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Heat-Induced Soy−Whey Proteins Interactions: Formation of Soluble and Insoluble Protein Complexes

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The aggregation behavior during heating of a solution containing soy protein and whey protein isolate (WPI) was studied using rheology, confocal microscopy, gel filtration, and electrophoresis. Soy/WPI mixtures formed gels at 6% total protein concentration with a high elastic modulus (G′) and no apparent phase separation. The ratio of soy to WPI was fundamental in determining the type of network formed. Systems containing a high soy to WPI ratio (>70% soy protein) showed a different evolution of the elastic modulus during heat treatment, with two apparent stages of network development. Whey proteins formed disulfide bridges with soy proteins during heating, and at low ratios of soy/WPI, the aggregates seemed to be predominantly formed by 7S, the basic subunits of 11S and β-lactoglobulin. Size exclusion chromatography indicated the presence of high molecular weight soluble complexes in mixtures containing high soy/WPI ratios. Results presented are the first evidence of interactions between soy proteins and whey proteins and show the potential for the creation of a new group of functional ingredients.

KEYWORDS: Soy proteins; milk proteins; aggregation

INTRODUCTION

In the 1970s, as a response to the increasing cost of and demand for traditional animal proteins, there was a growing interest in the utilization of soy proteins in food products. Soy protein isolates were added to replace nonfat dry milk, stabilizers, and sodium caseinate in the production of yogurt. Soy protein isolates were also used in the manufacture of coffee creamers, whipped toppings, and infant formulas to replace, totally or partially, milk proteins (*1*). However, technical challenges, especially the low functionality of the commercially available products and the beany flavor, limited the development of new beverage type products. Presently, there is a renewed interest in the study of soy protein functionality, in part because of the increased availability of good quality soy protein ingredients and, perhaps more importantly, because of the increased consumer demand for novel products containing soy proteins. This demand is at least in part caused by the health claims related to the consumption of products containing sufficient amounts of soy protein.

A limited number of studies are available on the interactions of soy proteins in mixed protein systems. In particular, the functional properties of soy proteins and milk proteins have been studied mainly from a product/process development prospective, for example, in comparative studies of milk-based and soy protein-based yogurt type products (*2*). Very little research has been reported on the characterization of the complexes formed between soy and milk proteins during processing of foods. A study of rennetting of milk in the presence of soy protein suggested that soy proteins may adsorb onto casein micelles or be entrapped within the casein micelle network, interfering with curd formation and causing a decrease in gel strength (*3*).

In mixed soy and milk protein systems, various types of aggregates may form during heating; however, the mechanisms of formation of such aggregates are quite unclear. The disagreements that currently exist in the literature can be mainly attributed to differences in the sources and the processing histories of the soy proteins employed in the various studies. Chronakis and Kasapis (*4*) studied the rheological properties of milk−soy protein gels using a commercial soy protein isolate (90% protein content). The isolate contained large molecular weight fractions and had low solubility, as a result of processing. In this system, phase inversion of the proteins was observed and the structure of the gels depended on the protein ratio: high amounts of soy (>11%) formed a continuous network, while at low soy−milk ratios, milk proteins formed a network containing soy protein inclusions. Comfort and Howell (*5*) also observed phase separation during heating at certain concentrations and ratios in soy protein mixes. This phase separation may have taken place because of the processing history of the protein. An interesting observation was that small amounts of soy protein added to whey protein result in an increase in gel strength.

Although phase separation of mixed soy/dairy protein has been shown during heating, no phase separation occurs in mixed gels prepared with glucono-δ-lactone (*6*). The addition of soy protein to skim milk changes the microstructure of the acid gels; the resulting gel network is less branched and more particulate than that formed by milk proteins alone. In addition, there is an increase in gel strength and pH onset of gelation when a small amount of skim milk is replaced with soy protein (*6*).

Heating of soy protein with milk protein forms large size aggregates (*6*). The aggregates could form via disulfide bridging

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between soy, whey proteins, and some of the caseins or via other noncovalent interactions occurring during heating. During acidification, these aggregates are incorporated in the protein network.

A better understanding of the formation of aggregates between soy proteins and milk proteins is needed to be able to optimize the texture and stability of food products containing both protein systems. The mechanism of aggregate formation and the types of interactions need to be further investigated. Understanding when milk proteins form aggregates with soy proteins may facilitate the design of a wide range of novel functional ingredients. The present research investigated the aggregation behavior of a mixed solution of soy and whey proteins and the formation of soluble and insoluble protein complexes.

MATERIALS AND METHODS

Protein Preparations. Soy protein was donated by the Solae Company (Alpha 5800, Solae Company Inc., St. Louis, MI). This protein was selected because of its relatively mild processing history. The protein was suspended in MilliQ water, stirred for 2 h, and stored overnight at 4 °C to allow complete hydration. The protein was dialyzed (Mw cut off 6000−8000, Fisher Scientific, Mississauga, Ontario, Canada) against high purity water for 24 h at 4 °C with six consecutive changes of water. After dialysis, the protein preparation was freeze-dried. The resulting protein preparation contained 84% (w/w) total protein (based on nitrogen content Dumas combustion method; Leco FP-528 Mississauga, ON, Canada). Whey protein isolate (WPI) was donated by Land O'Lakes (St. Paul, MN) and used without further purification.

The freeze-dried soy protein preparation and WPI were suspended (approximately 10% w/v) in MilliQ water containing 0.1 M NaCl and stirred for at least 2 h (the soy protein was dispersed with a hand held homogenizer, PowerGen 125, Fisher Scientific). The protein suspensions were adjusted to pH 7 using 1 M NaOH and then stored overnight at 4 °C to ensure complete hydration. Samples were brought to a room temperature and centrifuged at 8000*g* for 20 min at 23 °C with a temperature-controlled ultracentrifuge (Optima LE-80K Beckman Coulter TM, Mississauga, ON, Canada). The supernatants were collected, the total protein concentration was determined by DC protein assay (Bio-Rad, Mississauga, ON, Canada), and mixtures of the proteins were then prepared to the final concentration required.

Rheological Measurements. Mixed protein solutions were tested at a final protein concentration of 6% (w/v). Solutions containing soy to WPI ratios of 90/10, 70/30, 50/50, 30/70, and 10/90 were prepared, as well as controls containing 6% soy (100/0) and 6% WPI (0/100).

To determine the differences in the viscoelastic behavior of the mixtures during heating, measurements of the changes in storage (G′) and loss (G″) moduli over time and temperature were carried out using a controlled stress rheometer (AR 2000, TA instruments, United Kingdom). Measurements were performed using a concentric cylinder geometry (sample size was 5.25 mL) at a constant maximum strain of 0.01, an angular frequency of 0.63 rad s^{-1} (0.1 Hz), and a gap of 2 mm.

To prevent evaporation of the sample, a thin layer of mineral oil (around 0.5 mL) was put on the top of the sample. To induce gel formation, the mixtures were consecutively heated from 30 to 90 °C at a heating rate of 1 °C/min, kept at 90 °C for 1 h, and then cooled to 30 °C at a cooling rate of 1 °C/min. At the final temperature, a frequency sweep test was performed with a constant applied stress of 10 Pa (within the linear viscoelastic range of the samples). Differences in mechanical properties among soy/WPI ratios were evaluated by determining the frequency dependence of the values of G′ and G″. The slope of log G′ (or log G″) as a function of log ω (ω = frequency) indicated the frequency dependence and was calculated depending on the amount of soy protein in the mixture.

Statistical Analysis. Measurements were carried out in triplicate. Statistical analyses were performed by testing significant differences with SAS (version 8.2, Cary, NC) using analysis of variance and the Tukey test.

Microscopy. Samples were prepared using a method previously described by Roesch, et al. (*6*). In brief, small amounts of the samples were transferred to object glasses with a cavity, and a cover slip was placed over the sample and sealed with varnish. Object glasses were then wrapped in plastic and held in a water bath at 90 °C for 10 min and 1 h. Heat-induced gels were imaged with a confocal scanning laser microscope (Leica TCS SP2, Germany) using a triple dichroic filter (488/543/633 nm wavelength) and a green neon laser with an excitation wavelength of 543 nm. Samples were observed with a 100X/1.40-0.7 HCX PL APO oil-immersion objective (Leica, Germany) in reflectance mode with an emission wavelength of 535/560 nm.

Determination of Soluble Aggregates by Size Exclusion Chromatography. Three percent (w/v) of freeze-dried dialyzed soy protein or WPI was suspended in a buffer containing 0.05 M Tris and 0.1 M NaCl, pH 7.0, and stirred for 2 h. The soy preparations were also dispersed using a hand held homogenizer (PowerGen 125, Fisher Scientific) as previously described. The protein preparations were stored overnight at 4 °C to ensure complete hydration. After centrifugation of the bulk protein solutions (8000*g* for 20 min at 23 °C), the supernatants were filtered with 0.45 μm filters (Type HA, Millipore) and the protein concentration was measured with the DC protein assay (Biorad).

Solutions (1.4% w/v total protein) were prepared at two soy to WPI ratios, 30/70 and 70/30. After mixing, the samples were heated at 90 °C (with a raise time of 4 min) and held at this temperature for 10, 20, or 60 min. After heating, the samples were immediately cooled in an ice bucket to room temperature. Heated samples and unheated controls were centrifuged at 8000*g* for 3 min and then filtered with a 3 μm filter (Type HA, Millipore). Both pellets and supernatants were collected for further analysis. Control samples were also prepared by diluting soy or WPI protein solutions with buffer, to obtain 70 or 30% of the original protein concentration, and heated under the same conditions described above. Mixed protein samples and controls were also prepared in the presence of the disulfide blocker *n*-ethylmaleimide (NEM) and then heated at 90 °C for 30 min. The amount of NEM necessary was calculated based on the number of free SH groups present in the protein mixtures.

Size exclusion chromatography was carried out with a high-performance liquid chromatography Biologic Duo Flow system (Bio-Rad) with two columns connected in series (XK 1670, Amersham Biosciences, Uppsala, Sweden) using high-resolution Sepacryl S-500 (separation range 4×10^4 to 2×10^7, dextran standards) and S-300 (separation range 2×10^3 to 4×10^5, dextran standards, Amersham Biosciences) at a flow rate of 1 mL/min at room temperature. Aliquots (1 mL) of the centrifuged, filtered solutions were injected and eluted with a buffer containing 0.05 M Tris and 0.1 M NaCl at pH 7.0. The aggregate peaks were collected, dialyzed, and freeze-dried.

To determine if differences in composition existed between pellets and supernatants after centrifugation, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analyses were carried out on the various mixtures. The pellets collected after centrifugation were washed in buffer (0.05 M Tris and 0.1 M NaCl, pH 7.0) and centrifuged at 8000*g* for 3 min. Aliquots (12 mg) of pellet were dissolved in 300 μL of a buffer containing 0.020 M Tris and 0.002 M EDTA, pH 8.0, and mixed with sample electrophoresis buffer. Liquid samples (125 μL) were mixed with 200 μL of Tris−EDTA buffer at pH 8.0 and then mixed with sample buffer. Sample solutions were heated under continuous agitation to 95 °C for 5 min. Sample buffers were prepared for electrophoresis analysis under reducing (10% 2-mercaptoethanol, 2.6% SDS, and 10% bromophenol blue), nonreducing alkaline SDS (2.6% SDS and 10% bromophenol blue), and native (10% bromophenol, no SDS, and 2-mercaptoethanol) conditions. SDS−PAGE was carried out using the PhastSystem electrophoresis equipment (Amersham Biotech), with 20% homogeneous precast PhastGels. Coomassie blue R-350 was used for staining according to the manufacturer's instructions.

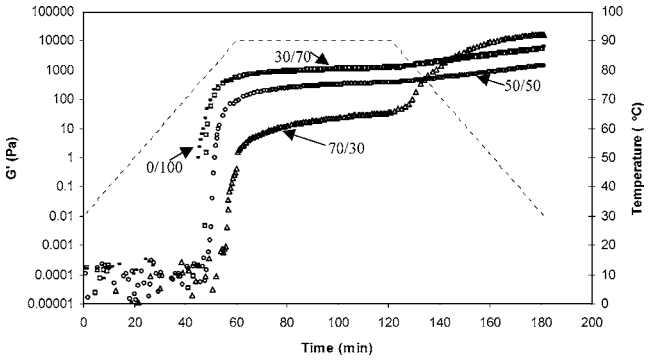

Figure 1. Development of elastic moduli (G′) as a function of time during heating of different soy/WPI mixes (6% total protein) in the presence of 0.1 M NaCl. The thin line represents the temperature change. Values are the average of three independent replicate experiments.

RESULTS AND DISCUSSION

Gelling Mixtures, Rheology and Confocal Microscopy. To determine if different ratios of soy to WPI affected the aggregation behavior during heating, the gel formation of protein solutions (6% total protein) was studied using a controlled stress rheometer. The development of the elastic modulus (G′) for soy−WPI mixtures heated in the presence of 0.1 M NaCl is illustrated in **Figure 1**. In general, changing the ratio of WPI affected the gelation behavior of the mixtures. The samples containing 6% total protein formed gels with heating, but WPI played a major role in network formation. Gelation was not observed in the absence of WPI (soy/WPI 100/0) or at high soy to WPI ratios (for example, 90/10). These results are in agreement with previously published research: A higher amount of soy protein is necessary to form visible gels; usually the minimum amount reported is 12% (*5*, *7*). Samples containing >30% WPI showed a different behavior than mixtures containing a ratio of soy to WPI of 70/30. While all of the samples (containing >30% WPI) showed an increase in G′ after 55 min at >70 °C, the increase in the G′ in the 70/30 samples seemed to level off a few degrees later than in the mixtures containing a higher ratio of WPI (89 °C instead of 81 °C). In addition, at a low concentration of WPI, after a few minutes of heating at 90 °C (70−80 min), lower G′ values were observed than in samples that contained higher WPI concentrations. In contrast with the other protein mixes, protein solutions containing 70/30 soy/WPI showed a second step increase in the elastic modulus during cooling, indicating that hydrogen bonds also played a major role in the formation of the network at these soy concentrations. With WPI > 50% in the mix, no differences were seen in the behavior of the soy/WPI solution as compared to the 100% WPI, confirming our observation that WPI was the component driving the formation of a network at 6% total protein concentration.

Frequency sweep tests were carried out immediately after cooling the final gels. There was very little frequency dependence for soy/WPI systems containing >50% WPI. As shown in **Table 1**, the slope of G′ and G″ as a function of frequency was not significantly different for samples containing >50% WPI, indicating no frequency dependence of G′ and G″. On the other hand, protein mixes containing 70/30 soy/WPI showed a significant difference in the slope of G′ and G″ and a higher frequency dependence than that of the other mixtures. This indicated that at 6% protein, the presence of soy proteins at high ratios as compared to WPI formed weak network structures. The final elastic moduli (G′) for all of the samples, with the exception of 70/30, showed no significant differences, suggesting a similar mechanical behavior and similar gel strength between all of the other gels. In contrast with previously reported results on soy protein−whey protein mixtures (*5*), no phase separation or phase inversion was observed in these samples. This disagreement between the present results and those previously reported may be caused by the absence of large aggregated material in these protein mixes: All samples were centrifuged and filtered before mixing, to eliminate the contribution to the aggregation from any large particles originally present in the protein preparations. The protein solutions had G′ comparable to those reported in previous work (*4*, *5*), despite the lower concentration (6% total protein) used in the present study.

Table 1. Frequency Dependence of G′ and G″ for Different Soy/WPI Ratios at 6% Protein Concentration[a]

soy/WPI mixtures	slope (G′)	slope (G″)
0/100	$0.080^{a,c}$	$0.079^{a,cd}$
10/90	$0.081^{a,c}$	$0.077^{a,cd}$
30/70	$0.080^{a,c}$	$0.071^{a,cd}$
50/50	$0.099^{a,cd}$	$0.092^{a,c}$
70/30	$0.120^{a,d}$	$0.055^{b,d}$

[a] Values are calculated from the slope of the log (G′ or G″) vs log frequency relationship and are means of three independent experiments. Differences were determined by the general linear model procedure with Tukey grouping. Different superscript letters indicate a significant difference. Letters a and b are within row and c and d are within column.

Confocal microscopy observations confirmed that different soy/WPI ratios affected the microstructure of the protein networks. The control samples containing only WPI showed a coarse network with a homogeneous structure (**Figure 2A**), in agreement with previous observations (*8*). A small amount of soy protein (soy/WPI 30/70) in the mix modified this homogeneous structure and caused the formation of large particulate aggregates. Under these conditions, soy proteins may form aggregates with WPI. Further addition of soy protein up to a 50/50 ratio of soy/WPI decreased the size of the gel pores (**Figure 2B−D**).

Figure 2E depicts the discontinuous network formed by the mix with 70/30 soy/WPI. This network showed large pores sizes, which could indicate phase separation. This difference in microstructure is related to the differences observed in the rheological behavior of the 70/30 soy/WPI samples as compared to the other mixed systems (30/70, 50/50). During heating of soy/WPI at this high ratio of soy, two separate aggregation steps were identified, the first at about 89 °C and the second step during cooling. The two stages may represent the aggregation of whey protein or of complexes of soy and whey proteins during heating, followed by the aggregation of the residual soy protein with cooling. The results obtained with samples heated at 90 °C for 60 min were also confirmed for samples heated at 90 °C for 10 min (images not shown).

Analysis of the Protein Aggregates by Chromatography and Electrophoresis. Although rheology and microscopy observations indicated that complexes may form between soy and WPI during heating and that the ratio of soy to WPI is important in determining the type of aggregates, size exclusion chromatography and electrophoretic analysis were carried out to better understand the interactions at the molecular level. Protein solutions were heated at a lower protein concentration (1.4% w/v total protein), and two ratios of soy/WPI were chosen, 70/30 and 30/70. Solutions containing the same amount of soy protein or whey proteins as present in the mixtures (either 70 or 30% of the total protein content) but in isolation were also analyzed.

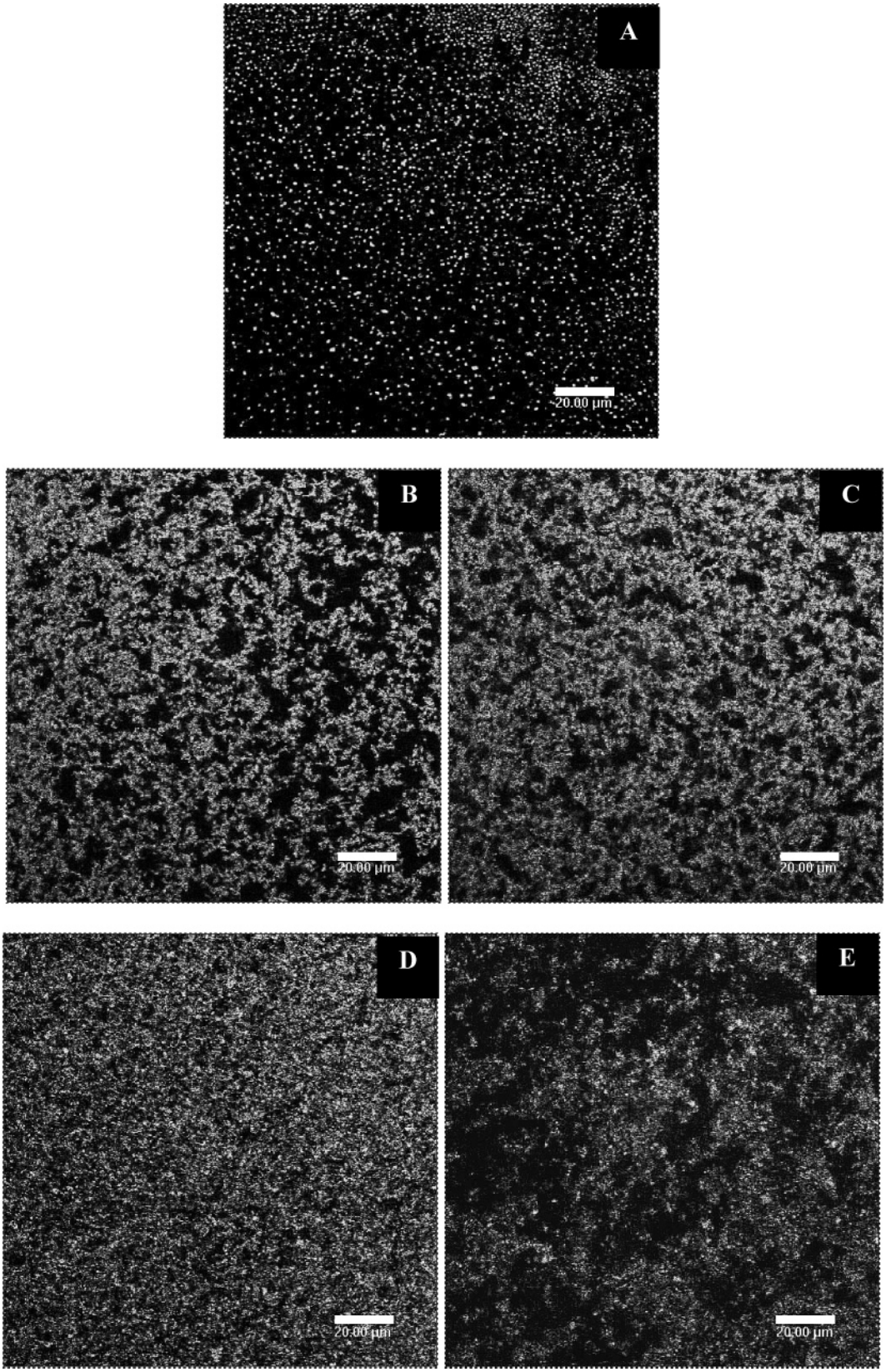

Figure 2. Confocal scanning micrographs of gels prepared with 6% protein after heating at 90 °C for 1 h. Images represent different soy/WPI ratios: 0/100 (**A**), 10/90 (**B**), 30/70 (**C**), 50/50 (**D**), and 70/30 (**E**); bar = 20 μm.

Presence of Low Amounts of WPI. **Figure 3** illustrates the chromatographic separation of a mixture of 70/30 soy/WPI after separation of the insoluble fraction by centrifugation. The soluble fraction of the protein mixture before heating showed the same elution behavior of the two control samples, containing the same concentration of either soy proteins or whey proteins but in isolation. After heat treatment, while the two control samples showed a decrease in the amount of soluble material eluting in the chromatogram, the sample containing both soy and whey proteins showed a large aggregate peak eluting at about 100 min (which corresponded to the void volume of the column, Mw > 10^7 Da). These results indicated that in the

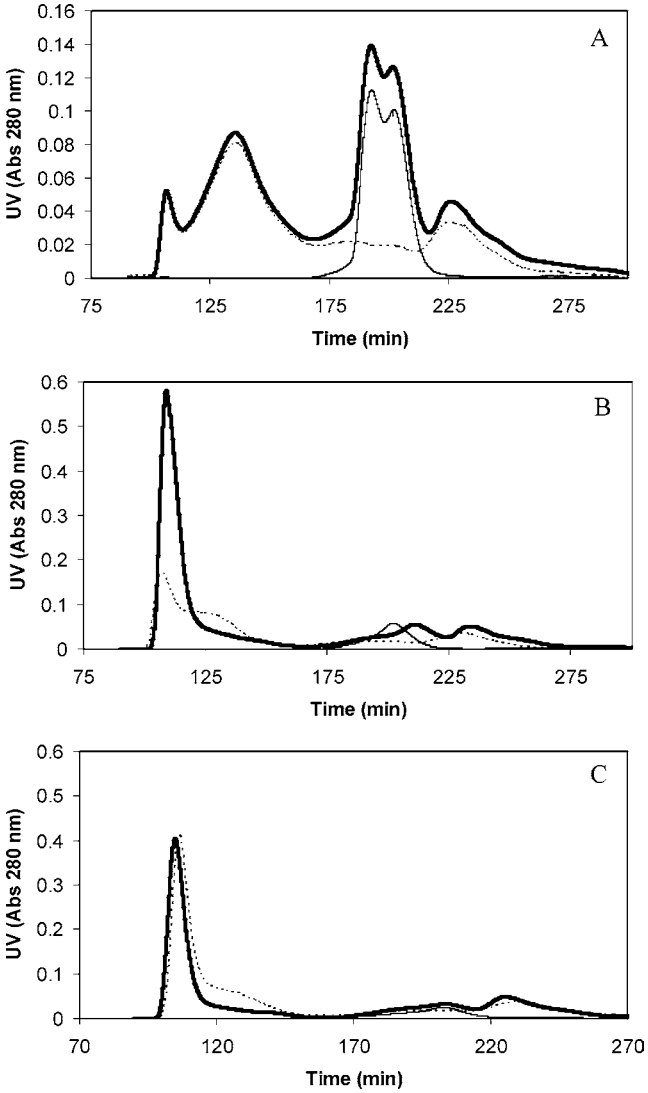

Figure 3. Protein elution profiles of soluble phases after centrifugation of 70/30 soy/WPI mixtures (solid thick line); WPI control solution containing the same amount of protein (30% of the total protein, solid thin line); soy control solution (70% of total protein, dotted line). Unheated samples (**A**), solutions heated at 90 °C for 10 min (**B**), and solutions heated at 90 °C for 60 min (**C**). Note the different scale on the *Y*-axis.

presence of a high ratio of soy protein to whey protein (70/30), soluble complexes formed with heat treatment. While after 10 min of heating the aggregate peak was larger in the heated mixture than in the soy sample heated in isolation, after 60 min of heating, most of the protein was present in the insoluble phase. After heating, no native whey protein was left in the soluble phase. The formation of soluble heat-induced aggregates of soy protein with whey protein may not only depend on time/temperature combinations but also by the amount of whey proteins available for the interactions.

Electrophoretic analysis of the aggregate peak fractions (eluting at about 100 min) confirmed that all soy protein subunits and whey proteins were present in the soluble aggregates (**Figure 4**). The protein complexes were mainly formed via disulfide bonds, and as shown in lane 2 of the electrophoresis gel, only a small amount of 7S subunits of soy protein migrated in the gel under nonreducing conditions.

Electrophoretic analysis of the precipitates and soluble fractions for the 70/30 soy/WPI mixture demonstrated that, after heat treatment, all of the subunits of soy protein as well as

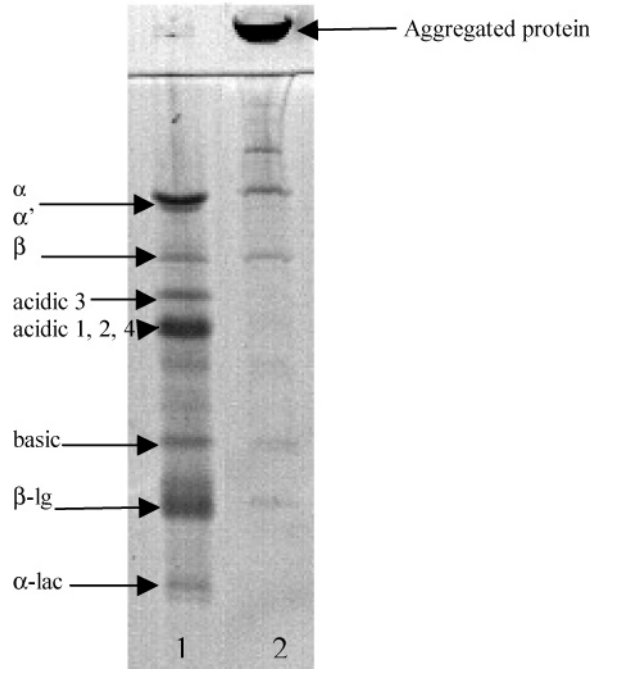

Figure 4. SDS–PAGE gel of the aggregate peak (eluted at 100 min) collected after size exclusion chromatography from a 70/30 mixture heated at 90 °C for 10 min. Lane 1, reducing conditions; lane 2, nonreducing conditions (no 2-mercaptoethanol added).

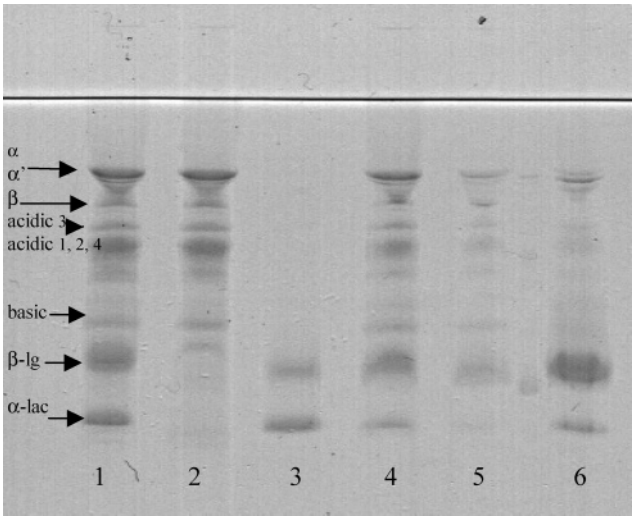

Figure 5. SDS–PAGE gel under reducing conditions of soluble and insoluble fractions (separated by centrifugation) for a 70/30 soy/WPI sample. Heating was performed at 90 °C for 10 min. Lane 1, supernatant of 70/30 soy/WPI; lane 2, supernatant of soy protein control; lane 3, supernatant of WPI control; lane 4, precipitate of 70/30 soy/WPI; lane 5, precipitate of soy protein control; and lane 6, precipitate of WPI control.

α-lactalbumin and *β*-lactoglobulin were present in both the soluble and the insoluble fractions (**Figure 5**). Up to 60 min of heating the soluble phase of the mixed solutions contained all of the soy subunits, *β*-lactoglobulin and α-lactalbumin, in agreement with the composition of the soluble aggregate peak (**Figure 4**). WPI seemed to be evenly distributed between the soluble and the insoluble phases of the 70/30 soy/WPI mixture. When soy protein was heated in isolation, very little soy protein was found in the insoluble fraction (lane 5, **Figure 5**). The behavior of soy protein in isolation was different than that of the same protein heated in the presence of whey protein (compare lanes 4 and 5 of **Figure 5**). Although these results

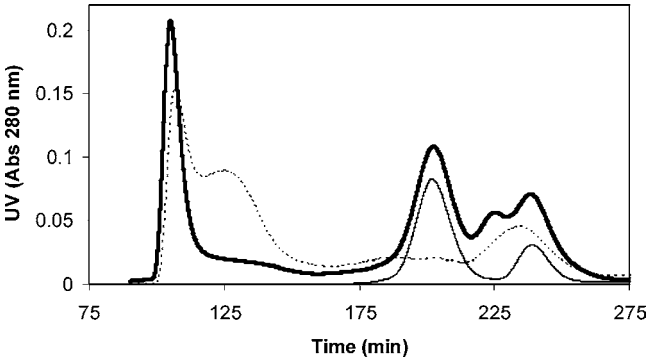

Figure 6. Protein elution profile of the soluble phases of soy control (70% of the total protein, dotted line), WPI control (30% of the total protein, solid thin line), and 70/30 mixture (solid thick line). Samples were heated at 90 °C for 10 min in the presence of NEM.

may suggest covalent disulfide bridging between β-lactoglobulin, α-lactalbumin, and the various soy protein subunits with heating, it cannot be excluded that soluble aggregates composed only of whey proteins could also be present.

To determine the role played by disulfide interactions in the mixtures of soy protein and whey proteins, the 70/30 soy/WPI samples were heated in the presence of NEM, a disulfide blocker. NEM was also added to the control solutions of soy protein and WPI. The chromatographic elution of the soluble fractions after heating at 90 °C for 10 min in the presence of NEM is shown in **Figure 6**. When compared to the chromatographic elution of samples heated under the same conditions in the absence of NEM (**Figure 3B**), it became apparent that NEM inhibited the formation of large soluble complexes. NEM inhibited whey protein aggregation, as native whey protein was still present in the soluble phase after heating. While whey protein aggregation was affected by NEM, soy proteins heated in isolation did not show changes in the elution behavior when treated with NEM, confirming that noncovalent interactions play a major role in the formation of soy protein aggregates.

Figure 7 illustrates the difference in the electrophoretic migration of the soluble and insoluble fractions of 70/30 soy/WPI after heating with or without NEM. Heating of soy proteins in isolation in the presence of NEM did not affect the electrophoretic of the soluble phase (lanes 2 and 5). On the other hand, the whey protein control solutions showed a higher amount of native protein in the soluble phase after heating in the presence of NEM (lanes 3 and 6). Analysis of the insoluble fractions indicated that α-lactalbumin formed complexes with β-lactoglobulin mainly via disulfide bridging. Only β-lactoglobulin was present in the insoluble fractions of WPI control after heating in the presence of NEM. In the 70/30 soy/WPI mixture, heating in the presence of NEM affected the amount of soluble soy protein present (lanes 1 and 4).

These results seemed to indicate that for samples containing soy/WPI in a 70/30 ratio, soluble complexes form via disulfide bridging. These soluble complexes contain whey proteins, but it is likely that some soy proteins participate in the aggregate. The precipitate fractions of the 70/30 soy/WPI mixture were different when heated with or without NEM (lanes 7 and 8). In agreement with what is shown during heating of WPI control samples, α-lactabumin was present in the insoluble phase only after disulfide bridging. In addition, the acidic and basic fractions of the soy 11S seemed to be present in lower amounts in the insoluble fraction when the protein was heated in the presence of NEM. When the insoluble fractions were analyzed under nonreducing conditions, some 7S as well as β-lactoglobulin

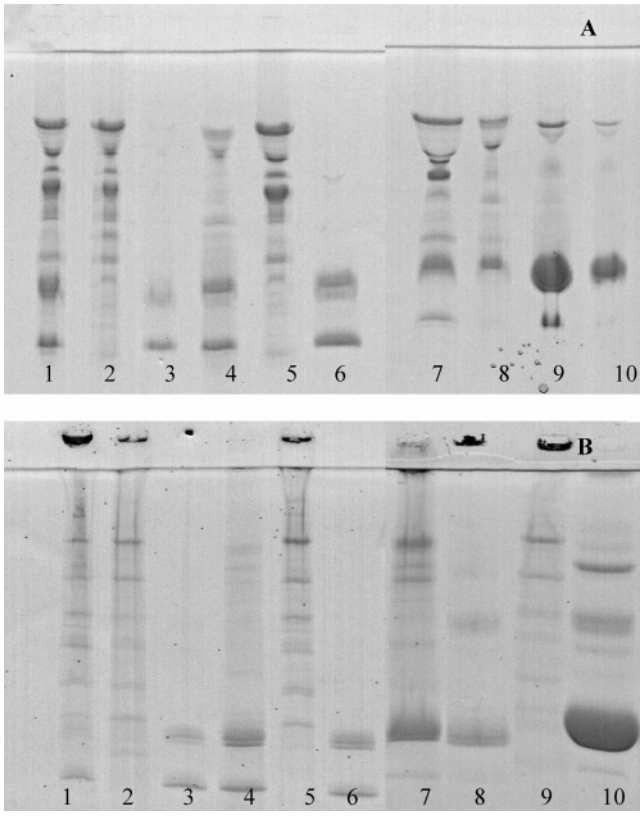

Figure 7. Gel electrophoresis of soluble and insoluble fractions (separated by centrifugation) of a 70/30 soy/WPI mixture heated at 90 °C for 10 min. Migration for samples treated under reducing conditions (**A**) and nonreducing conditions (**B**). Lane 1, supernatant mixture; lane 2, supernatant soy control; lane 3, supernatant WPI; lane 4, supernatant mixture with NEM added; lane 5, supernatant soy control with NEM added; lane 6, supernatant WPI control with NEM added; lane 7, pellet mixture; lane 8, pellet mixture with NEM added; lane 9, pellet WPI control; and lane 10, pellet WPI control with NEM added. Note that the pellet of soy controls did not contain enough protein to be analyzed.

(native or oligomers) migrated in the gel, demonstrating that noncovalent interactions also play an important role in complex formation, although disulfide bridging seemed to be necessary to form aggregates containing α-lactalbumin and 11S proteins.

Presence of Low Amounts of Soy Protein. **Figure 8** illustrates the difference in the elution profiles for the soluble protein fractions of a 30/70 soy/WPI mixture as compared to those of control samples containing the same amount of soy and WPI. The native WPI eluted at 180 min, with two unresolved peaks corresponding to β-lactoglobulin and α-lactalbumin. The soluble phase of unheated soy/WPI mixtures showed a chromatographic elution comparable to the control samples, similarly to what was observed for mixtures containing 70/30 soy/whey proteins, where the proteins did not show aggregation with mixing.

When WPI was heated in isolation, most of the protein precipitated during centrifugation. The supernatant showed only a small native peak mostly corresponding to α-lactalbumin. Differences were shown with time of heating, although after 10 min, most of the β-lactoglobulin was present in the precipitate. Soy protein samples (prepared at the same concentration of the mixture, i.e., 30% of the total protein) heated in isolation formed large soluble aggregates and showed no precipitate after centrifugation. These soluble aggregates eluted at the void volume peak (at about 100 min), corresponding to a molecular mass $>10^7$ Da, as already discussed for 70/30 soy/whey protein experiments (**Figure 3**).

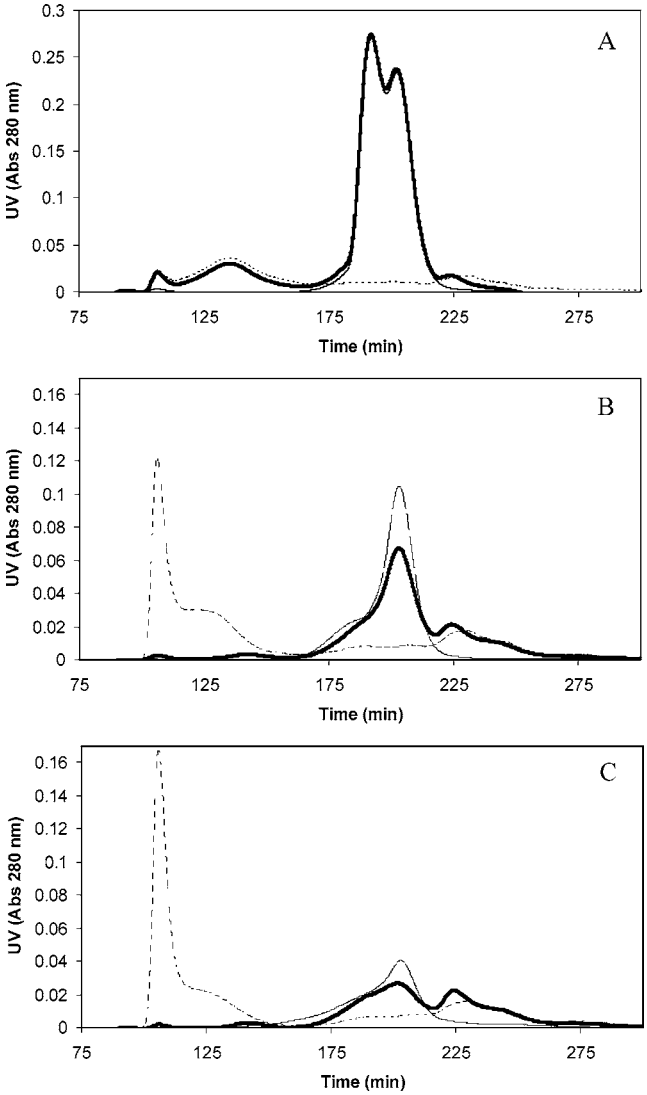

Figure 8. Protein elution profiles of soluble phases after centrifugation of 30/70 soy/WPI mixtures (solid thick line); WPI control solution containing the same amount of protein (70% of the total protein, solid thin line); and soy control solution (30% of total protein, dotted line). Unheated samples (**A**), solutions heated at 90 °C for 10 min (**B**), and solutions heated at 90°C for 60 min (**C**). Note the different scale on the *Y*-axis.

The soluble phase of the soy/WPI mixture at a ratio of 30/70 showed different elution profiles after heating. While soy protein in isolation formed large, soluble aggregates, the addition to whey protein reduced significantly the amount of soluble aggregate and induced precipitation. After heating and centrifugation, the peak present in samples containing only soy proteins was absent in the mixture, and the native whey protein peak (mostly α-lactalbumin) also decreased in size. After 10 min of heating, unlike samples with 70/30 soy/WPI, very little protein was left in the soluble fraction. At longer heating times, very little native whey protein was left in the soluble phase, and most of the soluble protein eluted in the excluded volume. It was concluded that, under these conditions, soy protein subunits were incorporated in covalent aggregates with whey proteins.

Differences in protein composition were shown in the soluble and insoluble phases when analyzed by SDS−PAGE (**Figure 9**). Most of the 7S and the basic subunit of 11S were present in the precipitate after heating with WPI. On the other hand, all of the soy protein subunits were still soluble when heated in isolation. When whey protein was heated in the presence of soy protein, more α-lactalbumin seemed to be present in the insoluble phase as compared to that of the heated WPI control.

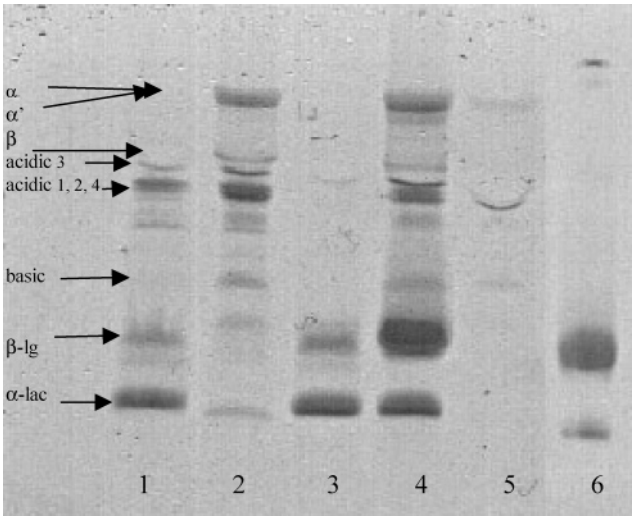

Figure 9. Gel electrophoresis of soluble and insoluble fractions (separated by centrifugation) of a 30/70 mixture heated at 90 °C for 10 min. Lane 1, supernatant mixture; lane 2, supernatant soy protein control; lane 3, supernatant WPI control; lane 4, pellet mixture; lane 5, pellet soy protein control; and lane 6, pellet WPI control.

These results are first evidence of the covalent interactions between soy protein subunits and β-lactoglobulin and α-lactalbumin. The ratio of soy protein/whey protein in the mixture is fundamental in determining the type of aggregates which form during heating. It is possible to hypothesize that when WPI is present at concentrations high enough to form aggregates and networks, soy proteins are incorporated in the aggregates, while at lower amounts of WPI, various types of soluble complexes may form, some containing only whey proteins and some containing mixed soy/whey proteins.

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