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Protein–Protein Interactions during High-Moisture Extrusion for Fibrous Meat Analogues and Comparison of Protein Solubility Methods Using Different Solvent Systems

KeShun Liu* and Fu-Hung Hsieh

Grain Chemistry and Utilization Laboratory, National Small Grains and Potato Germplasm Research Facility, Agricultural Research Service, U.S. Department of Agriculture, 1691 South 2700 West, Aberdeen, Idaho 83210, and Department of Food Science, University of Missouri, Columbia, Missouri 65211

Soy protein, mixed with gluten and starch, was extruded into fibrous meat analogues under high-moisture and high-temperature conditions. The protein solubility of samples collected at different extruder zones and extrudates made with different moistures was determined by 11 extraction solutions consisting of 6 selective reagents and their combinations: phosphate salts, urea, DTT, thiourea, Triton X-100, and CHAPS. Protein solubility by most extractants showed decreasing patterns as the material passed through the extruder, but the solution containing all 6 reagents, known as isoelectric focus (IEF) buffer, solubilized the highest levels and equal amounts of proteins in all samples, indicating that there are no other covalent bonds involved besides disulfide bonds. With regard to relative importance between disulfide bonds and non-covalent interactions, different conclusions could be made from protein solubility patterns, depending on the type of extracting systems and a baseline used for comparison. The observation points out pitfalls and limitation of current protein solubility methodology and explains why controversy exists in the literature. Using the IEF buffer system with omission of one or more selective reagents is considered to be the right methodology to conduct protein solubility study and thus recommended. Results obtained with this system indicate that disulfide bonding plays a more important role than non-covalent bonds in not only holding the rigid structure of extrudates but also forming fibrous texture. The sharpest decrease in protein solubility occurred when the mix passed through the intermediate section of the extruder barrel, indicating formation of new disulfide bonds during the stage of dramatic increase in both temperature and moisture. After this stage, although the physical form of the product might undergo change and fiber formation might occur as it passed through the cooling die, the chemical nature of the product did not change significantly.

KEYWORDS: Protein–protein interaction; protein solubility study; disulfide bonds; high moisture extrusion; soy protein

One promising and emerging technology for transforming vegetable proteins in general and soy proteins in particular into palatable and consumer-acceptable products is high-moisture (typically 50–80%) extrusion (*1*−*6*). The extrusion process uses a twin-screw extruder fitted with a long cooling die and produces a meat analogue having more resemblance to muscle food than low-moisture extrusion, such as thermoplastic extrusion, which produces expanded products that lack real fiber texture and need rehydration before use (*7*).

Yet, despite rapid development of extrusion technology in the past several decades, the way proteins interact during extrusion is poorly understood. Early work on protein–protein interactions during extrusion focused mainly on extrudates made by thermoplastic extrusion under low moisture content. Regardless of moisture levels, it has long been assumed that the protein is insolubilized and aggregated into a macroscopic structure due to molecular changes in the protein fraction. These changes are clearly complex, involving alteration of both covalent and non-covalent interactions (*7*−*9*).

With regard to relative importance of non-covalent interactions, intermolecular disulfide bonds, and possible formation of other covalent bonds for structural stabilization of extrudates, some controversy exists in the literature. Early work with spun soy fiber (*10*) pointed out action of hydrogen, ionic, and disulfide linkages. Later, Burgess and Stanley (*11*) and Smonsky and

* Author to whom correspondence should be addressed [e-mail keshun.liu@ars.usda.gov; telephone (208) 397-4162; fax (208) 397-4165].

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Stanley (*12*) discounted the involvement of disulfide bonding and suggested the formation of intermolecular peptide bonds during extrusion and its importance in forming the structure of extrudates. However, since then, many investigators (*9, 13−17*) have come to the conclusion that both disulfide bonds and noncovalent interactions are responsible for textural formation during extrusion and that there is lack of evidence of significant formation of intermolecular peptide bonds or other covalent bonds. The conclusion is based on a general observation that proteins could be resolubilized only from extruded products with extracting solutions containing an agent capable of destroying hydrogen bonds and hydrophobic interaction [such as sodium dodecyl sulfate (SDS), urea] plus an agent capable of disrupting disulfide linkages (such as 2-mercaptoethanol, dithiothreitol).

In a recent study (*6*), we examined the nature of protein–protein interactions in soy protein gels and extrudates of high-moisture extrusion by studying their protein solubility in selected extraction agents and found that the extrudates had a rather different solubility pattern compared to those of raw protein mixes and soy protein gels. The results supported the hypothesis that soy protein gels and extrudates both have the same types of chemical bonds, namely, covalent disulfide bonds and non-covalent interactions. It is the relative proportion of each type of bond in their structures that differentiates the two with respect to reversibility and structural rigidity. In forming protein gels during heat-induced gelation, non-covalent bonds play a dominant role over disulfide bonds, whereas in forming the fibrous structure of protein extrudates, non-covalent and divalent disulfide bonds are both important. However, like many studies conducted by previous investigators (*7, 9, 13, 14, 16, 17*), this recent study could not determine the relative importance of the two types of bonding in the extrudates or the stage of extrusion at which the chemical bonds undergo dramatic changes and formation.

A key reason for poor understanding of protein–protein interactions during extrusion is the inherent difficulty of studying the interactions. The most common approach is based on protein resolubilization by selective reagents with known mechanisms of protein solubilization (*9−11, 13−17*). The method is known as "protein solubility study". However, because of the complexity of chemical bonding responsible for protein structure, data interpretation based on a single or on dual reagents and comparison with a general salt buffer, as commonly practiced in the existing protein solubility methods, can be misleading. Furthermore, almost all of the studies compared only two sets of samples: raw mix and extruded products.

As a continuation of work on high-moisture extrusion, the present study was conducted, using protein solubility methods, with two sets of samples and three extracting systems. The first set consisted of samples collected at each extruder's zone, plus samples at the cooling die and final extrudate after a dead-stop run. Samples of the second set were extrudates made at three different moisture levels. The objectives were (1) to determine the relative importance between non-covalent bonding and covalent bonding in stabilizing extruded products and/or creating fiber structure, (2) to determine when these bonds form or change during extrusion, and (3) to compare protein solubility methods using different solvent systems. A study like this could shed some new light on the bonding nature of the protein–protein interactions that occur during food extrusion in general and high-moisture extrusion in particular. This could help clarify controversy in the literature, optimize production processes, and improve end product quality.

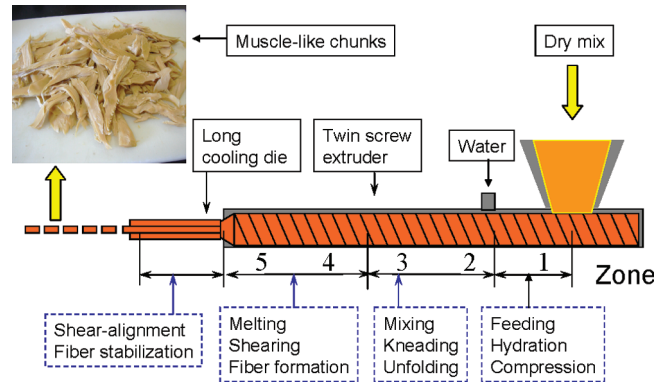

Figure 1. Scheme of a twin-screw extruder for high-moisture extrusion of proteinaceous materials into fibrous meat analogues.

MATERIALS AND METHODS

Chemicals. Urea, thiourea, Triton X-100, KH_2PO_4, and K_2HPO_4 were purchased from Fisher Scientific (Pittsburgh, PA). Dithiothreitol (DTT) and CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] were products of Sigma Chemical Co. (St. Louis, MO).

High-Moisture Extrusion. The material used for extrusion, the extruder, and extrusion conditions were described in detail in the previous publication (*5*), using the extruder and high-moisture extrusion process shown in **Figure 1**. Basically, we used a pilot-scale, corotating, intermeshing, twin-screw food extruder (MPF 50/25, APV Baker Inc., Grand Rapids, MI) with a smooth barrel and a length/diameter ratio of 15:1. At the end of the extruder, a long cooling die with a dimension of 60 × 10 × 300 mm (W × H × L) was attached. The clamshell style barrel is segmented into five temperature-controlled zones that are heated by an electric cartridge heating system. The barrel can be split horizontally and opened to enable rapid removal and cleaning of the barrel and the screws. The raw material consisted of soy protein, wheat gluten, and unmodified wheat starch in a weight ratio of 60:40:5. The extrusion temperature was kept at 170 °C, and three moisture levels were tested: 72.12, 66.78, and 60.11%.

The clamshell style of the extruder allowed us to run a dead-stop operation, which was conducted at the end of a run at the moisture level of 60.11%. At this moisture, products with well-defined fibrous structures were produced under the described extrusion conditions. The extrusion operation was intentionally shut down (dead-stop) after reaching steady state. The barrel was cooled using the maximum cooling capacity and opened immediately, and samples along the extruder barrel at each of the five zones and the cooling die and the extruded product were collected. The sample from zone 1 corresponded to the raw mixture. Zone 5 was the last zone adjacent to the cooling die (**Figure 1**).

Samples, 0.5–1 kg each, were collected for each treatment and zone section and immediately put into airtight plastic bags. Bags of samples were kept in a refrigerator at 4 °C until measurement. Duplicate samples were made for each treatment or zone section.

Design of Extracting Systems and Determination of Protein Solubility. A total of six types of selected reagents for protein solubility study were used: phosphate salts, urea, thiourea, DTT, Triton X-100, and CHAPS. The base extracting solution was 100 mM phosphate buffer (PB), pH 7.5, which is generally known to extract protein in its native state. Each of the remaining reagents, singly and in combination, was prepared with the phosphate buffer. If all of the above reagents are added together, the solution is known as isoelectric focus (IEF) buffer. This buffer is often used in proteomic studies for protein extraction. A total of 11 extracting solutions were made. They were categorized into three systems for easy comparison and discussion (**Table 1**). Each system 1 solvent contained only one reagent dissolved in the PB. Each system 2 solution contained two or more reagents dissolved in PB. System 3 solutions contained more than one reagent dissolved in PB, consisting of IEF and subtraction of one or more reagents from it.

Table 1. Eleven Extracting Solutions with Selective Reagents and Their Combinations for Protein Solubility Study of Samples of Dead-Stop Runs and Extrudates at Different Moisture Levels[a]

no.	solvent system	extracting solution	100 mM phosphate buffer, pH 7.5	8 M urea	50 mM DTT	2 M thiourea	2% TritonX-100	2% CHAPS
1	1A	phosphate buffer (PB)	X					
2		PB + urea	X	X				
3		PB + DTT	X		X			
4	1B	PB + thiourea	X			X		
5		PB + Triton	X				X	
6		PB + CHAPS	X					X
1	2	PB	X	X	X			
7		PB + urea + DTT[b]	X	X	X			
8		PB + thiourea + Triton + CHAPS[c]	X			X	X	X
9	3	isoelectric focus buffer (IEF)	X	X	X	X	X	X
10		IEF w/o urea	X		X	X	X	X
11		IEF w/o DTT	X	X		X	X	X
8		IEF w/o urea and DTT[c]	X			X	X	X
7		IEF w/o thiourea, Triton, and CHAPS[b]	X	X	X			
1		PB	X					

[a] PB, phosphate buffer; IEF, isoelectric focus; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. [b] PB + urea + DTT was the same as IEF w/o thiourea, Triton, and CHAPS. [c] PB + thiourea + Triton + CHAPS was the same as IEF w/o urea and DTT. However, for easy comparison within each solvent system, we used different names.

Table 2. Types of Interactions, Amino Acids Capable of Engaging in Them, and Reagents Able To Break the Interactions[a]

type of interaction	specific interaction	amino acids	reagents capable of breaking up the interaction
covalent	disulfide bonding	cysteine/cystine	oxidizing or reducing agents, e.g., performic acid, 2-mercaptoethanol, DTT, Na_2SO_3
non-covalent neutral	hydrogen bonding	asparagine, glutamine, threonine, serine, cysteine	strong H-bonding agents, e.g., urea, dimethyl formamide, thiourea, SDS
non-covalent neutral	hydrophobic interaction	tyrosine, tryptophan, phenylalanine, proline, methionine, leucine, isoleucine, valine, alanine, glycine	ionic and nonionic detergents, e.g., SDS, thiourea, Triton, CHAPS, sodium salts of long-chain fatty acids
non-covalent electrostatic	acid hydrophilic basic hydrophilic	aspartic acid, lysine, arginine, histidine, glutamic acid	acids, alkali or salt solutions

[a] SDS, sodium dodecyl sulfate; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Each solvent was used to extract proteins from two sets of samples. The first set consisted of samples collected from each zone of a dead-stop operation at moisture of 60.11%, which had been shown to produce extrudates with the best fiber formation (*5*). The second set consisted of extrudates obtained by extrusion under three different moisture contents: 72.12, 66.78, and 60.11%. Extraction was carried out at room temperature (about 23 °C) by using a home kitchen blender (Osterizer, 12 speeds, Sunbeam Products, Inc., Boca Raton, FL). Sample weight ranged from 0.5 to 3.0 g, whereas the extractant volume was kept at 50 mL. After blending at the highest speed for 3 min, the dispersion was centrifuged at 16000*g* for 15 min (Beckman J2-21 M/E centrifuge, using a JA-20 rotor, Schaumburg, IL). Solubilized protein in the supernatant was determined by a protein test kit, Coomassie Plus from Pierce (Rockford, IL). The total protein content in the original samples was measured by a combustion method based on an official method (*18*), using a protein analyzer (model FP-428, Leco Corp., St. Joseph, MI). Samples were measured for moisture content using a vacuum oven, based on an official method (*18*). The value was used for correcting to dry matter basis for protein contents. Duplicate analysis was carried out for protein extraction and moisture measurement for each sample. The final results were averaged, and standard deviation was calculated on the basis of four data points for each treatment.

Data Treatments and Statistical Analysis. Data were treated with the JMP software, version 5 (JMP, a business unit of SAS, Cary, NC) for calculating means and standard deviation and for analysis of variance to determine the effect of reagent(s), zone at the extruder barrel, product moisture, and their interactions on protein solubility. Tukey's HSD (honestly significant difference) test was also conducted for pair comparison.

RESULTS AND DISCUSSION

Basic Protein Chemistry and Actions of Selected Reagents. Although an enormous range of proteins exist in nature, they are all composed of the same relatively simple units: about 20 amino acids. The diversity of proteins comes about because the amino acids are arranged in different sequences, and those sequences are of different lengths. All amino acids have in common the presence of an α-amino group ($-NH_2$) and a carboxyl group ($-COOH$). Condensation of these groups is through formation of peptide bonds, leading to creation of polypeptides. The differences among amino acids lie in the side chains attached to the carbon atom between their carboxyl and amino groups. Side chains can be classified according to their capacity for interacting with other amino acids by different mechanisms. **Table 2** lists types of interactions, the amino acids capable of engaging in them, and reagents able to break the interactions.

Most previous studies on protein solubility of extrudates utilized three types of extraction reagents. The first type is a general salt buffer, such as phosphate buffer, which can extract protein only in its native state. The second type of reagent, such as urea or SDS, is known to break non-covalent interactions. The third type is a reducing agent, such as DTT or 2-mercaptoethanol, which breaks disulfide bonds. In this study, phosphate salts, urea, and DTT were selected to represent the three types of reagents, respectively. Furthermore, three additional reagents, thiourea, Triton X-100, and CHAPS, were used to further

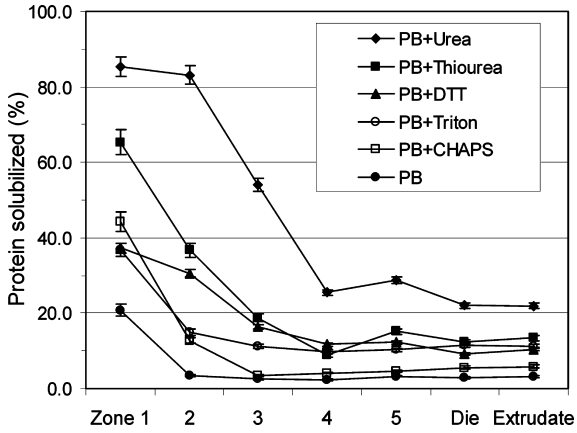

Figure 2. Protein solubilized by system 1 extracting solutions (refer to **Table 1**) from the samples collected at different zones of the extruder. PB, phosphate buffer; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

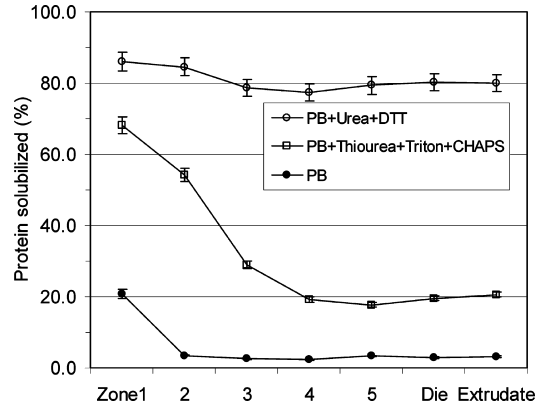

Figure 3. Protein solubilized by system 2 extracting solutions (refer to **Table 1**) from the samples collected at different zones of the extruder. PB, phosphate buffer; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

improve protein solubility. Urea and thiourea are both known to break non-covalent interactions, but substituted urea is more efficient than urea in breaking hydrophobic interaction, whereas urea is more efficient in breaking hydrogen bonds. Triton X-100 and CHAPS are zwitterionic and nonionic detergents, respectively. They also disrupt hydrophobic bonds. Uses of these reagents could help differentiate the relative importance among non-covalent interactions.

Protein Solubility in System 1 Solvents. Each system 1 solution contained only one reagent dissolved in PB. Furthermore, system 1 solutions could be further categorized into A and B types. System 1A refers to PB containing either urea or DTT, whereas system 1B refers to PB containing either thiourea, Triton X-100, or CHAPS (**Table 1**). As the raw mix passed through zone 1 to zone 5, the die, and ultimately was extruded as the final product, its temperature increased from room temperature to about 170 °C, its moisture content increased from about 8% in the premix to over 60% (*5*), and the amount of protein solubilized by all of the system 1 extracting solutions decreased (**Figure 2**). Among all of the extraction solutions, the PB solubilized the least amount of proteins in all zone samples tested. Also, using PB, there was a sharp decrease in the solubility value between zone 1 and zone 2 samples, with the rest of the zone samples remaining the same. Because PB could solubilize protein only in its native state, these observations indicate that the proteins in the raw mix were vastly denatured during their commercial preparation and that a small amount of proteins that remained in native state could be readily denatured as the raw mix passed through the extruder.

The highest solubility and the sharpest decreasing rate as the premix material passed through the extruder were associated with the PB + urea solvent (**Figure 2**). Slightly over 80% of protein could be extracted with this solvent from samples in zones 1 and 2. The sharpest decrease was with the samples from zones 3 and 4, reaching about 25% protein extraction level. This level was maintained throughout the remaining extrusion process. The curve immediately below the PB + urea curve resulted from PB + thiourea. The value decreased from about 65% at zone 1 to about 10% at zone 4, and again this level was maintained for the remaining process. The three curves between the PB curve and the PB + thiourea curve corresponded to solutions containing DTT, Triton, or CHAPS, respectively. They also decreased to a lower level within the first two to three zones and maintained the lower level throughout the remaining process. Thus, with a single reagent, the solubility for all of the system 1 solutions was rather low (<25%) for samples collected at zone 4 and beyond. In other words, when compared with the solubility curve of PB, adding any of the other five reagents (including DTT) only slightly improved solubility in samples collected at zone 4 and thereafter.

Data from system 1 solutions (**Figure 2**) supported a common belief that in order to form a rigid extrudate structure (not necessarily a fibrous structure), proteins have to be insolubilized during extrusion. The data also provide some information regarding which type of bond is important in forming the rigid structure. Using the PB curve as the baseline for comparison, it was observed that adding either urea or DTT improved protein solubility, but adding urea caused a little higher solubility than adding DTT. This leads us to believe that although both covalent disulfide bonds and non-covalent bonds are responsible for the formation of aggregate and/or fibrous structure in final extrudates, the non-covalent interactions appear to play a more important role.

Protein Solubility in System 2 Solvents. Unlike system 1 solutions, system 2 solutions contained two or more combined reagents dissolved in PB. When urea and DTT were both present, there was an overwhelming increase in protein solubility for all of the samples, as compared with that in PB (**Figure 3**). The curve was basically flat (little change) from zone 1 to the final extrudate. By comparison with **Figure 2**, clearly, the increase in protein solubility values was far beyond an additive effect of the two agents. There was a significant synergic effect of the two reagents ($p < 0.05$). In contrast, when thiourea, Triton, and CHAPS were present, the increase in protein solubility appeared to result from their additive action (**Figures 2** and **3**). No synergic effect was observed for these three reagents. There was a decrease in protein solubility by this solvent as the material passed through zone 1 to zone 4 of the extruder.

Many previous investigators used a combination of two types of reagents, one to break disulfide bonds and one to break non-covalent interactions, in their protein solubility studies and found a similar synergic effect. Jeunink and Cheftel (*13*) reported that extraction with the SDS-containing buffer solubilized 45 and 14% of the proteins of the initial and the extruded concentrates, respectively, whereas extraction with the buffer containing SDS plus DTT solubilized 73 and 79% of the proteins from the two samples, respectively. Hagar (*14*) found that for the extrudate of soy concentrate by thermoplastic extrusion under conditions specified in his study all but 2–4% of the protein could be solubilized by using a solvent containing urea and a disulfide

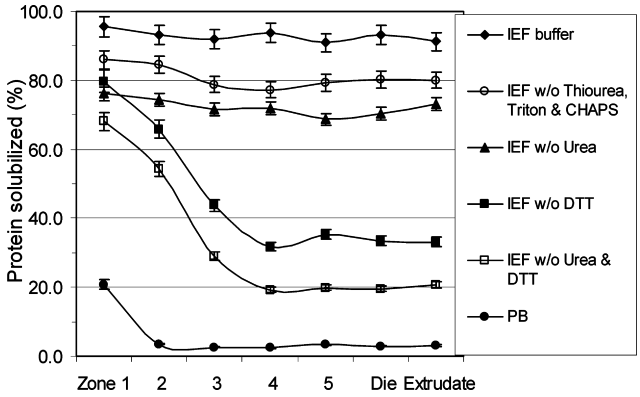

Figure 4. Protein solubilized by system 3 extracting solutions (refer to **Table 1**) from the samples collected at different zones of the extruder. IEF, isoelectric focus; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PB, phosphate buffer.

cleaving reagent, Na_2SO_3. This amount of total insoluble material was the same for extruded soy concentrate as for the unprocessed soy concentrate. He reasoned that the major forces responsible for insolubilization and rigid structure in the extrudate samples appeared to be hydrophobic interactions, hydrogen bonding, and covalent intermolecular disulfide bridges. It was unnecessary to involve other covalent interactions to explain the structure of soy protein extrudates. Aqueous solutions containing SDS and 2-mercaptoethanol were also found to almost completely dissolve protein in extruded soy protein products (*16*) as well as wheat products (*17*). On the basis of these observations, a common conclusion was that proteins aggregated primarily through nonspecific hydrophobic interaction and disulfide bond formation during extrusion.

The current observation with system 2 solutions once again confirmed the findings of these previous investigators. The difference was that the current study measured samples at different zones in the extruder, whereas all of the previous studies used only two types of samples: an initial raw material and a final extrudate. Regardless of this difference, data in **Figure 3** can only indicate that both types of bonding, disulfide bonds and non-covalent interactions, are important. Like previous studies (*5, 13, 14, 16, 17*), the present study with system 2 solutions could not determine the relative importance between non-covalent linkages and covalent disulfide bonds and the relative importance within the non-covalent interactions.

Protein Solubility in System 3 Solvents. For determining which type of bonding is more important during extrusion, system 3 extracting solutions (refer to **Table 1**) were made. The system 3 solutions were characterized by subtracting one or more reagents from an IEF buffer. With this system, data interpretation is based on comparison with the IEF buffer instead of a general salt buffer, such as PB, used by all of the previous investigators. This unique selection of extracting systems and unique data interpretation, in combination with the use of two sets of samples (samples collected at different zones and extrudate samples obtained under different moisture levels) turned out to be a rather important improvement for using protein solubility study to understand the true nature of protein–protein interaction in extruded protein products.

IEF buffer was a phosphate buffer containing all of the system 1A and system 1B reagents. It is commonly used in proteomic study and known to collectively break up all of the non-covalent bonds as well as disulfide bonds. Indeed, as shown in **Figure 4**, the IEF buffer not only solubilized the highest amount of proteins from all of the samples but also extracted essentially the same amount of proteins in all of the samples tested regardless of levels of temperature exposure history. On the basis of this observation, it can be concluded that the chemical bonding responsible for the rigid structure of protein extrudates is limited only to covalent disulfide bonds and non-covalent interactions. No other covalent bonding formed during high-moisture extrusion.

The curve immediately below the IEF buffer curve represents protein solubility by the IEF buffer without thiourea, Triton, and CHAPS, which is the same solution as PB + urea + DTT. Like the IEF buffer curve, it remained essentially flat for all of the samples as the product passed through the extruder. There was a constant difference in protein solubility between this curve and the IEF buffer curve. Because thiourea, Triton, and CHAPS are all known to break hydrophobic interactions, this constant difference would reflect the role of hydrophobic interactions, which appeared to remain constant during extrusion. The difference in protein solubility between the IEF curve and the IEF without urea curve was also constant. Because urea is known to break mainly H-bonds, we can reason that like hydrophobic interactions, H-bonding plays a role in holding protein secondary and tertiary structures, but it also remains unchanged during extrusion. This is in sharp contrast with the earlier conclusion based on **Figure 2** data, where urea was present as a single agent in PB, that non-covalent interactions, particularly H-bonding, play a major role in forming rigid texture of extrudates.

The IEF without DTT curve and the IEF without urea and DTT curve in **Figure 4** exhibited the sharpest decreases. Clearly, these additional data allow us to infer that the most important linkage that leads to insolubility, integrity of extrudate structure, and fibrous formation is disulfide bonding. The difference between these two curves resulted from the addition of urea and thus reflects the important but relatively more minor role of hydrogen bonding. Again, it was observed that the difference was constant, similar to the constant difference between the IEF buffer curve and the IEF without urea curve in the same figure. This further supports the notion that H-bonding does not increase as a result of extrusion. The bottom curve resulted from PB extraction. Its difference from the IEF buffer curve was largest and remained constant for all of the samples except the raw mix. This largest difference reflects the combined effect of all non-covalent bonds plus disulfide bond. As discussed before, the decrease in protein solubility by PB from zone 1 to zone 2 indicates that a small amount of native protein in the original protein mix underwent rapid denaturation as the moisture and temperature started to rise during the extrusion.

Changes of Bonding Nature during Extrusion. Figures 2−4 also address such a question as when chemical bonds underwent dramatic changes or formations during extrusion. For all of the curves that show decreasing patterns, the period of the sharpest decrease occurred when the mix passed from zone 1 to zone 4. This was also the time when both moisture and temperature increased rapidly. After passing zone 4, there were little change in protein solubility at late stages, even when the material passed through the cooling die. In the previous paper (*5*), based on visual examination and digital imaging of the samples collected at different barrel zones after the dead-stop run at 60.11% moisture content, fiber formation did not occur until the last zone of the extruder barrel (zone 5), and it became complete as the material entered and traveled through the cooling die. However, on the basis of protein solubility data in this study, during this late stage, although the physical form underwent change and fiber formation might occur, the chemical nature

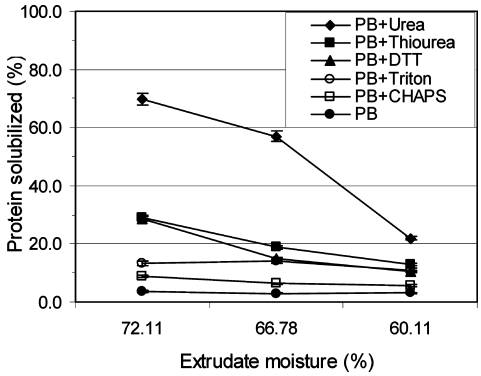

Figure 5. Protein solubilized by system 1 extracting solutions (refer to **Table 1**) from extrudates made with different moistures. PB, phosphate buffer; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

of the product did not change significantly. The current observation also indicates that the chemical reactions induced by high temperature and high moisture were instant, and there appeared to be no latent changes once the product reached and passed through the high-temperature zone.

Effect of Moisture Levels and Bonding Nature for Fibrous Formation. In the previous publication (*5*), a twin-screw extruder fitted with a long cooling die was used to extrude a mixture of soy protein isolate, wheat gluten, and starch at a barrel temperature of 170 °C with three levels of moisture: 72.12, 66.78, and 60.11%. It was found that among the products extruded under different levels of moisture, only the one extruded at the 60.11% moisture level produced a product having well-defined fiber orientation (**Figure 1**).

In the present study, for determining the effect of moisture levels on protein–protein interaction and its relationship to fiber formation, the 11 solutions in **Table 1** were also used to extract extrudates made at three different moisture levels. Note that only the extrudate made at a moisture content of 60.11% showed well-defined fibrous structure. Extrudates at the other two moisture levels showed less well-defined fibrous structure but were rigid enough to hold a chunk-type of structure. For the extrudate samples made at decreasing moisture contents, the protein solubilized by six system 1 solvents decreased (**Figure 5**) and the degree of fiber formation increased. Like the samples collected at different zones of the extruder barrel (**Figure 2**), the solubility value and the solubility decrease were both highest by using PB + urea as the extractant. This was followed by PB + thiourea and then PB + DTT, both exhibiting a similar decreasing pattern. PB + Triton and PB + CHAPS showed lowest solubility values and smallest solubility decreases. Data in **Figure 5** imply that for fiber formation further insolubilization of proteins is needed.

Also, like the observation with the samples collected at different zones, PB + urea + DTT exhibited a synergic effect for the three extrudate samples (**Figure 6**). However, when system 3 solutions were used, all curves were basically flat except for the two curves that corresponded to IEF buffer without DTT and IEF buffer without urea and DTT (**Figure 7**). These two curves decreased with decreasing extrudate moisture at similar rate, making them almost parallel. Interestingly, solubility values for the extrudate extruded at 72.11% by the two solutions were still much lower than those of the premix (zone 1 sample in **Figure 4**), 57 versus 80% for IEF without DTT solvent and 52 versus 68% for IEF without urea and DTT, indicating that formation of disulfide bonds in this sample occurred even though it did not have fibrous structure.

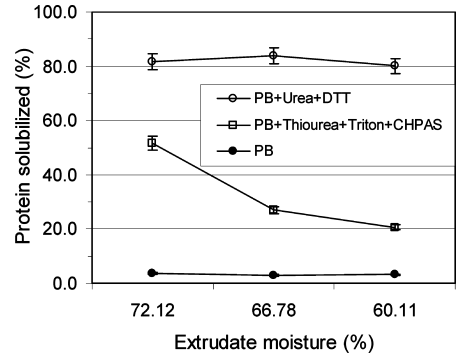

Figure 6. Protein solubilized by system 2 extracting solutions (refer to **Table 1**) from extrudates made with different moistures. PB, phosphate buffer; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

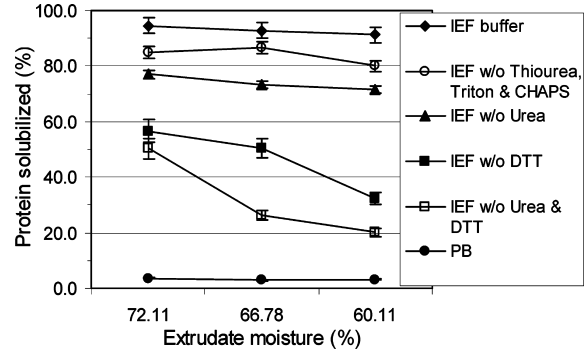

Figure 7. Protein solubilized by system 1 extracting solutions (refer to **Table 1**) from extrudates made with different moistures. IEF, isoelectric focus; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PB, phosphate buffer.

From all of these data, we can reason that disulfide bonding plays a more important role than non-covalent interactions in forming not only rigid structure but also the fibrous texture of final extrudates. Furthermore, to form a fibrous structure, additional formation of disulfide bonds is needed, as compared to the formation of a rigid and chunk-type structure.

Comparison of Protein Solubility Methods Using Different Solvent Systems. On the basis of the above observations on protein solubility studies with three solvent systems, with regard to relative importance between disulfide bonds and non-covalent interactions for holding the rigid structure and/or forming fibrous texture of extrudates, different conclusions could be made from protein solubility patterns, depending on the type of extracting systems and the baseline used for comparison. By using a single reagent system and the PB curve as a comparison baseline, urea gave the sharpest decreasing curve as well as the highest value in protein solubility, as compared with DTT and other single reagents (**Figures 2**, **5**). This observation would lead us to believe that non-covalent interactions are more important than disulfide bonding. Yet, when a solution containing both urea and DTT was used, and again compared with the PB curve, an overwhelming increase in protein solubility was observed (**Figures 3**, **6**). There was a synergic effect between the two (urea and DTT). The pattern leads us to believe that covalent disulfide bonds and non-covalent interactions are both important. Further work based on the IEF buffer system with omission of one or more reagents helped to unravel the synergic effect and gave changing patterns that were easier to interpret when the IEF buffer curve was used as a comparison baseline instead of the PB curve (**Figures 4**, **7**). Results indicate that disulfide bonding is more important for holding the rigid structure and/

or forming fibrous texture of extrudates. There were virtually no changes in non-covalent bonding (both hydrogen bond and hydrophobic interactions) during extrusion.

It is believed that the method based on the IEF buffer system and data interpretation based on comparison with the IEF buffer instead of a general salt buffer are the right ones to conduct protein solubility study. Therefore, the last conclusion drawn from the protein solubility data using system 3 solutions is upheld; that is, although non-covalent bonding has important roles in forming protein secondary and tertiary structures, it is disulfide bonding that plays a primary role in not only holding the rigid structure but also forming the fibrous texture of extrudates. This new conclusion is supported by an early patent reporting textural improvement of extruded soy proteins with the addition of elemental sulfur or sulfur-containing adjuncts during extrusion (*19*).

Furthermore, the above observations point out pitfalls and limitation of protein solubility studies conducted by many previous researchers, which have used single or dual reagent systems and made comparison with a general buffer solution. The pitfall of the protein solubility methodology, as shown in this study, is that data interpretation actually depends on extraction systems and a baseline used for comparison. The limitation of the existing protein solubility methods based on single or dual reagent systems for investigating protein–protein interactions in extruded products is that it cannot differentiate the roles between non-covalent bonds and disulfide bonds (*5, 13, 14, 16, 17*). The observations also explains why controversy exists in the literature regarding protein–protein interactions during extrusion.

Finally, on the basis of the findings of this study, in designing a protein solubility study of a food or nonfood system, the following procedure is strongly recommended: (1) start with a buffered solution that contains all of the reagents to break all of the possible bonds, (2) develop other extracting solutions by subtracting one or more reagents from this all-dissolving buffered solution, and (3) compare solubility values of other extractants with that of the all-dissolving buffered solution. This recommended protein solubility methodology is suitable for the evaluation of protein–protein interactions not only in extruded products but also in other processed products, such as muscle foods, surimi-based products, tofu, cheese, and gels.

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