protein isolate.

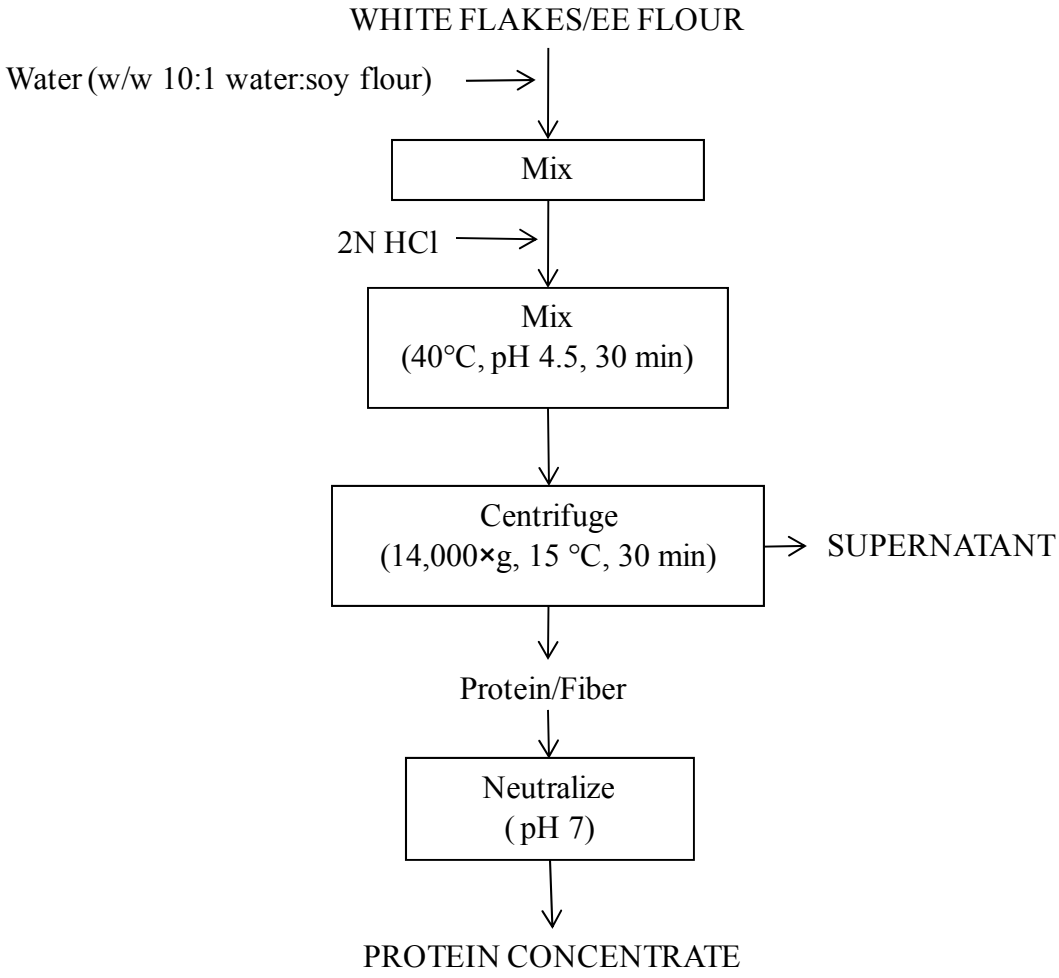

FIG. 1. Procedure for producing soy protein concentrate using the acid-wash method.

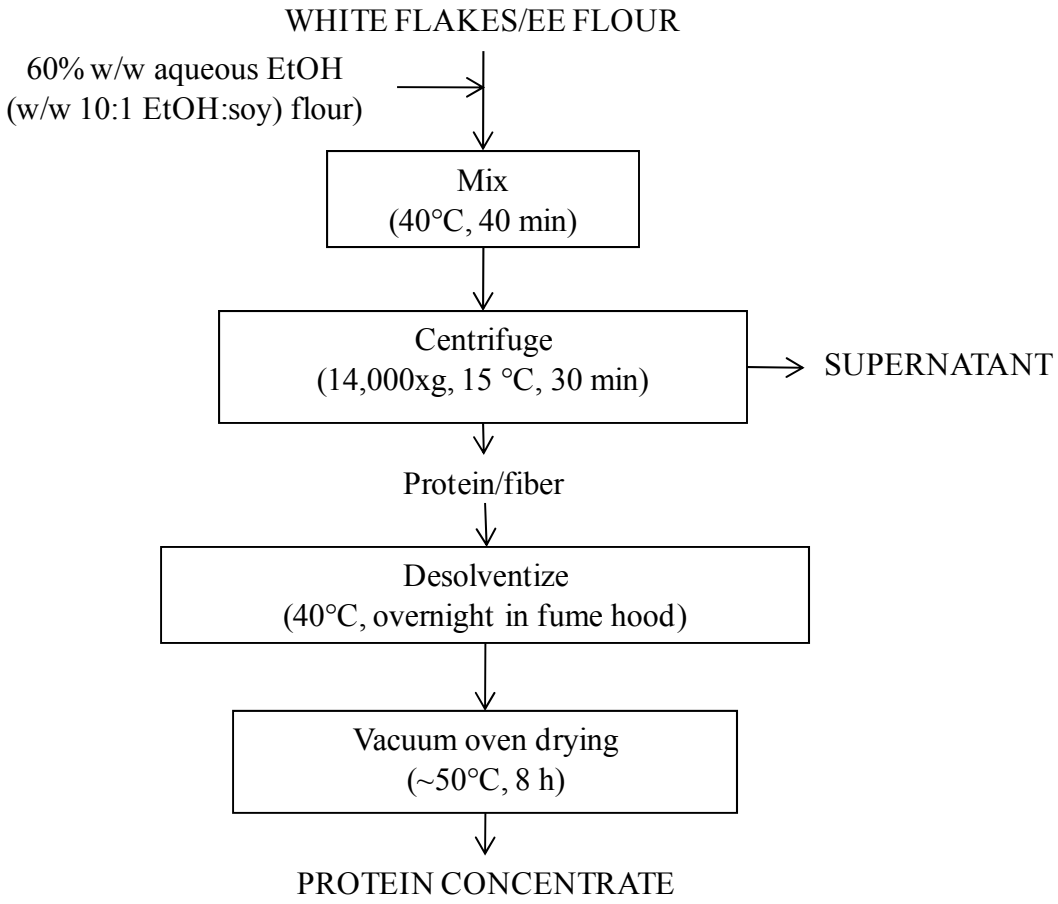

FIG. 2. Procedure for producing soy protein concentrate using the alcohol-wash method.

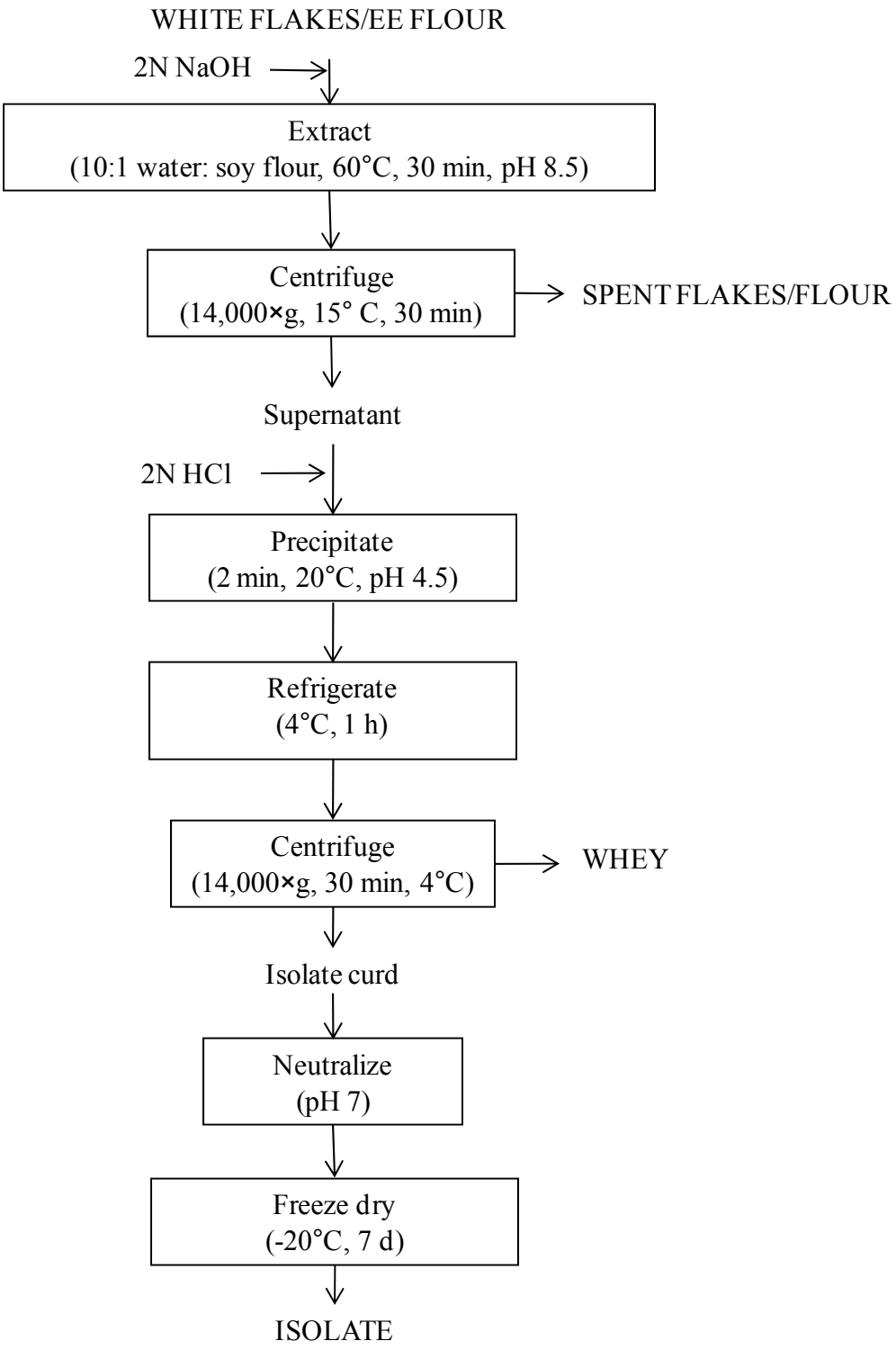

FIG. 3. Procedure for producing soy protein isolate.

TABLE 1.

Acid-washed and Alcohol-washed Soy Protein Concentrates (SPC) and Their Functionalities [a]

Process/Starting material	Yield (%)		PC [a]	Dispersibility (%)		EC [a,b]	Foaming property		
	SPC	Protein	(%)	Solids	Protein		FS [a,b]	FC [a,b]	*K* value [b]
Acid-washed SPC									
White flakes	72.35	90.93	68.28	38.38	53.72	132.54	0.32	1.45	0.00025
EE60	78.45	95.16	53.86	38.35	47.51	99.00	0.09	0.76	0.00148
EE35	78.16	96.03	60.38	18.88	17.42	127.75	0.20	1.36	0.00097
Alcohol-washed SPC									
White flakes	78.89	98.04	67.53	8.31	5.70	25.76	0.25	1.50	0.00067
EE60	83.98	98.65	52.16	16.08	6.26	19.77	0.21	1.28	0.00086
EE35	81.49	97.56	58.83	12.15	3.93	18.14	0.06	0.55	0.00288
P and LSD value									
Wash	<0.0001	<0.00001	0.0031	<0.0001	<0.0001	<0.0001	0.0054	0.0332	<0.0001
Sample	<0.0001	0.0106	<0.0001	<0.0001	0.0008	0.003	<0.0001	<0.0001	<0.0001
Interaction	0.0001	0.0084	0.5322	<0.0001	0.0009	0.0107	<0.0001	<0.0001	<0.0001
$LSD_{0.05}$ for sample	0.56	1.61	0.97	1.35	3.02	9.93	0.03	0.06	0.0002
$LSD_{0.05}$ for wash	0.45	1.32	0.79	1.10	2.47	8.11	0.03	0.05	0.0002

[a] PC, protein content; EC, emulsification capacity, FS, foaming speed; FC, foaming capacity; EE60, soy flour processed by extruding-expelling and having a protein dispersibility index (PDI) of ~60; EE35, same as EE60 except PDI was ~35.

[b] EC: g soybean oil/25 ml 2% slurry; FS: ml/sec; FC: ml/ml; *K* value: $ml^{-1} \cdot sec^{-1}$.

TABLE 2.

Compositions of Starting Materials, Alcohol-washed SPC, and SPI

Product	Protein (%)	Total carbohydrate (%)	Crude fat (%)	Crude fiber (%)	Ratio of β-conglycinin to glycinin
Starting materials					
White flakes	54.34	25.79	3.07	3.94	-[a]
EE60	44.40	23.26	16.73	3.96	-
EE35	49.14	24.01	9.84	4.16	-
Alcohol-washed SPC					
White flakes	67.53	16.17	3.40	4.39	-
EE60	52.16	15.78	22.23	5.17	-
EE35	58.83	17.02	11.96	5.54	-
P value	<0.0001	0.81	<0.0001	0.008	-
$LSD_{0.05}$	1.59	4.72	0.53	0.58	-
SPI					
White flakes	87.53	5.07	3.24	<0.02	0.72
EE60	80.82	5.08	11.69	<0.02	0.57
EE35	79.61	7.61	9.21	<0.02	0.47
P value	0.002	0.80	0.002	-	-
$LSD_{0.05}$	3.33	1.20	3.31	-	-

[a] Not determined. For abbreviations see Table 1.

TABLE 3.

SPI Prepared from Different Materials and Their Functionalities [a].

Product	Yield (%)		PC	Dispersibility (%)		EC	Foaming property		
	SPI	Protein	(%)	Solids	Protein		FS [a,b]	FC [a,b]	*K* value [b]
White flakes	45.48	73.27	87.53	95.69	94.07	248.42	0.63	1.69	0.00011
EE60	33.45	60.89	80.82	100.00	100.00	272.28	0.62	1.67	0.00014
EE35	24.98	40.46	79.61	100.00	100.00	316.42	0.63	1.67	0.00013
P value	<0.0001	<0.0001	0.002	0.035	<0.0001	0.021	0.94	0.064	0.001
$LSD_{0.05}$	0.87	2.78	3.32	3.19	0.75	42.7	0.08	0.02	1.00E-5

[a] For abbreviations and units see Table 1.

Chapter 3. Effect of Alkali on the Refunctionalization of Soy Protein by Hydrothermal Cooking

A paper published in the Journal of American Oil Chemists' Society[1]

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Running title: Alkali hydrothermal cooking of EE meals

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ABSTRACT: The effects of hydrothermal cooking (HTC) at alkaline conditions on refunctionalization of heat-denatured protein of extruded-expelled (EE) soy meals and on preparation of soy protein isolate (SPI) from EE soy meal were determined. Two HTC setups, flashing-out HTC (without holding period) and HTC with holding for 42 s at 154°C, were evaluated. Alkali (NaOH) addition dramatically enhanced the refunctionalization of EE meal having an initial protein dispersibility index of 35. The more alkali added, the more refunctionalization occurred. Extensive refunctionalization was achieved at 0.6 mmol alkali/g EE meal and additional improvement was small with more alkali. For both HTC setups, the solids and protein yields of SPI from alkali-HTC treated EE meals were significantly higher than those from HTC without alkali addition. The yield of protein as SPI increased from 40 to 82% after HTC treatment at 0.6 mmol alkali/g EE meal compared with no alkali addition. The emulsification capacities of SPI after alkali-HTC were similar to those from HTC without alkali. SPI from holding-tube HTC-treated EE meals had higher emulsification capacities than those prepared by flashing-out HTC.

KEY WORDS: Alkali hydrothermal cooking, emulsification capacity, extruded-expelled soybean meal, foaming properties, functional properties, protein, protein dispersibility, refunctionalization, soy protein, soybean meal.

The use of soy protein ingredients by the food industry is increasing rapidly owing to health benefits, low cost, and versatile functionalities. Most soy protein products are manufactured from highly soluble (flash-desolventized) hexane-defatted soybean meal. Solvent extraction is capital intensive and unsuitable for processing small amounts of identity-preserved soybeans. Extrusion-expelling (EE) is a promising technique that presses the oil from the whole or cracked seeds and is suitable for processing small lots of identity-preserved seeds (1, 2). EE differs from traditional screw pressing in that a dry extruder replaces steam-heated stack cookers or rotary dryers. Compared with solvent-extracted white flake, EE protein meals that are processed into EE soy flour having much more heat exposure and protein denaturation [15-60 vs. 80-90 protein dispersibility index (PDI)], contain significantly more residual oil (6-12% vs. ~1%), having lower levels of heat-sensitive antinutritional factors (trypsin inhibitors) and enzymes (such as lipoxygenase), and possess a pleasant nutty flavor. Typical EE meal contains 50% protein and 6% oil, and 90% of its trypsin inhibitors are inactivated (1). The use of protein ingredients prepared from EE meal in foods, however, is limited by their poor functional properties and low yields of soy protein isolate (SPI), a direct consequence of heat denaturation of the protein.

Our previous work showed that SPI could be prepared from EE meals; however, the yields were low (the SPI yield and protein yield were only 25 and 40%, respectively) (6). Inspired by the work of Johnson (3) and Wang and Johnson (4), we demonstrated hydrothermal cooking (HTC) could be used to refunctionalize heat-denatured proteins of EE meals (5). We also demonstrated the feasibility of using HTC as a pretreatment in extracting soy protein products, such as soy protein concentrate (SPC) and isolate (SPI), from EE meals; the yields of SPI and protein were 36 and 53%, respectively.

We have observed that the viscosity of a slurry of alcohol-washed SPC, which contains highly denatured protein as a consequence of exposure to alcohol, dramatically increased with mild heating and alkali addition. We also observed similar behavior with EE meals. We hypothesized that the combination of alkali, high shear, and high temperature achieved during HTC can be used to refunctionalize EE soybean meal. The objective of this work was to determine the effects of different alkali addition on the major functional properties of EE meal, as well as SPI yield and properties prepared from treated EE meal.

EXPERIMENTAL PROCEDURES

EE meal and alkali treatments. Typical EE meal, EE35 (with PDI of 35), was prepared from cracked and dehulled commodity soybeans (Stine Seed Co., Adel, IA) by using processing conditions previously described (6). A slurry containing 20% meal solids was prepared by mixing with a Biomixer^{TM} handheld mixer (ESGE Ltd., Mettlen, Switzerland). Alkali (2 N NaOH) was added at 0.2, 0.4, 0.6, and 0.8 mmol/g EE meal (dry-weight basis) levels immediately before being pumped to a Stephan mill (Type MC15, A Stephan u. Söhne GmbH & Co., Hameln, Germany) for grinding and mixing.

HTC setup and treatment conditions. A Moyno pump (2MI type SSQ; Robin and Myers, Inc., Springfield, OH) was connected to a steam infusion hydroheater (size 300 type B; Hydrothermal Co., Milwaukee, WI) where culinary-grade steam (~90 psi, 6.5 kg/cm^2) was infused into the protein slurry to achieve instantaneous heating and high shear. The slurry feed rate was maintained at 1.5 kg/min for all treatments. Two HTC setups were used (Fig. 1). For one HTC treatment, the heated slurry was merely flashed out and was designated as flashing-out HTC. In this case, the cooked protein slurry was discharged

directly into a flash chamber at atmospheric pressure without any holding tube or backpressure after the steam infusion. The slurry temperature was about 104°C for 2 s. For a more severe heat treatment, we placed a holding tube after the hydroheater. The holding tube was 4.48 m long (2.54 cm i.d. and 2.66 cm o.d.), and provided 42-s residence time (5). A backpressure valve, after which the sample passed to the flash tank, was used to control the steam pressure and thus temperature. The cooking temperature was maintained at 154±1°C and monitored by using thermocouples and a data logger. This treatment was designated as holding-tube HTC. These conditions were selected based on our previous work (5). The cooked slurry exiting the flash chamber was immediately cooled to <40°C by discharging into a stainless-steel beaker in an ice bath. All treated samples were refrigerated at 5°C until being analyzed.

Only flashing-out HTC was used to evaluate the effects of alkali addition on the refunctionalization of EE meals because of the availability of data on flashing-out refunctionalization of EE meals in our previous work for comparison (5). The alkali-treated samples were neutralized to pH 7 before determining functionality. Both flashing-out and holding-tube HTC with 0.2 and 0.6 mmol alkali/g meal were used to produce refunctionalized materials for SPI preparation.

SPI preparation. SPI was prepared using methods described by Lusas and Rhee (7) with minor modifications (Fig. 2). Protein extraction was carried out at the pH obtained after HTC with 0.0, 0.2 and 0.6 mmol alkali/g meal addition, diluting to 1:10 solids/water ratio and stirring for 30 min at 60°C as in our previous work (6). The pH values of the protein slurries under various conditions are shown in Table 1.

Characterization of functional properties. All compositional data are reported on dry-weight basis. Moisture was determined by using oven-drying at 130°C for 3 h. Protein content was measured by using

AOAC method 993.13 (8) with a Rapid N III analyzer (Elementar Analysesysteme GmbH, Germany) and 6.25 as the N conversion factor. Solids and protein dispersibilities were the dry matter and protein, respectively, in the supernatant relative to the total dry and protein matter after centrifuging a 10% suspension at 1050 × *g* at 5°C for 5 min (3).

Emulsification capacity was measured by a method based on Swift *et al.* (9) where 25 ml of a 2% solids dispersion was placed into a 400-mL plastic beaker, and a hand-held Biomixer™ was used at high speed (1,200 rpm) to emulsify the protein suspension with a commercially refined soybean oil, which was introduced at a rate of about 0.5 g/s. Emulsification capacity was the amount of oil that caused phase inversion. The inversion point was detected by observing a sudden separation of oil and water phases, an abrupt decline in viscosity, and a change in pitch of the noise from the mixer. A fat-soluble dye, Red Fat 7B, was added to the oil at about 4 ppm to make the end point more easily observed.

To quantify foaming properties, a foaming column made by fusing a fritted ceramic disk into a graduated glass column was used (10). Nitrogen gas was purged at 16.7 mL/s to produce 300 mL of foam from 100 mL of 1% solids sample suspension. Three measurements were recorded: time to produce 300 mL of foam (t_f, in s), volume of sample suspension incorporated into foam at the end of the foaming period (V_{max}, in mL), and time used for one-half of the foamed suspension to drain back ($t_{1/2}$, in s). From these measurements, three foaming parameters were calculated: foaming capacity (FC) = $300/(16.7 \cdot t_f)$, which was expressed as mL of foam formed per mL of N_2 purged; K value = $1/(V_{max} \cdot t_{1/2})$, in unit of 1/(mL·s), which was used to describe foaming stability (higher value indicates lower stability); and foaming rate, V_i = V_{max}/t_f, in unit of mL/s, i.e., the rate of liquid incorporation into foam.

SDS-PAGE. SDS-PAGE was performed by using a method similar to that of Jung *et al.* (11). Soy protein samples and standards (glycinin and β-conglycinin, provided by P. Murphy,, Department of Food Science and Human Nutrition, Iowa State University) were dissolved in a 2× sample buffer (125 mM THAM at pH 6.8, 0.2% SDS, 20% glycerol, 5.0 M urea, and 0.01% bromophenol blue) to achieve 1 mg/mL protein concentration. Storage and resolve gels contained 4 and 13% polyacrylamide, respectively. The loading volumes for samples and standards were 5 and 15 μL, respectively. Electrophoresis was conducted at 200 V for 45 min by using a Mini-PROTEIN® 3 Cell electrophoresis device (Bio-Rad Laboratories, Inc., Hercules, CA). Gels were stained for 1 h with a solution of methanol/acetic acid/water (50:10:18, by vol) and 0.22% Coomassie blue and destained for 4-5 h in methanol/acetic acid/water (50:10:40 by vol) solution. After washing with water, the gels were packed and dried overnight in a fume hood.

Experimental design and data analysis. A two-factor factorial design was used to evaluate the effects of the two HTC setups (flashing-out HTC and holding-tube HTC) at three alkali addition levels (0, 0.2, and 0.6 mmol alkali/g meal) on protein refunctionalization and SPI preparation. Each treatment was replicated two times. All SPI preparations and functional property measurements were performed in triplicate. Statistical analysis was performed using General Linear Model procedures of SAS 8.02 (12).

RESULTS AND DISCUSSION

pH of protein dispersion at different alkali additions. To understand pH effects, all pH values were measured at the same solids concentration (1:10 solids/water ratio or 9.1% dry solids) at 25 and 60°C after

stirring for 30 min as was used for extracting SPI (Table 1). After HTC, the pH dropped about 0.4 units compared to those without HTC treatment. The reduction in pH probably occurs because alkali-HTC dispersed or dissolved some of the heat-denatured protein, exposing more ionizable amino acid side chains of the protein to the aqueous environment, thus increasing the buffering capacity of the protein.

One potential concern of alkali HTC is the high pH environment and possible protein degradation. It was reported that at pH >9, cystine could be degraded to form dehydroalanine, which may further react with lysine to produce lysinoalanine, whose toxicological property is not yet fully understood (13, 14). Kidney cell enlargement was observed in rats due to lysinoalanine, but at the consumption dose of 1.5 mg/kg of body weight per day, no adverse effect was observed. When free lysinoalanine was fed to quail, mice, hamsters, rabbits, dogs, and monkeys, no kidney effects were observed (14). Research has so far failed to provide a definitive conclusion on its toxicity. Lysinoalanine is present in most proteinaceous foods or ingredients, including heated milk, cooked chicken, simulated cheese, cooked egg whites, hydrolyzed vegetable protein, milk powder, and casein (14).

Effect of alkali concentration on HTC refunctionalization of EE meals. Alkali-HTC improved solids and protein dispersibilities, emulsification capacity and foaming stability (lower *K* value indicates better stability) of EE35 meals over the control that was not HTC treated nor exposed to alkali (Fig. 3). Maximum solids and protein dispersibilities were achieved by carrying out alkali-HTC at 0.6 mmol alkali/g meal. Protein dispersibility may have accounted for most of the solids dispersibility since the two curves and the relative improvement were similar, and the other components may not have responded to alkali treatment.

Although there were some significant differences in emulsification capacities among different alkali concentrations, the differences were probably not sufficient to be practically significant. There were no differences in foaming capacities among different alkali concentrations during HTC treatment; however, these foaming capacities were lower than that of EE meal without any treatment (Fig. 3), and we could not explain this outcome. For foaming stability, the maximum effect was achieved at the alkali addition of 0.6 mmol/g meal, where *K* value was the lowest.

Preparation of SPI after alkali HTC refunctionalization of EE meal. For both flashing-out and holding-tube HTC, alkali addition significantly improved the solids and protein yields of SPI compared with HTC without alkali (Fig. 4). The improvements were more significant when compared to the initial meal without HTC. Higher alkali addition resulted in higher SPI yields. The protein concentrations in SPI prepared from flashing-out HTC and holding-tube HTC ranged 75-76% without significant differences among them. Although these protein concentrations were significantly lower than for the SPI from EE meals without HTC treatment (80%), the difference was only about 4-5%. Because these SPI products do not meet the protein concentration minimum requirement of 90% protein, they cannot be marketed as “soy isolate”. We believe that the lower protein purity (than SPI requires) was the result of the presence of significant levels of residual oils and carbohydrates in SPI, similar to the SPI prepared from EE meals without HTC treatment (6).

There were no statistically significant differences in SPI yield, protein yield and protein content for the two HTC setups (Table 2). The maximum SPI protein yield (82%) was achieved with holding-tube HTC at 0.6 mmol alkali/g meal. This protein yield was more than twice as much as from the untreated EE

meal (~40%) and 1.7 times more than from HTC-treated EE meals without alkali (~48%).

Functionalities of SPI produced from alkali-HTC refunctionalized EE flour. HTC setup had a significant effect on emulsification capacity (Table 2). SPI from holding-tube HTC had higher emulsification capacity than did SPI from flashing-out HTC, and the difference was about 100 g oil/g SPI, which was 25% higher than flashing-out HTC. The level of alkali addition had little effect on emulsification capacity within the flashing-out HTC or the holding-tube HTC treatment groups (Fig. 5). The emulsification capacities of SPI from holding-tube alkali-HTC-treated EE meal were lower than that from EE meal without any treatment (633 g oil/g). One possible explanation is that SPI recovered from the original EE meal (with very low yield) consisted of mainly native proteins, thus gave high emulsification capacity. HTC treatment had most likely denatured all the remaining native proteins. It was not clear why SPI from holding-tube HTC had much higher emulsification capacity than did the SPI prepared by flashing-out HTC under both neutral and alkaline conditions.

We speculate that since EE meals contain both native and heat-denatured proteins, HTC decreased the emulsification capacity of the native proteins and at the same time increased the emulsification capacity of the denatured protein by disintegrating the large heat-denatured protein aggregates. The overall emulsification capacity of SPI was the combination of the two opposing actions. Compared with flashing-out HTC, holding-tube HTC exposed the protein to a much higher temperature for a longer period. This may have been more effective in breaking up the aggregates and thereby resulting in a protein product with an improved balance of hydrophobic and hydrophilic surfaces. Although the emulsification capacity was the same for SPI from HTC without alkali, alkali-HTC resulted in much higher SPI yield.

HTC setup and alkali addition significantly affected both foaming capacity and foaming stability (*K* value) of the SPI (Table 2). The more alkali was added, the higher was the foaming capacity, especially for holding-tube HTC. The foaming capacities of SPI prepared from HTC-treated EE meals were higher than that for the SPI from original EE meal. Holding-tube HTC increased foaming capacity more than flashing-out HTC at the same alkali concentration, but the increases were not great (Fig. 5). Alkali addition of 0.2 mmol/g meal significantly decreased foaming stability of SPI compared with that of original EE meals, but when alkali addition was increased to 0.6 mmol/g meal, foaming stability was improved and was similar to SPI from original EE meal (Fig. 5). Since the foaming properties are related to the surface behavior of the proteins at the interface between solution and air of the foam bubbles, the exact mechanism is difficult to understand except that alkali-HTC probably changed the conformations of the soy protein molecules and resulted in modified surface properties. It is worth noting that foaming stability of SPI from HTC-treated EE meal without alkali decreased (*K* value increased) compared with that of SPI from original EE meal, similar to the trend in emulsification capacity (Figs. 5 A and 5 C). This may be explained by the denaturation of the residual native protein in the original EE meal during HTC without alkali treatment.

Characterization of SPI by SDS-PAGE. The initial objective in determining the SDS-PAGE profile of the SPI was to see whether protein decomposition or hydrolysis occurred during alkali-HTC. No major peptide decomposition was found in SDS-PAGE profiles (Fig. 6). The difference in protein band density of SPI between flashing-out and holding-tube HTC at the same alkali concentration was not great. Alkali-HTC treatment decreased the band densities of major protein subunits. At higher alkali addition

(especially 0.6 mmol alkali/g meal), the subunit bands became lighter and at the same time, a darker residual band remained on the top of the gel, indicating more protein was not dissolved even in the presence of strong denaturation agents (SDS, urea) and reducing agent (2-mercaptoethanol). The accumulation of proteins at the gel top suggested that strong interactions between protein and protein, or protein and non-protein components (possibly carbohydrates) were formed. These interactions, however, did not affect the extraction of the protein or protein functionalities. There were considerable non-protein components, including carbohydrates, in SPI from EE meals. The SPI prepared from original EE35 meal contained 80% protein, 8% carbohydrates (by phenol-sulfuric acid method), 9% crude fat (by acid hydrolysis method), and no crude fiber (6). Typical SPI from alkali-HTC contained 76% protein.

Overall, alkali addition enhanced the refunctionalization of EE meal and dramatically increased the protein yield of SPI, which had similar or improved functionalities compared to SPI from HTC without alkali addition. Holding-tube HTC resulted in SPI with better functional properties than flashing-out HTC.

ACKNOWLEDGMENTS

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Figure captions:

FIG. 1. Schematic diagram of the two HTC setups (flashing-out and holding-tube HTC).

FIG. 2. Procedure for producing soy protein isolate (SPI) from alkali-HTC-treated extruded-expelled (EE) soybean meal.

FIG. 3. Effects of alkali (NaOH) addition and flashing-out-HTC on the major functional properties [solids dispersibility (A), protein dispersibility (B), emulsification capacity (C), foaming capacity (D), and K value (E)] of EE35 meals, which has a protein dispersibility index of 35. Points with the same letters within the same chart are not significantly different (P =0.05).

FIG. 4. Comparisons of solids yields, protein yields, and protein purities of SPI from flashing-out (A) and holding-tube (B) HTC-treated EE meals at different alkali (NaOH) concentrations. Points with the same letters within and across charts are not significantly different (P = 0.05).

FIG. 5. Effects of alkali-HTC on emulsification capacities (A), foaming capacities (B), and foaming stabilities (C) of SPI. Points with the same letters within the same chart are not significantly different (P = 0.05).

FIG. 6. SDS-PAGE profiles of SPI from alkali-HTC treatments (α′, α, and β are the major subunits of β–conglycinin, acidic (A), and basic (B) subunits are the major components of glycinin).

TABLE 1.

pH of Protein Slurries (9.1% solids) at Different Alkali Concentrations before and after Hydrothermal Cooking (HTC)

Location of alkali addition	Alkali concentration (mmol/g meal)		
	0	0.2	0.6
Before HTC, 25°C	6.7	9.4	11.4
Before HTC, 60°C	6.4	8.3	10.0
After HTC, 60°C	6.4	7.9	9.6

TABLE 2.

P-Values for Treatment Effects on SPI Preparation and Major Functional Properties[a]

Treatment	d.f.	SPI Yield	SPI Protein Content	SPI Protein Yield	EC[a]	FC[a]	*K* Value[a]
HTC setup	1	0.257	0.659	0.480	<0.0001	0.0027	0.016
Alkali addition	2	<0.0001	0.074	<0.0001	0.620	0.0003	0.0005
HTC*alkali addition (interaction)	2	<0.0001	0.761	0.0002	0.694	0.378	0.038

[a] EC, emulsification capacity; FC, foaming capacity; *K* Value, foaming stability; SPI, soy protein isolate.

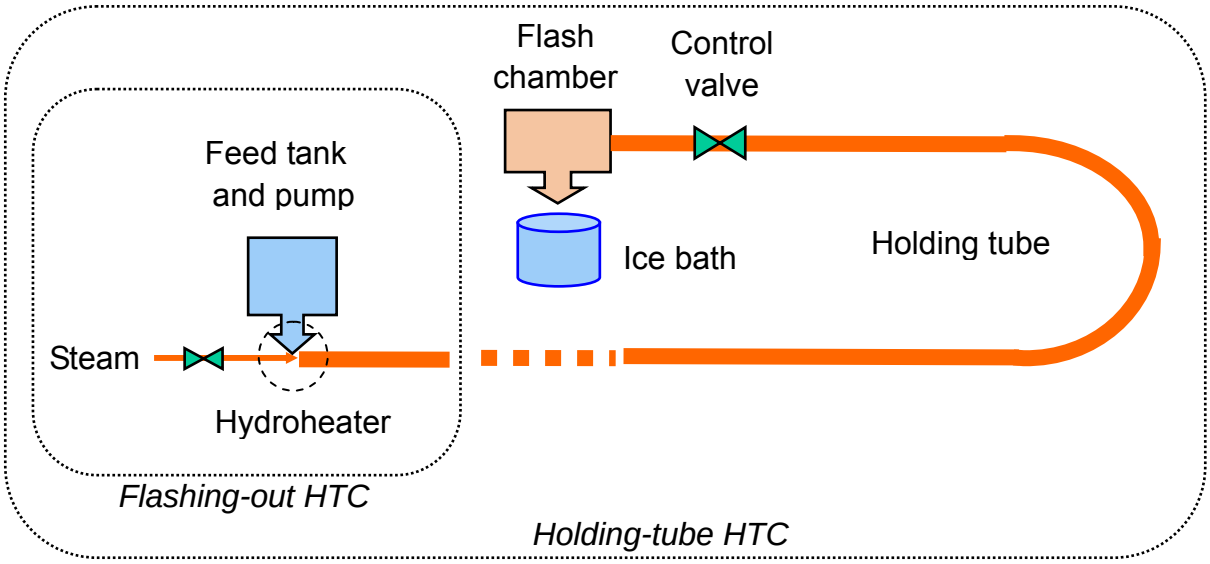


FIG. 1. Schematic diagram of two HTC setups (flashing-out and holding-tube HTC).

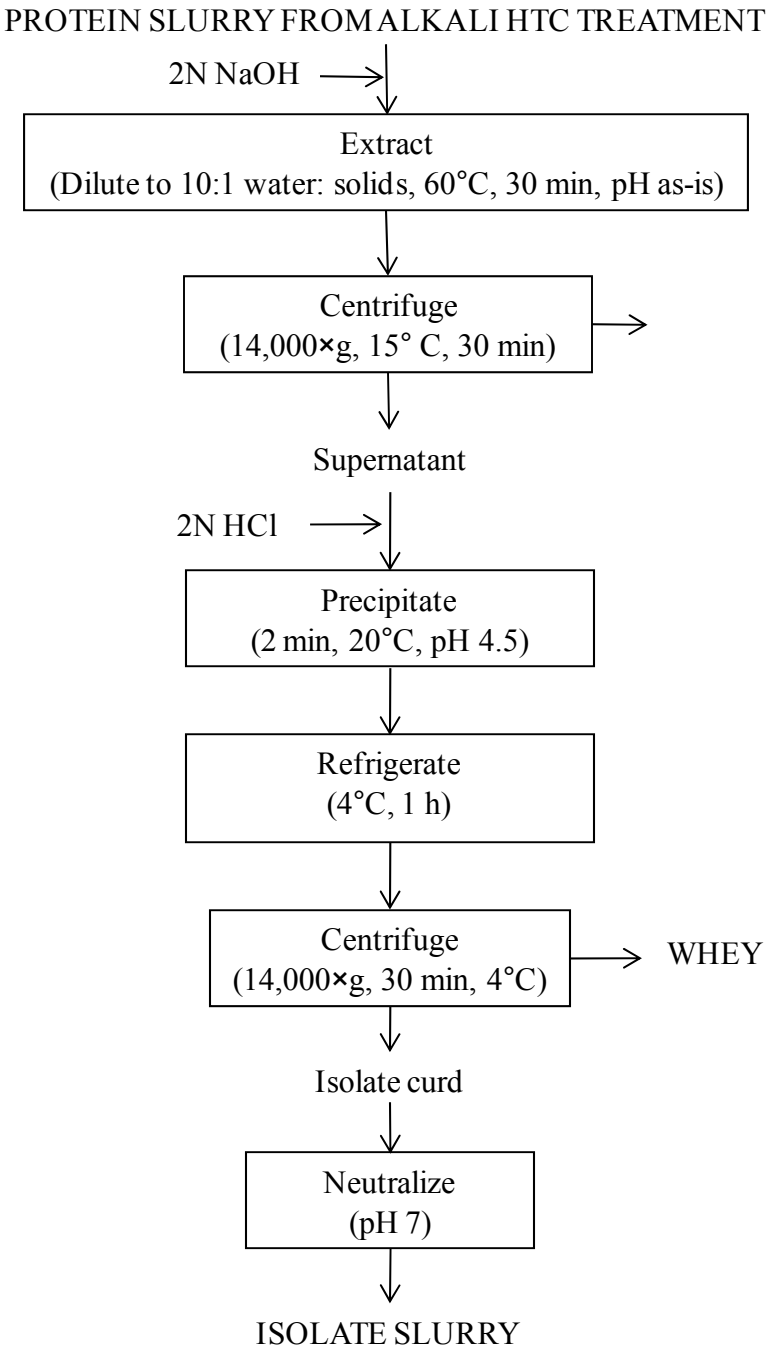


Fig. 2. Procedure for producing soy protein isolate (SPI) from alkali-HTC-treated extruded-expelled (EE) soy meal.

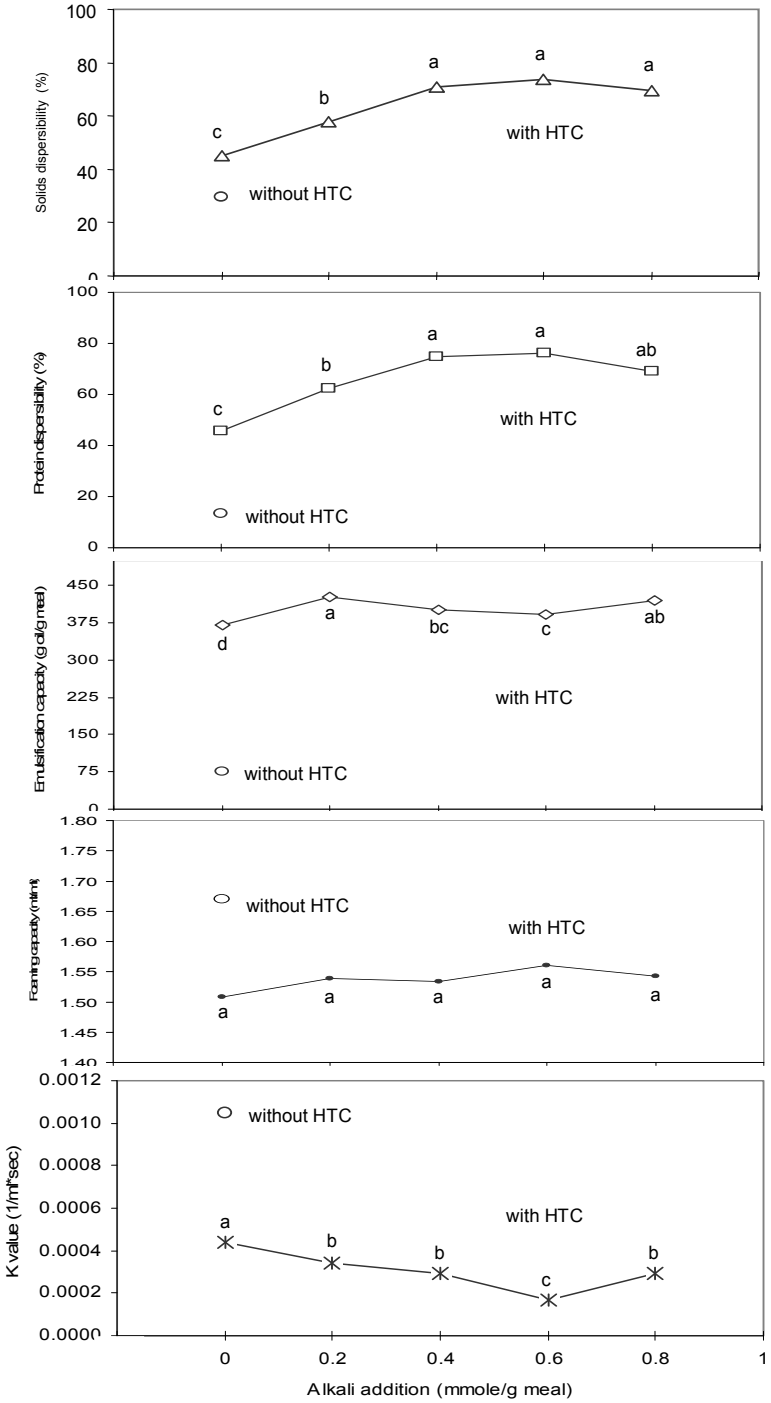


FIG. 3. Effects of alkali (NaOH) addition and flashing-out-HTC on the major functional properties [solids dispersibility (A), protein dispersibility (B), emulsification capacity (C), foaming capacity (D), and K value (E)] of EE35 meals, which has a protein dispersibility index of 35. Points with the same letters within the same chart are not significantly different (P = 0.05).

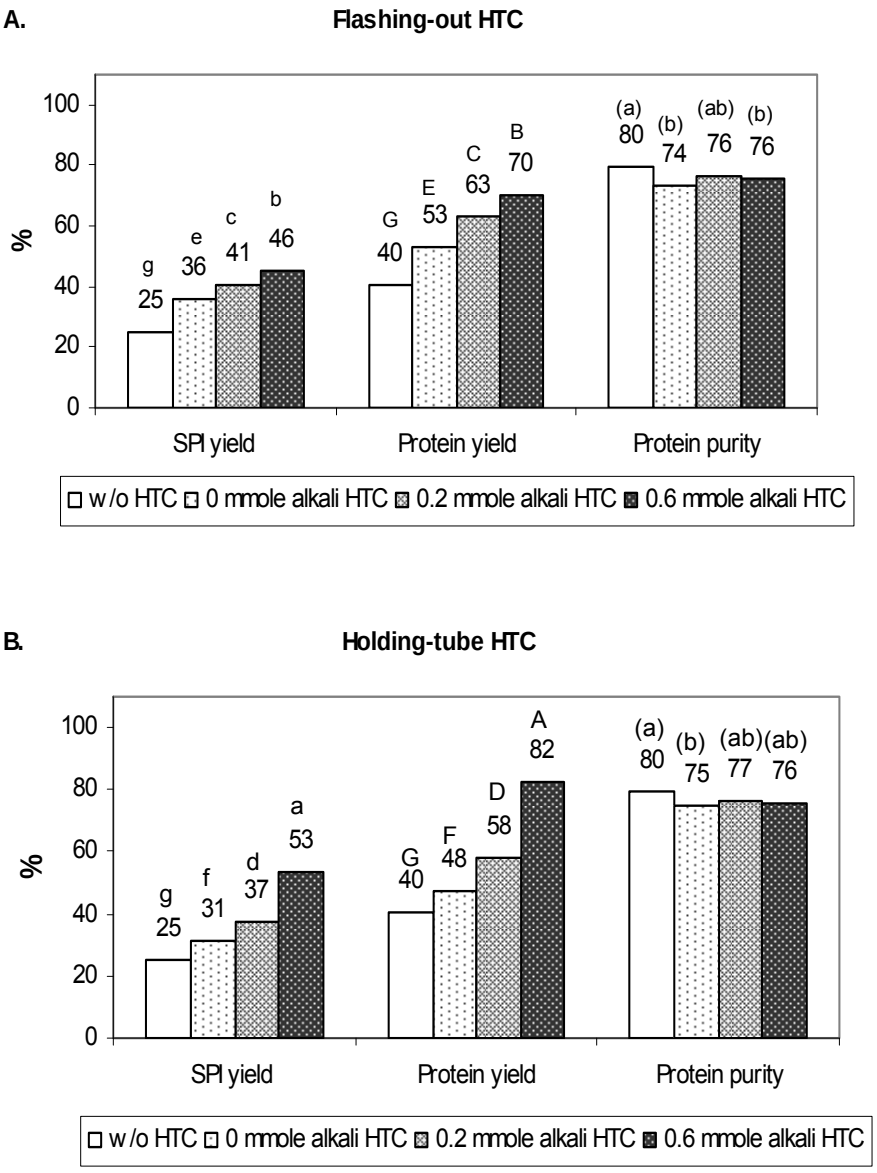
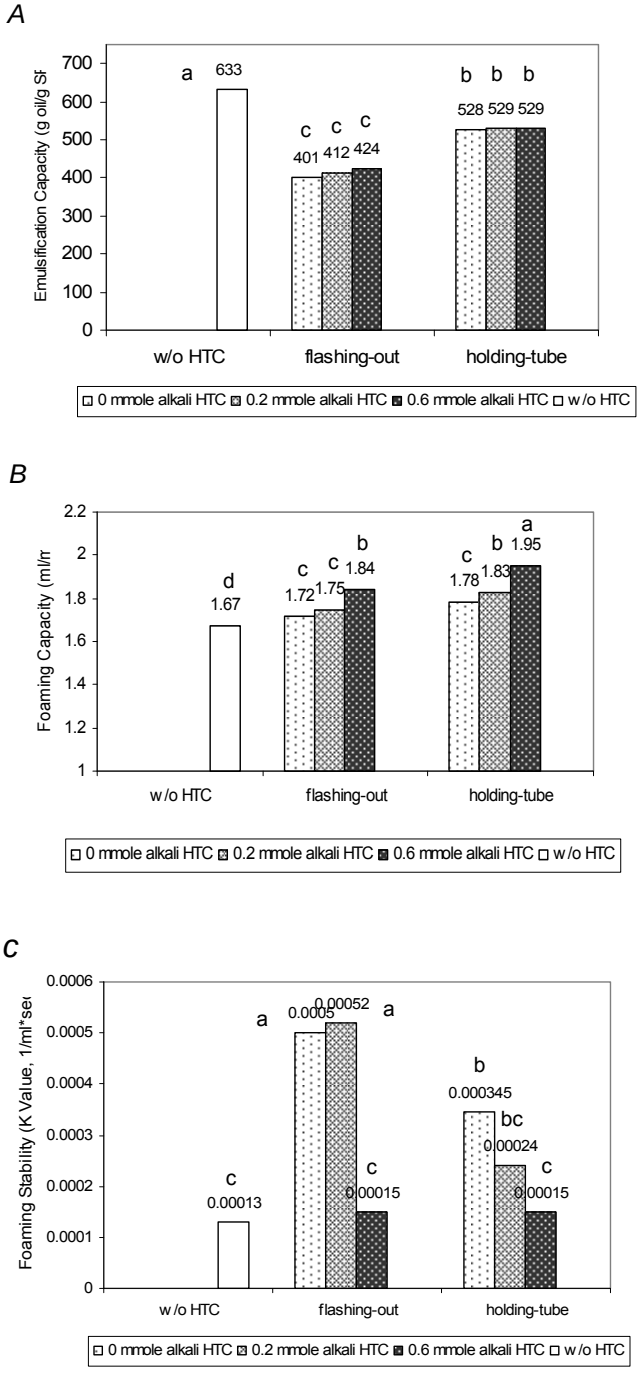


FIG. 4. Comparisons of solids yields, protein yields, and protein purities of SPI from flashing-out (A) and holding-tube (B) HTC-treated EE meals at different alkali (NaOH) concentrations. Points with the same letters within and across charts are not significantly different (P = 0.05).



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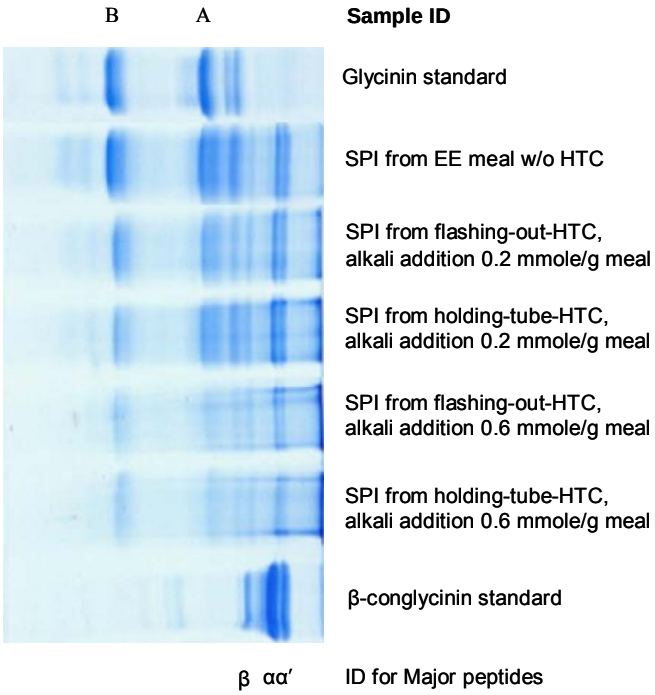

FIG. 6. SDS-PAGE profiles of SPI from alkali-HTC treatments (α′, α, and β are the major subunits of β–conglycinin, acidic (A), and basic (B) subunits are the major components of glycinin).

Chapter 4. Mechanism for Refunctionalizing Heat-Denatured Soy Protein by Alkaline Hydrothermal Cooking

A paper published in the Journal of American Oil Chemists' Society[1]

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Running title: Protein refunctionalization by alkali hydrothermal cooking

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ABSTRACT: Using extrusion heat-denatured soy protein isolate (SPI) as a model, the mechanism for refunctionalizing of heat-denatured soy protein by hydrothermal cooking (HTC) with alkali was studied. Heating causes soluble protein to form insoluble protein aggregates. Treating heat-denatured soy protein with alkali dispersion without HTC increased solubility and viscosity by dissolution of a portion of the protein aggregates and swelling of the large protein particles. This suspension was more stable to solids separation than that of original untreated heat-denatured protein, but it was less stable than the protein suspensions that were refunctionalized by water dispersion with HTC or alkali dispersion with HTC. Water dispersion with HTC disrupted the large aggregates into smaller aggregates. The viscosity and total number of particles in the system also increased dramatically. The most significant effect was achieved with alkali dispersion (0.6 mmol NaOH/g) with HTC. The solubility increased from 4 to about 80% at neutral pH, and viscosity (at zero shear rate) increased by more than 1,000 times compared with extrusion heat-denatured SPI. Alkali-dispersion (0.6 mmol NaOH/g) with HTC dissolved most of the protein particles, decreasing the particle number by a factor of almost 100. The suspensions of heat-denatured soy protein became much more stable after HTC as shown by particle settling velocities. The most effective treatment was alkali dispersion (0.6 mmol NaOH/g) with HTC, followed by water dispersion with HTC. The soy protein slurry refunctionalized by alkali dispersion (0.6 mmol NaOH/g) with HTC formed soft, translucent gels.

KEY WORDS: Alkali dispersion, extrusion, heat-denatured protein, hydrothermal cooking, particle-size distribution, protein solubility profile, settling velocity, soy protein isolate, Stokes' law, viscosity.

Our previous work showed that hydrothermal cooking (HTC), especially when the protein was dispersed in alkali (0.2-0.8 mmol/g meal) prior to HTC, improved the functional properties, such as solids dispersibility, emulsification capacity and foaming properties, of extruded-expelled (EE) soybean protein meals (1, 2). The protein yield of soy protein isolate (SPI) after alkali dispersion with HTC (alkaline HTC) of EE soybean meal was improved by twofold (from 40 to 81%) compared with the original meal, whereas the protein yield after water dispersion with HTC (HTC without alkali) was 53%. We hypothesized that alkali dispersion with HTC increases the extractability of the protein and the stability of the resulting protein suspension (as SPI) by dissolving the broken-up aggregates pieces. The objective of the present work was to determine the changes of protein solubility or dispersibility, particle size of the denatured protein aggregates, viscosity, density differences between the dispersed and continuous phases etc, after various HTC treatments, in order to reveal the mechanism during HTC. To eliminate interference from nonprotein components in EE meal, such as fiber and residual oil, we chose native SPI (with protein content > 90%) as a model to study the mechanism of protein refunctionalization and improvement in SPI yield by alkali dispersion with HTC.

EXPERIMENTAL PROCEDURES

Materials. Native SPI (spray-dried without other intentional heat denaturation or additives) prepared at the Center for Crops Utilization Research, Iowa State University, was used as purified soy protein. A co-rotating laboratory-scale Leistritz Micro-18 twin-screw extruder (American Leistritz Corp., Sommerville, NJ), with the screw having a low shear configuration (18 mm diameter, 30 L/D ratio), was

used to denature the protein in SPI. To make SPI extrudable, additional water was injected into the middle of the barrel. The maximum amount of water that could be added into native SPI before extrusion was only about 20%, which was less than required for trouble-free extrusion. The water feed rate was about 14 g/min. Screw rotation speed was set at 200 rpm. Moistened SPI was fed with a metering feeder (Accurate Inc., Whitewater, WI) at about 4 g/min. The internal temperature profile was 60, 90, 130, 130, and 130˚C from the feed inlet to the die outlet. The extrudates were dried at ambient temperature for 2 d and then ground with a Magic Mill III Plus high-speed flour mill (Magic Mill, SSI Division., Salt Lake City, UT). The flour was then passed through a 40-mesh sieve and kept at ambient temperature to equilibrate the moisture content (5.1%). The protein dispersibility index (PDI) of the heat-denatured SPI was 10.3 as measured by NP Analytical Laboratories (St Louis, MO) by using AOCS official Method Ba 10-65 (3).

Treatments. Five treatments (Fig. 1), identified as "water-dispersed heat-denatured SPI without HTC", "alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC", "alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC", "water-dispersed heat-denatured SPI with HTC", and "alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC" were used. Water-dispersed heat-denatured SPI without HTC was SPI after extrusion cooking to heat-denature the protein and dispersion in water at 10% (db: dry basis). Alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC was prepared by dispersing extrusion heat-denatured SPI as a 10% dry matter suspension, adjusting the pH to 8.5 with about 0.2 mmol NaOH/g SPI, and stirring at 60˚C for 30 min as in conventional SPI extraction (4). Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC was prepared following the same procedure as alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI

without HTC, except more alkali (0.6 mmol NaOH/g SPI) was added. This trial was designed to determine the effect of alkali since the same amount of alkali was used as in alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC. Water-dispersed heat-denatured SPI with HTC had HTC carried out on slurries of extrusion heat-denatured SPI without alkali addition. Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC was treated the same as water-dispersed heat-denatured SPI with HTC, except 0.6 mmol NaOH/g SPI was added before HTC.

For all HTC treatments, a Moyno pump (2MI type SSQ; Robin and Myers, Inc., Springfield, OH) was connected with a hydroheater (size 300 type B; Hydrothermal Co., Milwaukee, WI) where culinary-grade steam (~90 psi pressure, 6.5 kg/cm^2) was infused to give the heat and shear treatment. The samples (20% slurry) were infused with steam in the hydroheater, then passed to a holding tube that provided 42-s residence time at 154ºC. The slurry was then released into the flash chamber. The cooking temperature was adjusted by a control valve located between the holding tube and the flash chamber; the temperature was monitored by a thermocouple (1). All HTC-treated SPI were cooled to ambient temperature, neutralized to pH 7, and stored at 5°C until analyzed.

Sample analyses. The protein solubility profile was determined as the percentage of solublized protein in the supernatant of 1% (db) dispersions in a series of pH values (2.5-10.5) after centrifugation. The pH was adjusted to specific levels with 2 N HCl or 2 N NaOH. After stirring for 1 h, the pH values of the suspensions were adjusted again, stirred for an additional 15 min, and centrifuged at 10,000 ×*g* at 20ºC for 10 min. The protein content of the supernatant was determined by using the Biuret method (5) with BSA (Sigma, St. Louis, MO) as the standard. The initial protein content (N × 6.25) was determined by

using a Rapid N III analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

The particle-size distribution and mean diameter of the dispersed particles of protein aggregates were measured by using a Mastersizer particle analyzer (Malvern Instruments Inc., Lombard, IL). The viscosity profiles of 10% sample (db) dispersions were measured at 25ºC by using a HAAKE RheoStress viscometer RS150 (Gebrüder HAAKE GmbH, Karlsruhe, Germany).

To be able to use Stokes' Law to explain our stability observations, we estimated "density difference", which is the difference between the density of dispersed particles and the density of the continuous phase. The method was derived from a centrifugation procedure, in which the density and mass of the supernatant under a series of centrifugation forces were measured and the data fitted with a linear model to extrapolate to an estimated density difference.

Our initial trial showed that the number of protein aggregates changed dramatically after alkali dispersion with HTC (alkaline HTC). The protein slurry was diluted to 1% in 20% aqueous glycerin and treated with 0.1% Coomassie Brilliant Blue G-250 to stain the protein. The suspension was spread onto a microscopic slide in a thin layer, and the slide was observed microscopically. Images were captured with a Zeiss Axioplan-2 microscope system (Carl Zeiss, Inc., Thornwood, NY). A magnification of 20× was selected because the range of particle sizes was easily observed. The particles were counted in a fixed area as seen on a computer screen. Dark field microscopy was used to highlight the particles from the background. For each sample, three photos were randomly selected. The particle number report was the mean of the three counts. We assumed that the particles were uniformly distributed in the suspension, thus the particle number along any direction will be the same and the "particle number" may be used as a

parameter across different dimensions. A simple mathematical function was used to convert particle number from count per area to count per volume (Fig. 2), and the particle concentrations were expressed millions/mL in a 1% protein slurry (Table 1).

Experimental design and data analysis. All treatments were completely randomized with three replications. Statistical analysis was performed using General Linear Model procedures of SAS 8.02 (6).

RESULTS AND DISCUSSION

Protein solubility profiles. All samples had U-shaped solubility curves and isoelectric points (pI) of about pH 4.5, which is typical for soy protein (Fig. 3). As expected, extrusion heat denaturation dramatically reduced the solubility of spray-dried SPI. At pH values of 6.5 and 10.5, the solubilities of heat-denatured SPI decreased from 44 to 3% and 74 to 9%, respectively, compared with spray-dried SPI. Alkali dispersion without HTC increased the solubility of extrusion heat-denatured SPI. When heat-denatured SPI was treated by alkali dispersion (0.2 mmol NaOH/g) without HTC, the solubility increased only slightly compared with water-dispersed heat-denatured SPI without HTC. When treated with more alkali [alkali dispersion (0.6 mmol NaOH/g) without HTC], solubility increased much more. For example, at pH 6.5 the solubility of alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC increased from 12 to 37%. The increase in solubility suggests that, although the amount of alkali added was critical, mild heating (60˚C) in alkali for a prolonged time (30 min), as in traditional SPI preparation, was not sufficient to solublize or disperse the heat-denatured protein. This was especially true for the low alkali addition (0.2 mmol NaOH/g SPI).

When treated with HTC at neutral pH (water dispersion with HTC), the solubility of heat-denatured SPI dramatically increased. For example, at pH 6.5, the solubility increased from 3 to 35%. Solubility was near that of spray-dried SPI without extrusion heat denaturation. The solubility steadily increased with increasing pH to 52% at pH 10.5 (water-dispersed with HTC). The solubility increase was greater in water-dispersed with HTC than that of alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC, which increased only from 37 to 43%. Thus, when comparing the solubilities resulting from the various treatments, HTC without alkali can refunctionalize heat-denatured soy protein considerably.

The most dramatic protein resolublization occurred with alkali dispersion (0.6 mmol NaOH/g) with HTC. At pH 6.5, the solubility soared from 4% of that of water-dispersed heat-denatured SPI without HTC, to 80%. The solubility remained nearly constant as pH increased. Solubility of the alkali-dispersed HTC material was much higher than that of spray-dried SPI. The solubility profile of spray-dried SPI (before extrusion) was between that of water-dispersion with HTC and alkali-dispersion with HTC. Spray-drying and subsequent storage may have reduced protein solubility. At pH 5.5, alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC had considerable solubility (about 28%) compared with SPI with other treatments (all below 6%). The reason is unknown. Apparently, high pH induced rapid solublization or dispersion of denatured SPI.

Settling velocity. Many liquid foods are suspensions of solid particles in liquid; these suspensions are termed sols. Maintaining a uniform dispersion of solid particles in the liquid continuous phase is critical to product quality. Over time, the solid particles will settle as a result of gravity. Estimates of the mean settling velocity of the particles are indicators of how stable the liquid suspension or food

preparation will be during distribution and storage. Stokes' law can be used to estimate settling velocity of dispersed particles. Several factors influence settling velocity, including the size of particles (diameter), the density differences between the particles (dispersed or discontinuous phase) and the liquid (continuous phase), and the viscosity of the medium (continuous phase). We calculated settling velocities of the dispersed protein aggregates after various treatments based on the estimates of the parameters in the Stokes' law.

The order of increasing mean particle diameters of the protein particles in the treated suspensions was as follows: water-dispersed heat-denatured SPI with HTC < alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC < water-dispersed heat-denatured SPI without HTC < alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC < alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC (Fig. 4). The mean particle diameter increased after alkali dispersing without HTC.

The particle size distributions were normal on the log scale for all treatments except for water-dispersed heat-denatured SPI with HTC, which was a bi-normal distribution (Fig. 4). After treatment with alkali alone (alkali-dispersing without HTC), the peaks of protein particle distribution shifted to the right indicating larger size. More alkali addition (0.6 vs 0.2 mmol NaOH/g SPI) resulted larger particles (Fig. 4). Although alkali dispersion with mild heating may dissolve the small particles and partially dissolve the large particles of heat-denatured SPI, the effect of swelling may be more dominant. The net result was increased mean diameter of the dispersed particles. The more alkali was added, the more swelling there was, although more protein was also dissolved, as shown by the solubility data (Fig 3).

Water-dispersed heat-denatured SPI with HTC caused a bi-normal distribution. One peak (at about 500 μm) appeared at the right side of the original heat-denatured SPI, the other (at 25 μm) showed up on the far left side. The peak at the right side suggests that while most of the large particles were disrupted into smaller particles by the high shear force, some resistant particles survived the treatment and swelled in the aqueous system even without alkali addition, because their mean diameters were about 500 μm, larger than that of original heat-denatured SPI (345 μm). The peak at the far left side suggests the majority of disrupted aggregates had diameters around 25 μm. This observation supports our hypothesis that high shear during HTC disrupts the large aggregates of heat-denatured protein into smaller ones (2).

Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC reduced the mean particle size compared to the particles of water-dispersed heat-denatured SPI without HTC. The small particles may have been dissolved and the large particles were disrupted and partially dissolved by the combination of alkali solublization and high shearing force during HTC. Although alkali dispersion (0.6 mmol NaOH/g) with HTC may cause protein aggregates to swell in the same fashion as alkali dispersion without HTC, the swollen particles must have been short-lived because the high shear force, high temperature, and alkali to which they were subjected during treatment also disrupted and solublized the particles. Therefore, the mean particle size was reduced.

It should be noted that particle-size distribution and mean particle diameter were based on the remaining dispersed particles. The treatments changed not only the particle diameter or size distribution but also the total number of particles in ways such that the final products showed significantly different properties (Fig. 5). The protein particles in the slurry of heat-denatured SPI settled quickly, leaving a

nearly clear solution in the upper portion. After alkali dispersion without HTC, the two layers did not clearly separate and the particles were better dispersed, although there were visible precipitates in the slurry. This was probably a result of more protein in the solution as well as the swelling of the protein particles. After water dispersion with HTC, the dispersion had a distinctive white milky color and a homogenous viscous texture. The SPI suspension after alkali dispersion (0.6 mmol NaOH/g) with HTC formed translucent to transparent soft gels after neutralization (the print characters behind the gel are clearly visible in Fig. 5). These observations cannot be explained by mean particle size and size distribution alone.

The number of particles must be quantified in order to address the significant differences in sample properties. The results of particle number concentration are shown in Table 1. Alkali dispersion without HTC increased the particle number compared to water-dispersed heat-denatured SPI, possibly because of partially dissolution and physical disruption of the aggregates. The most dramatic changes occurred after HTC. The particle number concentration (millions/mL of 1% slurry) increased 200-fold after water-dispersing with HTC, but decreased by almost 100-fold after alkali dispersion (0.6 mmol NaOH/g) with HTC compared with water-dispersed heat-denatured SPI without HTC. HTC disrupted particles, producing smaller ones, whereas alkali largely dissolved the proteins. The combination of alkali and shear at high temperature disrupted the aggregates and then dissolved the proteins more efficiently.

The viscosity profiles of the protein slurries after different treatments are shown in Figure 6. All samples exhibited typical non-Newtonian shear-thinning. Many models can describe viscosity behaviors of pseudoplastic materials of this type, such as using the power law (Ostwald-de Waele), Carreau-Yasuda,

Cross, Ellis, Meter, and the like (7). Usually the more comprehensive and better-fitting the model is, the more independent parameters are required. A simple logarithmic conversion was performed to describe the relationship between viscosity and shear rate measured in our experiment (Fig. 7). All samples showed downward linear relationships between shear rates of 10 to 500 s^{-1} after log-log conversion. Even the slurry of water-dispersed heat-denatured SPI without HTC was a shear-thinning, non-Newtonian pseudoplastic suspension. The alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC, water-dispersed heat-denatured SPI with HTC, and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC exhibited almost perfect linear relationships (R^2 = 0.9995, 0.9982, 0.9989, and 0.9984, respectively). Therefore, the power-law model was a good fit.

After heat denaturation, the viscosity of spray-dried SPI dispersion decreased. Viscosities of all the HTC or alkali-dispersed heat-denatured SPI increased compared to untreated heat-denatured SPI. The viscosity of alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC increased slightly. The increases were higher for the water-dispersed heat-denatured SPI with HTC and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC, where the viscosities were near that of spray-dried SPI dispersions. The viscosity increase of alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC was the greatest, followed by alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC, water-dispersed heat-denatured SPI with HTC, and alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC. Since the lines are above each other, there is little possibility that their relationship could change beyond the shear rate range used in the analysis. It is well known that the power law model

does not fit reality well at extreme shear rates (for example, zero and infinite shear rate). When a particle settles through a liquid phase under the force of gravity, there will be a near zero-shear rate. A viscosity value near zero-shear is needed for the Stokes' law particle settling velocity calculation. We regarded the shear rate of 1 s^{-1} as sufficiently low to represent the value at zero-shear. A zero-shear rate viscosity was extrapolated based on power law prediction from Figure 7.

The density difference in Stokes' law is defined as the mean density of particles (dispersed phase) minus the density of the continuous phase (we used the density of the supernatant after centrifugation at 15,000×g for 15 min as the estimate for the density of the continuous phase) at ambient conditions. Because settling particles can precipitate under centrifugation, the mean density of the settling particles at different centrifugal speeds (rpm) were calculated by using the following formula, assuming the sum of the volume of precipitate and supernatant after centrifugation equals to the volume before centrifugation:

$$\text{Average density of settling particles} = \frac{\text{mass of precipitate}}{\text{volume of precipitate}} =$$

$$\frac{\text{mass of precipitate}}{\text{total volume of slurry before centrifugation} - \text{volume of supernatant}}$$

The density difference between dispersed particles and continuous phase is centrifugation speed-specific when using this formula. An approach was needed to derive the density difference under gravity alone. We found a near-linear relationship between centrifugal speed (rpm) and density difference for heat-denatured SPI. We assumed that the relationship between density difference and centrifugation

rpm is linear for our samples. Therefore, extrapolation was used to calculate the density difference at the centrifugal speed of zero (under gravity, when slurry is free to settle). Figure 8 shows one example of density difference calculation. When speed = 0, density difference = 0.059 kg/m^3.

The accuracy of this method depends on the clear separation of precipitate and the measurement of supernatant volume. Considerable errors, especially for the viscous samples, were observed. The errors were so significant that sometimes no linear relationship was observed for certain samples. Another problem which may compromise the result is the small density difference between hydrated protein and continuous phase, especially considering the fact that many water molecules will hydrate the dispersed protein or indispersible aggregates or be physically trapped inside the protein matrix.

Nevertheless, this method did provide useful information. The approximations were acceptable on further consideration that the density difference in this experiment had smaller impact on the settling velocity calculation than other parameters, either because of less relative change or the low power in the formula.

Stokes's law (settling velocity) calculations. $V = 2 \cdot g \cdot d^2 \cdot \Delta\rho / (9 \cdot \mu)$, where V = settling velocity of protein particles, unit m/s; g = gravitational constant, 9.81 m/s^2; d = mean diameter of particles, m; $\Delta\rho$ = mean density difference between particles and water, kg/m^3; μ = viscosity of the suspension, kg/(m·s) [note: 1 N·s/ m^2=0.1 kg/(m·s)]. The number of days it takes for the particles to settle a distance of 10 cm were also calculated to compare the relative stabilities of the dispersions after different treatments.

Tables 2 shows that water-dispersed heat-denatured SPI with HTC and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC had dramatically reduced the settling velocities of the protein

particles. The effect was achieved mostly by the increase in viscosity of the slurry. The settling velocity results generally agreed with observations of the dispersions. The particles of water-dispersed heat-denatured SPI without HTC settled most quickly, followed by particles in alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC, water-dispersed heat-denatured SPI with HTC, and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC. The difference between alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC and water-dispersed heat-denatured SPI with HTC may be too small to be significant. It is very important to emphasize that for the alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC, not only a majority of the particles were solublized but also the solution formed soft translucent or transparent gels where few protein aggregates survived, thus no settling was observed.

It's worth noting that the viscosity term in Stokes' equation is theoretically for the continuous phase or the medium. We used viscosity of the suspension for calculation, because it is difficult to define and justify a reasonable medium for all samples and treatments. Centrifugation could be used to remove the insoluble protein particles for measuring medium viscosity, but the speed to use was an unsettled question because of the different sizes of protein particles present in different samples. We assumed that the contribution of the dissolved protein to viscosity is much greater than the contribution of the insoluble protein particles; therefore our suspension viscosities may be a slight overestimation of the true medium. As a validation, the water-dispersed heat-denatured SPI without HTC had much lower viscosity than the rest of the samples (Table 2), and such low viscosity was primarily produced by the insoluble protein particles.

Although water dispersion with HTC considerably refunctionalized heat-denatured soy protein and alkali dispersion without HTC partially refunctionalized heat-denatured soy protein, their individual effects were less significant than the combination of HTC and alkali dispersion, in which HTC and alkali work synergistically to refunctionalize heat-denatured soy protein. These effects were achieved by disrupting the large particles by high shear and high temperature and by dissolving the disrupted protein particles with alkali. The majority of the particles were dissolved and a translucent gel was formed, making soy protein stable and functional. The solubility of refunctionalized soy protein increased 20-fold and the viscosity (at zero shear rate) was 1,000 times greater than that of extrusion heat-denatured SPI.

Another potential implication of alkaline refunctionalization of heat denatured soy protein is that the treated protein product may have improved protein digestibility and bioavailability, therefore, a high-quality protein may be produced by this processing technique.

ACKNOWLEDGMENTS

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TABLE 1.

Particle Concentrations in SPI Slurries after Different Treatments[a]

Treatment	Without HTC			With HTC	
	Water-dispersed heat-denatured SPI	Alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI	Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI	Water-dispersed heat-denatured SPI	Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI
Particle concentration in 1% slurry (million/ml)	14.3	76.2	5.2	2920	0.2

[a]SPI, soy protein isolate; HTC, hydrothermal cooking.

TABLE 2.

Settling Properties when Using Linear Extrapolation to Calculate Density Difference[a]

Treatment	Mean particle diameter (10^{-6}m)	Viscosity $\mu_{(\text{shear rate}=0)}$ (10^{-3} kg/ms)	Density difference (kg/m^{-3})	Settling velocity (10^{-9} m/s)	Time to settle 10 cm (d)
Water-dispersed heat-denatured SPI without HTC	345	52.3	0.050	247	4.70
Alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC	385	327	0.028	27.9	41.4
Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC	417	1480	0.021	5.5	211
Water-dispersed heat-denatured SPI with HTC	184	1300	0.103	5.8	198
Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC	227	72300	gel	Gel	gel

[a]For abbreviations see Table 1.

Figure Captions

FIG. 1. Diagram of hydrothermal cooking (HTC) treatments for protein refunctionalization and analyses used to study the mechanism of the refunctionalization.

FIG. 2. Schematic figure showing how particle concentration was calculated.

FIG. 3. Protein solubilities of heat-denatured SPI after different treatments.

FIG. 4. Particle-size distributions and volumetric mean diameters of heat-denatured SPI after different alkali and HTC treatments. Means followed by the same letters are not significantly different ($P \leq 0.05$).

FIG. 5. Photographs of 10% dispersions of heat-denatured SPI after different treatments.

FIG. 6. Viscosity profiles of heat-denatured SPI after different treatments.

FIG. 7. Viscosity and shear rate relationships of heat-denatured SPI after different treatments.

FIG. 8. Estimation of density difference under gravity using centrifugation method.

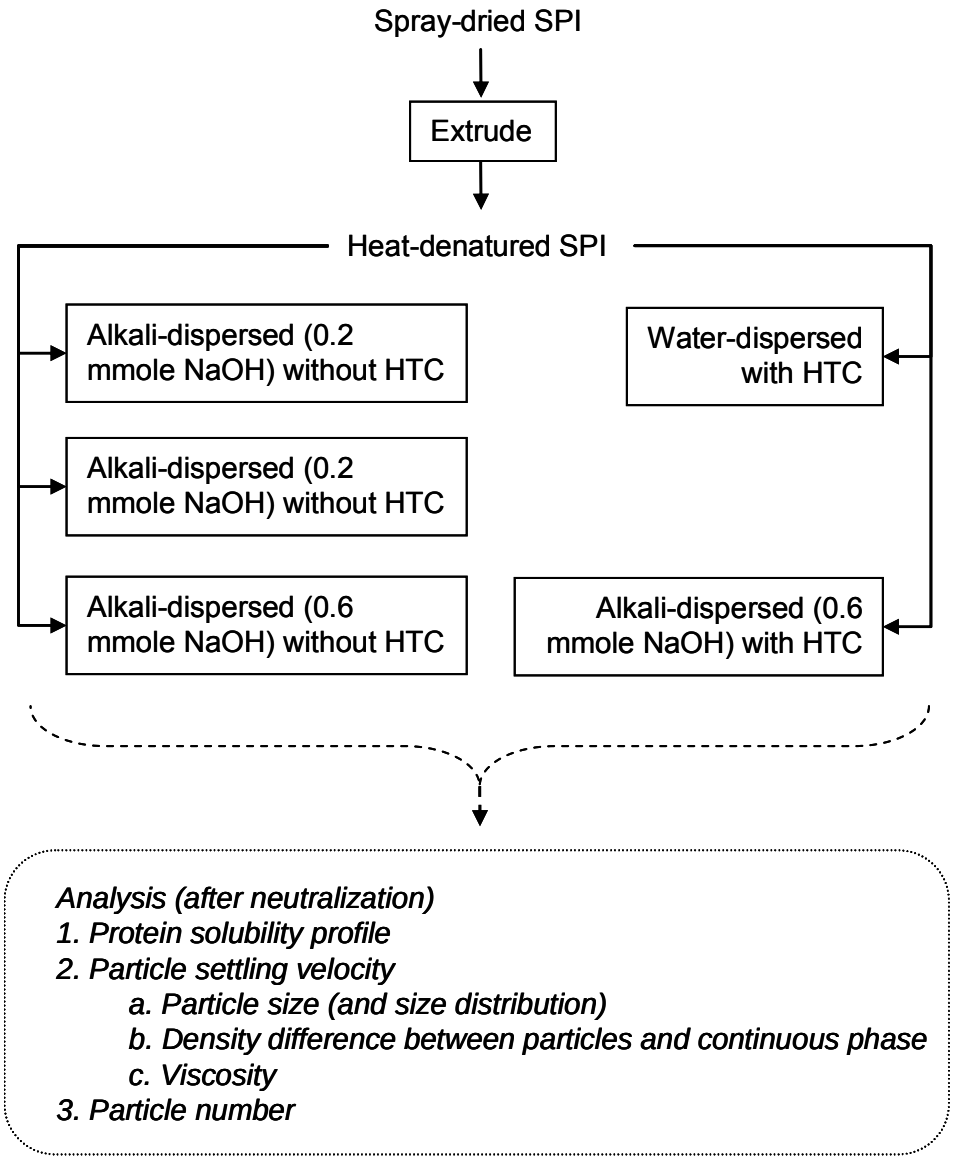
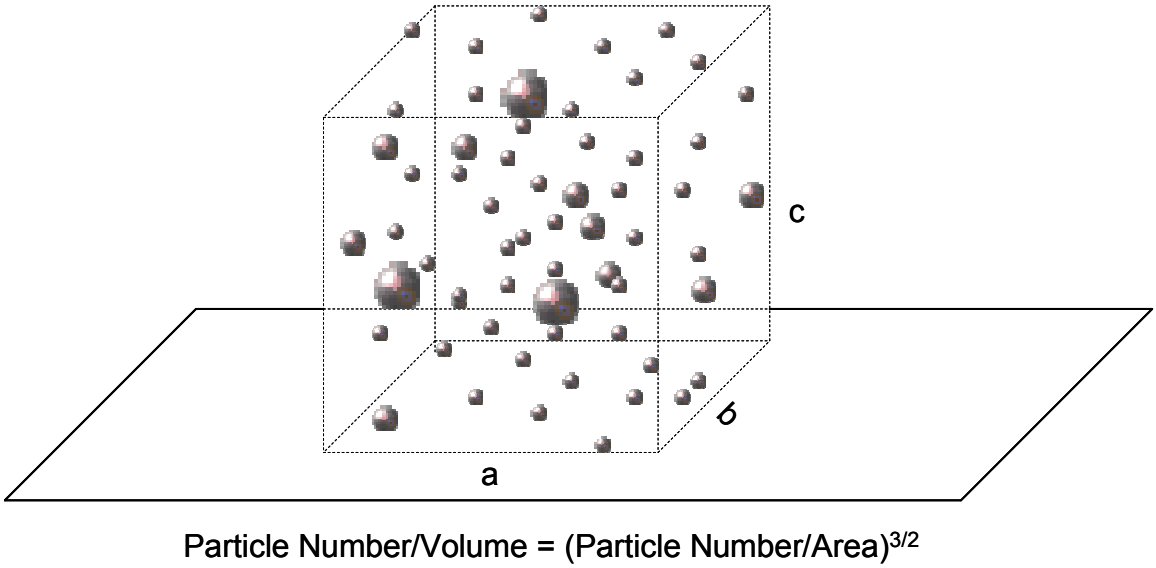


FIG. 1. Diagram of hydrothermal cooking (HTC) treatments for protein refunctionalization and analyses used to study the mechanism of the refunctionalization.



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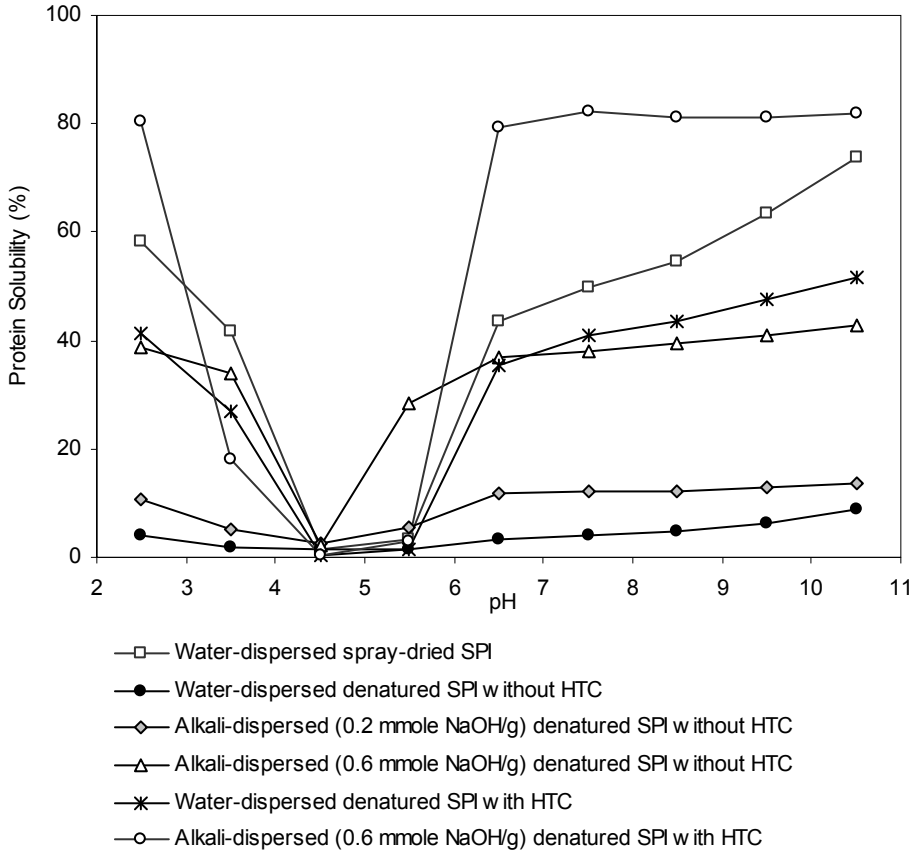

FIG. 3. Protein solubilities of heat-denatured SPI after different treatments.

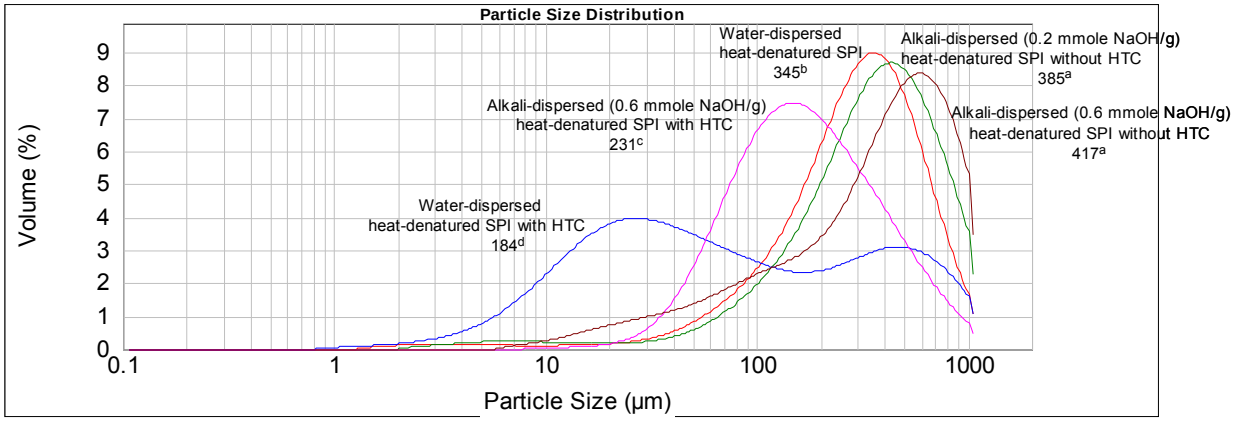

FIG. 4. Particle-size distributions and volumetric mean diameters of heat-denatured SPI after different alkali and HTC treatments. Means followed by the same letters are not significantly different ($P \leq 0.05$).

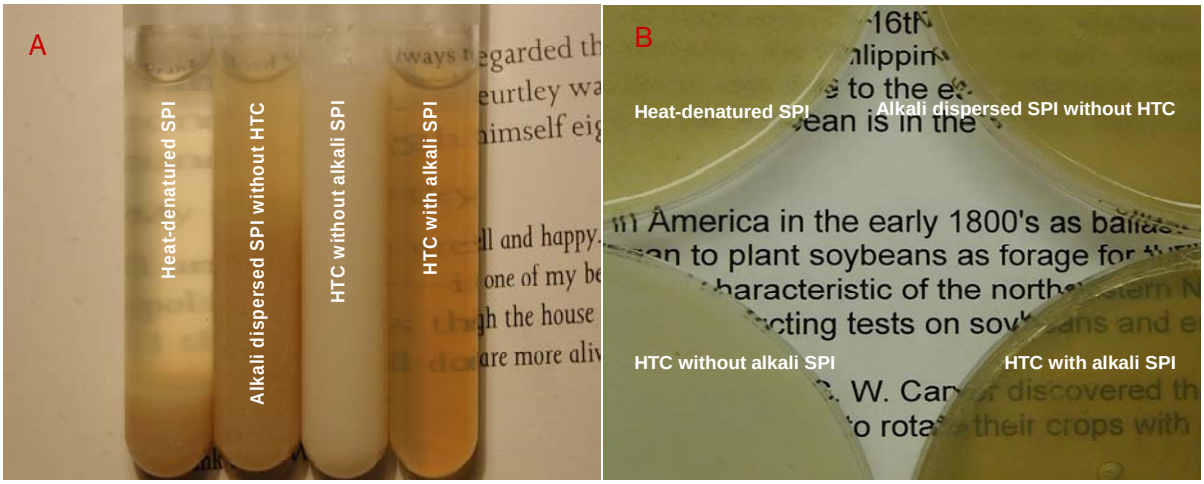

FIG. 5. Photographs of 10% dispersions of heat-denatured SPI after different treatments.

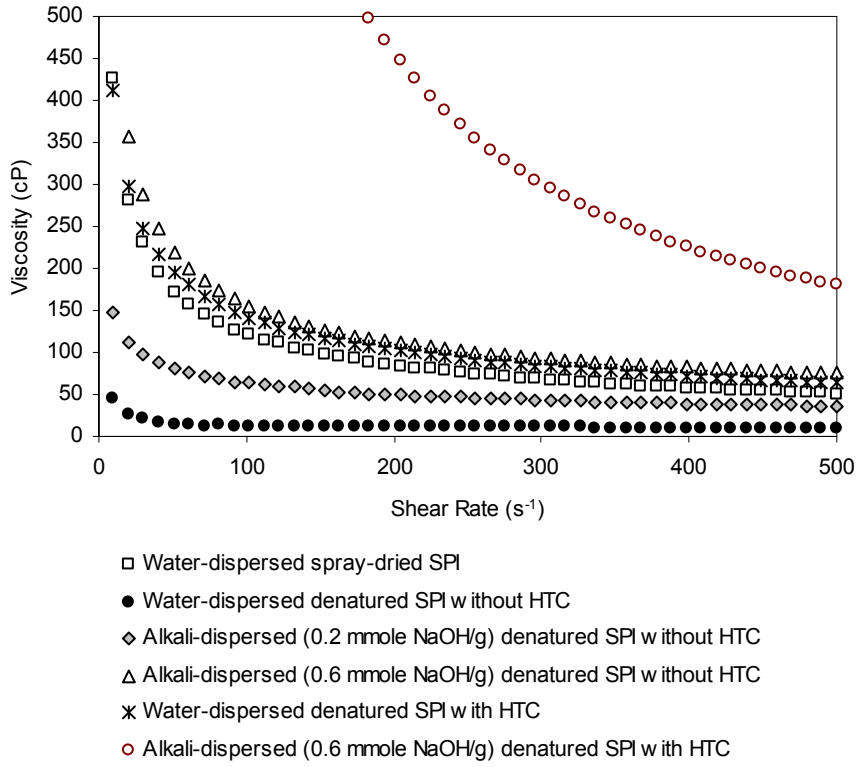

FIG. 6. Viscosity profiles of heat-denatured SPI after different treatments.

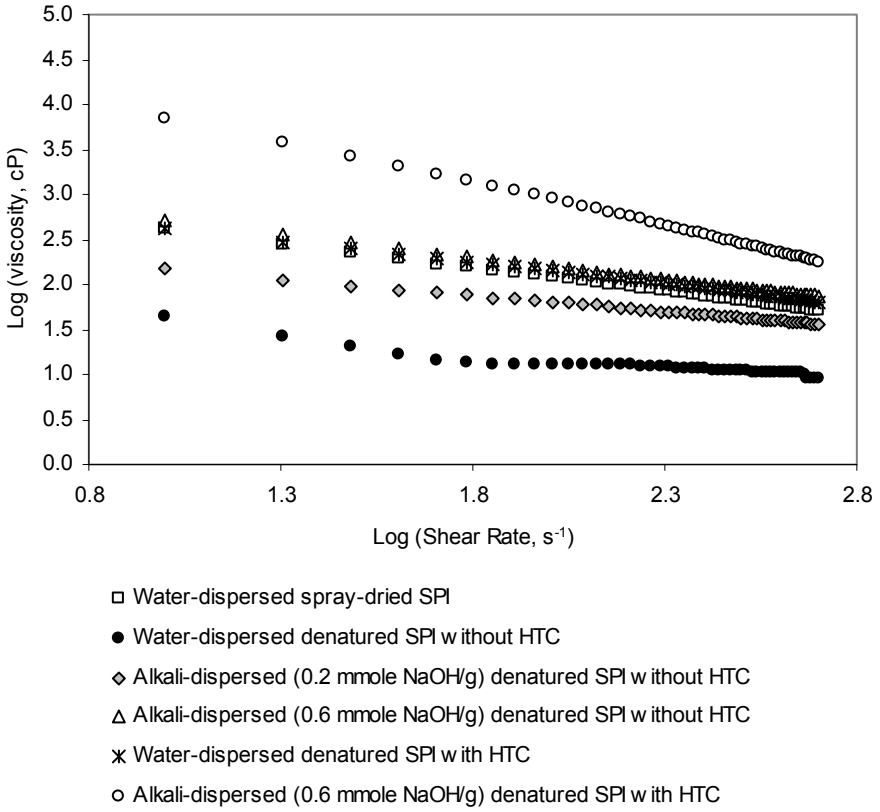

FIG. 7. Viscosity and shear rate relationships of heat-denatured SPI after different treatments.

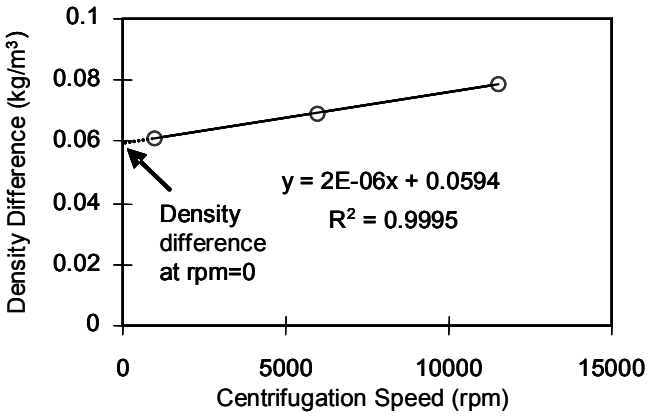

FIG. 8. Estimation of density difference under gravity using centrifugation method.

GENERAL SUMMARY

HTC with both setups (with-holding-tube and flash-out) significantly affected the major functionalities of EE protein meals. EE meals with lower PDI had greater improvement. When using the with-holding-tube setup at 154°C cooking temperature, most functional properties (especially solids and protein dispersibilities and emulsification capacity) improved by using short residence time (about 42 s). With longer cooking times, their functionalities tended to decrease but were still higher than those without HTC treatment. Only foaming capacity and stability of EE meal with PDI of 35 showed significant improvement. Flashing-out HTC had similar effects on refunctionalization of EE meals. For example, the solids dispersibility, protein dispersibility and emulsification capacity of EE meal with PDI of 35 were improved 2.0, 4.4 and 2.1 times after the flashing-out HTC treatment.

Both acid-washed and alcohol-washed SPC prepared from EE meals had high yield but low protein purity, presumably due to the higher residual oil content in EE meals compared to white flakes. Alcohol-washed SPC had lower functionalities compared with acid-washed SPC. The SPI prepared from EE meals had lower yield and protein purity than SPI prepared from white flakes. The protein purity was about 80%, which cannot be classified as commercial SPI, but the SPI from EE meal with PDI of 35 had similar or better functionalities than that prepared from white flakes. SPI yield increased as PDI of the starting materials increased and the SPI from EE meals had higher glycinin to β-conglycinin ratio than SPI from white flakes indicating that the extent of heat-denaturation during EE processing directly impacted the properties and protein extractabilities of EE meals. EE meals are suitable for preparing SPC.

Preparation of an SPI-like protein product from EE meal is feasible. The relatively low yield may be offset by the additional value resulting from identity-preserved EE processing.

Extraction of SPI after EE meal was refunctionalized by HTC at neutral pH had limited benefits; however, when alkali was added during HTC, refunctionalization was dramatically enhanced. Refunctionalzation increased with increasing alkali addition during HTC, but after adding 0.6 mmol alkali/g meal, the trend plateaued. When treated by HTC with alkali addition, the EE meal with PDI of 35 had much higher protein yield than EE meal without any treatment or with HTC without alkali addition. The more alkali added, the higher the SPI protein yield achieved from refunctionalized EE meals. The highest SPI protein yield was obtained at with-holding-HTC with 0.6 mmol alkali/g meal, which was 82% of the protein, a two-fold increase from that of the original EE meal, which was 40%.

The more alkali used, the higher foaming capacity and stability. Emulsification capacity of SPI prepared from alkaline-HTC-treated EE meal was slightly lower than that from original EE meal. One needs to bear in mind that SPI protein yield was more than doubled, to 82%, after alkaline-HTC, which means the majoritiy of heat-denatured protein in EE meal with PDI 35 was refunctionalized and recovered as SPI with emulsification capacity rival to the native proteins. SPI from alkaline-HTC-treated EE meal had higher yield (82 vs 73%) and higher emulsification capacity than SPI from white flakes (530 vs 500 g oil/g SPI).

Using extrusion heat-denatured SPI as a model, we showed that heat generated during extrusion caused soluble protein to form insoluble protein aggregates. Alkali-dispersing only (without HTC) dissolved a small portion of the denatured protein while the rest swelled. HTC without alkali disrupted the

large aggregates into smaller ones, resulting a milky homogenous slurry. The viscosity and total number of particles in the system increased dramatically. The most significant effect was achieved by HTC with alkali addition (0.6 mmol NaOH/g). The protein solubility profile showed that at neutral pH the solubility increased from 4% to about 80%, as a result the viscosity (at zero shear rate) increased by more than 1,000 times compared to the original denatured SPI. HTC with alkali dissolved most of the protein particles, the particle count decreased by almost 100 times. HTC and alkali had synergic effect on the refunctionalization of heat-denatured protein.

We concluded that SPI preparation from EE meals is technically feasible. HTC significantly improved the major functional properties of EE meals by disrupting the large heat-denatured protein aggregates into a stable suspension of smaller aggregates. Adding alkali during HTC can dramatically increase refunctionalization. Most of the protein particles dissolved. The treated samples can be used to make SPI-like soy protein products with high yield and good functionalities.

HTC was effective in refunctionalizing the heat-denatured EE meals. This technique can be used to produce highly functional EE meal or used as a pretreatment for extracting value-added soy protein products, such as SPI-like soy proteins with high protein content, good functionalities, and unique characteristics including non-GMO, all natural or other identity-preserved (IP) claims. Such products offer the food industry more choices to make specialty food products that health-conscious customers are constantly looking for.

Recommendations for future research

Fundamental research. Use purified glycinin and β-conglycinin proteins, pre-denatured by alcohol and heat, respectively, to investigate the behaviors of the individual proteins during HTC processing with or without alkali. The association/disassociation of subunits and different levels of protein structure changes under different conditions can be investigated by using techniques including ultracentrifugation, tryptophan fluorescence, and far- or near- Ultraviolet Circular Dichroism (CD). A micro-scale HTC system may be used in order to reduce the amount of glycinin and β-conglycinin.

Processing research. Food-grade ammonia can be used as alkaline agent in the HTC process. Ammonia is volatile, can be easily removed by spray-drying. Because there is no acid precipitation or neutralization, no additional salt will be introduced in the products nor will pH denaturation occur. Since this technique will not involve lowering the pH to the isoelectric point, no methanethiol will form, which was found to be only produced during acid precipitation and is one of the most potent odor compounds in commercial SPI (Boatright, W. L., Q.X. Lei, and C.J. Stine, Sulfite Formation in Isolated Soy Proteins, *J. Food Sci. 71*:C115–119, 2006). The resulting product should have milder flavor and better functionalities compared with existing commercial products. For example, ammonia-HTC can be used in SPI preparation. The process can start with alcohol-washed SPC. Right after ammonia-HTC, the fiber fraction in the slurry can be removed by centrifugation. The supernatant is spray-dried while hot. By coupling HTC to the spray-dryer, most of the energy used in HTC can be utilized. All the processing can be operated in a continuous, sealed system. Since HTC is used in the conventional acid-precipitation SPI production already, this technique can streamline the conventional process.

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