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Study of the Effect of Soy Proteins on the Acid-Induced Gelation of Casein Micelles

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The objective of this research was to understand whether addition of soy protein to milk protein affects the properties of acid-induced casein gels. Different samples were prepared by suspending casein micelles pellets in milk serum containing soy proteins or whey proteins as well as mixtures of the two proteins. Glucono-δ-lactone was added, and the changes in apparent size (in diluted systems) as well as the viscoelastic properties of the mixtures were measured. Size exclusion chromatography was also carried out to characterize the soluble phase of the various mixtures before and after heating. Soy protein affected the gelation of the mixtures; however, not to the same extent as whey proteins, which dominated formation of the network in soy−whey−casein systems. It was concluded that, up to a critical ratio of soy/whey proteins, soy proteins can be incorporated in the mix without a significant change in structure of the casein gels.

KEYWORDS: Acid-induced casein gels; soy proteins; gelation

INTRODUCTION

Soy proteins are often employed as ingredients in food products, which also contain milk proteins. A better understanding of the interactions between soy and milk proteins would increase the opportunities for food manufacturers to create new products, employing the combined functionality of the two protein ingredients (*1*). In particular, the effects of changes of pH and temperature on the aggregation of soy and milk proteins have been extensively studied on the proteins in isolation (*2*−*9*); however, very little is known about the changes occurring when soy proteins and milk proteins are heated and/or acidified together.

During acidification of milk several changes occur: the calcium phosphate is released from the casein micelles, the outer layer of the micelles collapses, decreasing the steric stabilizing effect of κ-casein, and the charge repulsion between the casein micelles is minimized. Casein micelles aggregate when the pH approaches the isoelectric point of the caseins (*10*, *11*). Heat is an important step in the manufacture of acid milk gels as it affects the pH of aggregation as well as the structure and texture of the final gel (*11*−*13*). During heating of milk whey proteins form aggregates and interact with the casein micelles, depending on the extent of treatment (*12*). These interactions occur via disulfide exchange reactions with other whey proteins (β-lactoglobulin, α-lactoalbumin, BSA) and the caseins (κ- and α_{s2}-casein) (*14*−*16*). Formation of these protein complexes in milk and the specific association of denatured whey proteins with the casein micelles are dependent on pH (*7*, *17*).

The major proteins present in soy are glycinin and β-conglycinin. These are heterogeneous proteins varying in their subunit composition (*18*). These two proteins form gels with heating and cooling and show different gelling properties. It has been shown that β-conglycinin denatures at a lower temperature than glycinin, and glycinin forms stronger gels than β-conglycinin (*19*). The gel strength is related to pH and the presence of ions, as these conditions affect the formation of soy protein aggregates (*20*). Heat-induced gels at pH > 6.0 are weaker than those formed at pH < 6.0 (*6*). Not all the protein subunits (α, α', and β for β-conglycinin and acidic and basic for glycinin) present in soy protein participate equally to gel structure formation. It has been reported that at acidic pH all proteins participate in the network, while at high pH few acidic subunits of 11S take part in the network (*6*).

The soy proteins glycinin and β-conglycinin show distinct gelation behavior when acidified with glucono-δ-lactone. Glycinin forms gels at a faster rate than β-conglycinin (*9*, *21*, *22*). In addition, recent atomic force microscopy observations have shown that glycinin forms larger aggregates during acidification than those formed by β-conglycinin (*23*). The differences in the onset of gelation between the two proteins are attributed to their different isoelectric points (*24*). The physicochemical properties of the gels and the pH of gelation can be modulated by varying the combination of the two storage proteins (*9*).

To facilitate development of new products containing combinations of soy and milk proteins, a better understanding of the role played by the individual proteins during processing of mixed systems is needed. There seems to be disagreement on the existence of supramolecular aggregates of soy proteins and milk proteins. The contrasting reports on the interactions between these two protein groups result from differences in the type of soy proteins used and the different processing conditions of each study. In most studies reported to date on mixed protein

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systems, commercial sources of soy proteins were used without further purification (*25*−*27*).

It has been shown that addition of soy protein isolate to milk improves the textural properties of yogurt (*1*). The authors reported that addition of soy protein isolate is more effective than sodium caseinate in increasing viscosity and gel strength and reducing syneresis of yogurt gels. Addition of soy proteins decreases the strength of rennet curd when added to milk (*26*). It was suggested that soy proteins may be adsorbed onto the casein micelles or simply be entrapped in the casein network, hindering formation of stronger casein−casein interactions. In a study on the heat-induced gelation of soy proteins mixed with milk proteins it was reported that soy proteins do not interact with milk proteins (*25*). Recently, it was demonstrated that soy proteins affect the textural and structural properties of acid-induced casein gels (*27*). Addition of soy protein to skim milk causes an increase of the elastic modulus and the pH onset of gelation.

The objective of the present research was to better understand the role played by soy proteins in the aggregation of casein micelles during acidification and whether milk/soy protein complexes are responsible for the changes in structure and viscoelastic properties of the acid-induced gels which have been described in earlier reports (*1*, *27*). To better understand protein−protein interactions at the molecular level and identify the type of milk/soy protein complexes formed, only the soluble fraction of a commercial soy protein concentrate was used.

MATERIALS AND METHODS

Sample Preparation. Instant low-heat skim milk powder, donated by Parmalat (Toronto, ON), containing 33% (w/w) protein and a soy protein concentrate (Alpha 5800 Solae Inc., St. Louis, MO) containing 76% (w/w) protein were used in this study. Protein concentration was determined by the Dumas combustion method (Leco FP-528, Mississagua, ON). Alpha 5800 was chosen because of its high solubility and relatively mild processing history.

Soy protein concentrate was suspended in high-purity water, stirred for 2 h, and stored overnight at 4 °C. The protein was then extensively dialyzed (molecular cut off 6000 Da, Fisher Sci., Mississauga, ON) against high-purity water (5 exchanges, at least 100 volumes) for 36 h at 4 °C and freeze-dried. After dialysis the dry soy protein contained 84% (w/w) total protein, as determined by the Dumas combustion method.

Skim milk powder was suspended (12% solid total solids w/v) in high-purity water containing 0.02% sodium azide (to avoid microbial growth), stirred for 2 h, and incubated overnight at 4 °C to ensure complete hydration. One part of the milk was then equilibrated to room temperature and circulated continuously through a prep/scale−TFF ultrafiltration cartridge filter (Millipore Corp., Bedford MA) using a peristaltic pump to obtain milk permeate. Casein micelles were separated from whey protein by centrifuging 120 mL aliquots of skim milk at 60 000*g* for 40 min at 23 °C with a temperature-controlled ultracentrifuge (Optima LE-80K Beckman Coulter, Mississauga, ON). The precipitate was then resuspended in milk permeate with a hand-held homogenizer (PowerGen 125, Fisher Scientific) and centrifuged again to ensure that casein micelles were depleted of whey proteins. Supernatants from the first centrifugation, containing whey proteins, were collected and filtered with 0.45 and 0.22 μm nylon filters (Type HA, Millipore), and the total protein concentration was determined by DC protein assay (Bio-Rad, Mississauga, ON, Canada).

The freeze-dried soy protein preparation was suspended (approximately 4% w/v) in milk permeate, dispersed with a hand-held homogenizer (PowerGen 125, Fisher Scientific), and stirred for at least 2 h. After overnight storage at 4 °C to ensure complete hydration, samples were brought to room temperature (23 °C) and centrifuged at 8000*g* for 20 min at 20 °C. The supernatant was then collected, and the total protein concentration was determined by DC protein assay. The soy protein suspension was brought to the same concentration measured in the supernatants containing whey proteins (0.6% w/v).

To study the effect of soy protein during acidification of casein micelles, different samples were prepared by suspending pellets containing casein micelles in milk serum containing soy protein or whey protein alone as well as mixtures of the two proteins. Specifically, casein micelles were suspended with soy proteins dissolved in permeate or supernatants containing whey proteins. Pellets containing casein micelles were also suspended in permeates containing a final protein concentration of 0.6% (w/v) but with varying ratios of soy to whey proteins (0.42% soy proteins/0.18% whey proteins or 0.18% soy proteins/0.42% whey proteins, corresponding to a 70/30 and 30/70 ratio). These mixtures were prepared by mixing soy protein dissolved in permeate and supernatants containing whey proteins and soluble non-micellar-casein. Control samples were also prepared suspending the pellets containing casein micelles in milk permeate (without soy or whey proteins) or in permeates containing only 0.18% or 0.42% (w/v) soy proteins or whey proteins. All samples were brought to the same final volume, corresponding to that of the original milk before centrifugation. The samples were dispersed with a hand-held homogenizer, stirred, and left overnight at 4 °C. All mixtures were homogenized with two passes through a single-stage homogenizer (Avestin Emulsiflex C-5, Ottawa, ON) at 21 MPa. The protein samples were poured into glass containers, and aliquots (200 mL) of each sample were heated at 90 °C for 10 min (after a come up time of 4 min) in a thermostatically controlled water bath. After heating, the samples were immediately cooled in an ice bath to room temperature (23 °C).

Acidification. Concentrations of glucono-δ-lactone (GDL) (Sigma-Aldrich Co., St. Louis, MO) between 1.5% and 1.6% (w/v) were used to acidify heated and unheated samples containing only casein micelles, casein micelles/soy proteins, casein micelles/whey proteins, and casein micelles/soy−whey proteins to obtain a final pH of 4.6 after incubation for 4 h at 30 °C. Because of the different buffering capacity between the samples, the amount of GDL necessary to acidify each mixture was determined by performing preliminary experiments with different concentrations of GDL, so that comparable acidification rates between samples could be obtained. The acidification experiments were carried out in triplicate.

Aggregation Studies Using Dynamic Light Scattering. Changes in particle size during acidification of the unheated and heated samples were determined under diluted conditions in milk permeate using dynamic light scattering at a 90° angle (System 4700, Malvern Instruments Inc., Southborough, MA) equipped with a LHRP-1202 He−Ne laser with a wavelength of 633 nm and a nominal output power of 12.0 mW (Research Electro-Optics Inc., Boulder, CO). Aliquots (100 μL) of the samples were diluted in 25 mL of milk permeate previously filtered with a nylon 0.2 μm syringe filter (Millex-GV Millipore, Bedford, MA). After dilution, GDL (0.5% (w/v) was added to the diluted samples and stirred for 1 min (*27*). Samples (3 mL) were poured into polystyrene cuvettes and measured with dynamic light scattering at 30 °C. The temperature was controlled by a peltier heating system. Changes in apparent diameter during acidification were observed over time. In a parallel sample kept at the same temperature, the pH was measured. The results presented are the average of two separate experiments.

Rheological Measurements. To determine the differences in the viscoelastic behavior of heated and unheated protein solutions containing casein micelles/soy, and casein micelles/whey, casein micelles/soy−whey, and only casein micelles during acidification, measurements of changes in storage modulus (G') and loss modulus (G'') over time were carried out using a controlled stress rheometer (Advanced Rheometer AR 1000, TA instruments, New Castle, DE).

Rheological analysis was performed using a conical concentric cylinder geometry (20 mL sample size, 5920 μm fixed gap, 15 mm radius, 14 mm rotor outer radius, and 42 mm cylinder immersed height) at a constant temperature of 30 °C, controlled by a temperature-controlled water bath. Measurements were carried out with a constant maximum strain of 1% and a frequency of 0.5 Hz. Appropriate amounts of GDL were added to the mixtures, and after stirring for 1 min, a 20 mL amount was transferred to the rheometer. A water trap was used to minimize evaporation during the measurement. Values of pH and time

corresponding to the onset of gelation were obtained when $G' \geq 1$ Pa (*11*). After 4 h, a frequency sweep test was performed applying a constant stress (within the linear viscoelastic range of the samples, as determined by a stress sweep test). Differences in the mechanical properties among samples were evaluated by determining the frequency dependence of the values of G' and G'', calculating the slope of a log/log plot. Rheological measurements were carried out in triplicate. Statistical analyses were performed by testing significant differences with SAS (version 8.2, Cary, NC) using ANOVA and Duncan test for equal means.

Determination of Soluble Aggregates by Size Exclusion Chromatography. Unheated and heated samples were centrifuged at 26 000*g* for 1 h, and supernatants were carefully separated and filtered with a 3 μm syringe filter (Type HA, Millipore). Aliquots (1 mL) of the centrifuged, filtered solutions were injected and eluted with a buffer containing 50 mM Tris, 0.1 M NaCl at pH 7.0.

Size exclusion chromatography was carried out with a high-performance liquid chromatography Biologic Duo Flow system (Bio-Rad, Hercules, CA) with two preparative columns connected in series (XK 16/70, GE Biosciences, Baie D'Urfé, QC), packed with high-resolution Sephacryl S-500 (separation range from 4×10^4 to 2×10^7 Da, as dextran standards) and Sephacryl S-300 (separation range from 2×10^3 to 4×10^5 Da, as dextran standards) (GE Biosciences) at a flow rate of 1 mL/min at room temperature. The aggregate peaks were collected, dialyzed, and freeze-dried.

To determine if there were differences in protein composition of the aggregated peaks separated by chromatography, SDS-PAGE was carried out on these fractions. The freeze-dried samples were suspended in buffer to a final concentration of 0.03 mg/mL of freeze-dried powder and 20 mM Tris, 2 mM EDTA, pH 8.0, 10% 2-mercaptoethanol, 2.6% SDS, and 10% bromophenol blue. Samples were heated under continuous agitation to 95 °C for 5 min. SDS-PAGE was carried out using the PhastSystem electrophoresis equipment (GE Biosciences) with 20% homogeneous precast PhastGels (GE Biosciences). Coomassie blue R-350 (GE Biosciences) was used for staining according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The effect of soy protein on the acid-induced aggregation of casein micelles was studied by observing the changes in apparent diameter of samples diluted in milk permeate containing GDL. **Figure 1** illustrates the apparent diameter of the mixtures as a function of pH for heated and unheated protein mixtures containing only casein micelles or casein micelles with 0.6% soy protein or whey protein. In general, it was observed that the apparent diameter of the protein particles decreased with decreasing pH until a certain pH, where the protein particles started to aggregate. The decrease in the apparent diameter continued until a pH of about 5.0 in the unheated and heated casein micelles (**Figure 1A**) and in the unheated mixtures containing whey proteins (**Figure 1B**). **Figure 1A** indicates clearly that the micelle size decreased with pH until about 5.5 and then showed another decrease between 5.2 and 5.0 before aggregating. This behavior can be attributed to the collapse of the casein micelle's steric stabilizing layer because of reduction of charge repulsion as the pH approaches the isoelectric point of the caseins (*8*). The decrease in size seemed to slow down between pH 5.5 and 5.0; however, it is important to note that the casein micelles were separated by centrifugation, resuspended (perhaps causing some destabilization of the micelles), and extensively diluted in permeate before acidification.

Heating of the mixtures containing whey proteins affected the pH of aggregation: the protein particles showed a decrease in diameter until pH 5.3 and then showed an earlier onset of aggregation, compared to the same mixture, unheated (**Figure 1B**). The decrease in size of the protein particles in these unheated samples was larger than in the heated samples, as the heated samples aggregated much earlier. It is understood that during heating whey proteins form soluble complexes with κ-casein, and these complexes are responsible for the higher pH of aggregation of the heated samples.

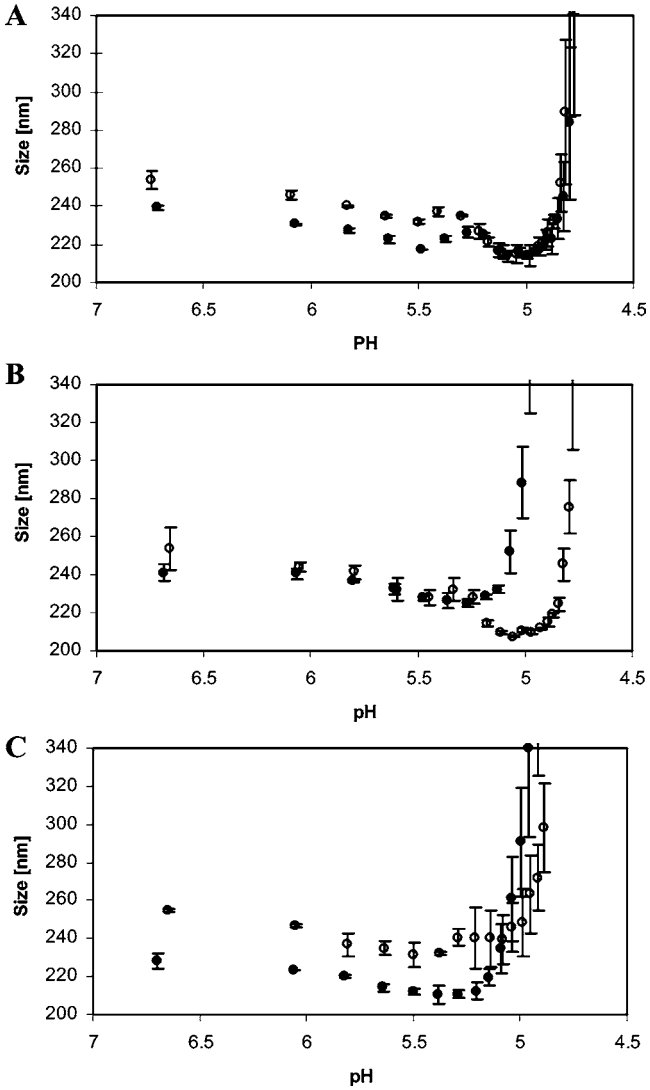

Figure 1. Apparent diameter of different protein mixtures diluted with milk permeate containing GDL as a function of pH during acidification: casein micelles suspended in milk permeate with no protein added (A); casein micelles/whey proteins (B); casein micelles/soy proteins (C); unheated mixtures (○); heated mixtures (●). Values are average of two replicate samples. Bars represent standard error.

When casein micelles were mixed with soy proteins, heated samples showed a similar behavior during acidification to that of heated mixtures containing whey proteins (**Figure 1C**). There was no significant difference in the pH onset of aggregation between heated and unheated mixtures containing casein micelles and soy proteins and heated mixtures containing casein micelles and whey proteins.

A more detailed view of the size changes of the mixtures (relative to the initial size at pH 6.8) is shown in **Figure 2**. A similar decrease in the size of the protein particles was shown for all samples, although the pH of aggregation was higher for mixtures containing soy (both heated and unheated) and heated mixtures containing whey proteins. The difference in the pH onset of aggregation in heated samples containing whey proteins is attributed to the whey protein denaturation and the presence of caseins−whey protein complexes (*7*). However, the reasons for the earlier onset of aggregation of soy proteins/casein

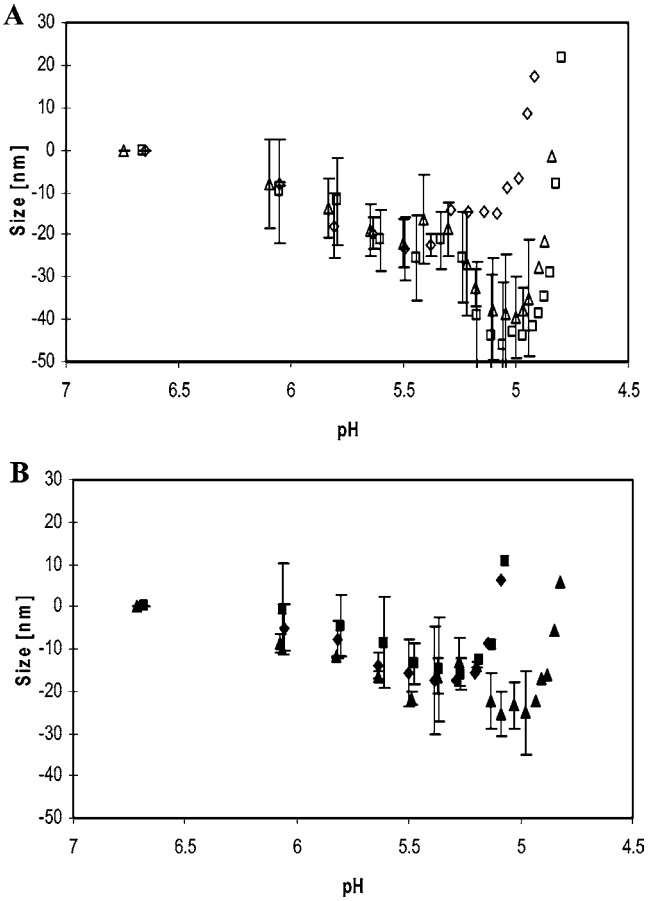

Figure 2. Size change in apparent diameter of unheated (A) and heated (B) protein mixtures diluted with milk permeate as a function of pH during acidification with GDL: casein micelles/soy proteins (◇, ◆); casein micelles/whey proteins (□, ■); casein micelles suspended in milk permeate with no protein added (Δ, ▲). Values are average of two replicate samples. Error bars are shown only before the aggregation point.

micelles mixtures are not yet known. These findings may suggest that the mechanisms that induce an earlier destabilization of the casein micelles are similar in the case of soy proteins as in whey proteins (after heat treatment). The early onset of aggregation of mixtures containing soy proteins could be attributed in part to soy protein interactions with the casein micelles and formation of large protein aggregates mainly by 11S (*23*). However, this would not explain the similar pH onset of aggregation of unheated casein micelles/soy mixtures. A more likely cause for the similar behavior between soy and heated whey proteins is a destabilization of the casein micelles caused by protein aggregates (soy aggregates or whey protein aggregates) at an earlier pH. Soy proteins have a higher isoelectric point than casein micelles, and therefore, they would precipitate at pH values > 5.3. It has also been reported that after heating, whey protein complexes precipitate in this pH range (*28*). The similar behavior of whey and soy mixtures would suggest that the aggregation is driven by the casein micelles and their interactions with the denatured proteins.

These results on the early pH onset of aggregation in soy−casein systems were in agreement with earlier studies of acidified skim milk containing soy protein (*27*). Heated or unheated mixtures containing skim milk and soy proteins showed a similar behavior, with a pH of aggregation higher than that reported for heated skim milk (*27*). However, the data reported in this study show no difference between soy mixtures and whey mixtures after heating. This could be caused by the higher amount of soy protein used in the previous study or, most likely, by the composition and processing history of the soy protein sample used. In this study, a soluble fraction of the same commercial sample was used. It is important to note that these studies are conducted in extremely dilute conditions; therefore, they may not fully explain the reactions occurring in the undiluted samples.

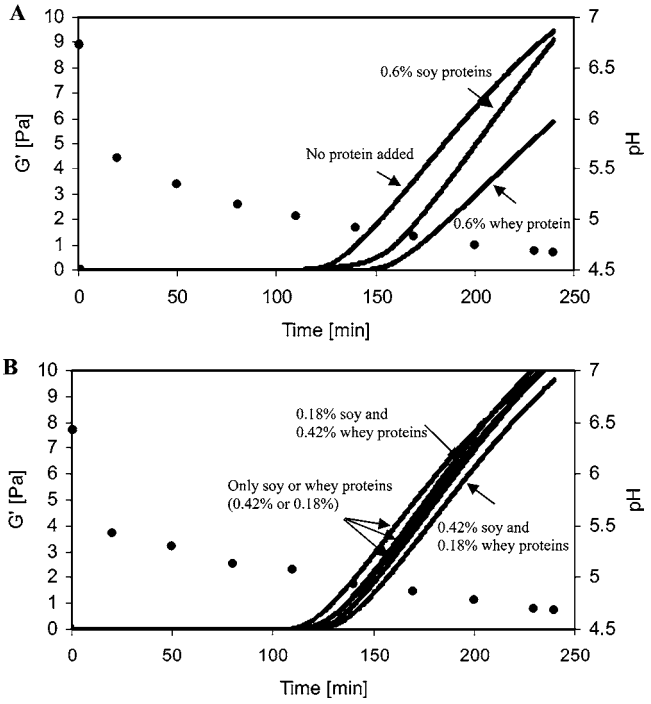

Figure 3. Development of the elastic modulus (*G*′) as a function of time during acidification with GDL at 30 °C for unheated mixtures containing casein micelles with soy proteins (0.6%), casein micelles with whey proteins (0.6%), or casein micelles suspended in permeate with no protein added (A), casein micelles containing 0.42% soy proteins and 0.18% whey proteins, casein micelles containing 0.18% soy proteins and 0.42% whey proteins, and casein micelles with only soy proteins (0.42% and 0.18%) or only whey proteins (0.42% and 0.18%) (B). Measured values of pH are also indicated (●).

While the initial particle size of unheated mixtures containing casein micelles and soy proteins was not different from those of heated and unheated controls (casein micelles only), heated samples containing casein micelles and soy protein showed a significantly smaller apparent diameter. This decrease in diameter is not fully understood and is in contrast with data reported on heat treatment of soy protein alone, where soy proteins show an increase in the size of aggregates after heating (Roesch and Corredig, unpublished data). It could be hypothesized that the presence of casein micelles may affect the heat-induced interactions of soy proteins and perhaps induce formation of smaller aggregates of soy proteins or containing both caseins and soy proteins. This difference in size between soy−casein mixtures before and after heating has not been previously reported.

To determine the effect of the addition of soy protein on the acid-induced aggregation of casein micelles in undiluted systems, gel formation of the protein mixtures before and after heating was studied using a controlled stress rheometer (**Figures 3** and **4**).

All unheated protein mixtures, regardless of the type of protein present in the mix, formed very weak gels and showed an increase in the elastic modulus (*G*′) after about 110 min, corresponding to pH 5 (**Figure 3A,B**). In general, there was no

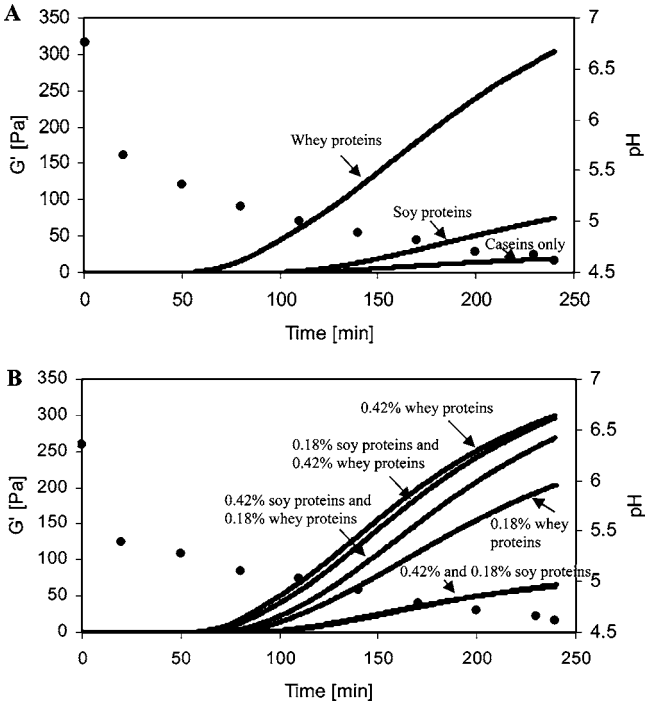

Figure 4. Development of the elastic modulus (G') as a function of time during acidification with GDL at 30 °C for heated mixtures at 90 °C for 10 min containing casein micelles with soy proteins (0.6%), casein micelles with whey proteins (0.6%), or casein micelles suspended in permeate with no protein added (A), casein micelles containing 0.42% soy proteins and 0.18% whey proteins, casein micelles containing 0.18% soy proteins and 0.42% whey proteins, and controls containing casein micelles with only soy proteins (0.42% and 0.18%) or only whey proteins (0.42% and 0.18%) (B). Measured values of pH are also indicated (●).

difference in the gelling behavior between mixtures containing soy protein, whey protein, or a mixture of the two. **Table 1** summarizes the average values for the time of onset of gelation (defined as when $G' \geq 1$ Pa) as well as the values of G' measured at 1 Hz with a frequency sweep test performed at the end of the experiment (after 4 h, at pH 4.6). In unheated samples there was no significant difference in the time of onset of gelation with the exception of casein micelles and whey protein mixtures, which showed a significantly longer time of gelation. It is important to note that centrifugation and washing of the casein micelles and recombination of whey with the washed pellet may have somewhat affected the equilibrium of the original milk. All systems showed a rather slow onset of gelation and very weak gels. Unheated mixtures containing soy proteins did not show an earlier onset of gelation, as suggested by the results reported from dynamic light scattering (**Figure 1**). Data reported in **Table 1** also indicated that the average G' measured at 1 Hz for unheated samples containing casein micelles with soy proteins or a mix of soy and whey proteins were not significantly different from the control samples. It was concluded that neither soy protein nor whey proteins affected the acid-induced gelation of casein micelles in unheated samples. The low value of G' indicated that a very small number of linkages are formed during acidification in unheated mixtures. It is important to consider that these results could vary depending on the processing history, composition, and amount of soy proteins present in the system. It may be possible to hypothesize that an aggregated soy protein isolate would interact with the other proteins in the mixture differently than what is shown in this study where soluble proteins were used.

In contrast with the results on unheated mixtures, in heated mixtures soy proteins and whey proteins affected formation of the gel network (**Figure 4**). Under these experimental conditions, addition of soy proteins or whey proteins to casein micelles influenced the evolution behavior of G' and showed an early onset of gelation.

Compared to control mixtures containing only casein micelles, the heated mixtures containing only soy proteins showed a faster onset of gelation and a higher average G' value (**Table 1**). As a higher G' value was observed for heated samples containing soy protein compared to the same samples not subjected to heating, it is possible to hypothesize that an interaction occurs between caseins and soy proteins, but no data is yet available to support this hypothesis. No significant differences were shown between samples containing a different concentration of soy protein (0.18% or 0.42%). These results seemed to suggest that soy proteins, under these conditions, do not play a significant role in formation of the gel network.

After heating, all mixtures containing casein micelles and soy proteins showed a slower onset of aggregation and lower G' values than mixtures containing whey proteins. The faster onset of gelation and high G' of the heated samples containing whey proteins is attributed to formation of complexes between κ-casein, α_{s2}-casein, and whey proteins and complexes of denatured whey protein (*7*, *15*, *16*, *28*−*30*).

When soy proteins and whey proteins where combined together with casein micelles (**Figure 4B**), after heating the mixtures showed a faster onset of gelation than control samples containing only casein micelles or casein micelles with only soy protein. The amount of soy protein present in the mixture did not seem to affect the onset of aggregation or the value of G' measured at 1 Hz (**Table 1**). The mixtures containing soy proteins and whey proteins showed a similar behavior to the corresponding whey protein controls (i.e., casein micelles with whey proteins added at 70% and 30% of the total protein). These results demonstrated that the changes in the aggregation and gelation behavior of the mixtures compared to casein micelles alone were driven by the whey proteins. However, at high ratios of whey proteins to soy proteins, the presence of soy proteins did not seem to affect the G' value as there was no significant difference between samples containing 0.6% whey proteins, 0.42% whey proteins, 0.42% proteins, and 0.18% soy proteins. These are important observations because they suggest that a small replacement or incorporation of soluble soy proteins in dairy systems may not affect the gelation behavior of the mixture. These results demonstrated once again the importance of composition and processing history of the soy proteins. In fact, there are some discrepancies from earlier reports on the effect of addition of a commercial soy protein isolate on the acid-induced gelation of skim milk (*27*).

To better understand if the overall nature of the gels was different between samples prepared with different proteins, a frequency sweep test was performed at the end of the acidification experiment. **Figure 5** summarizes the average values of G' and G'' as a function of the oscillatory frequency for acidified mixtures containing whey proteins and soy proteins. Both heated and unheated samples showed a similar mechanical behavior indicating the particulate nature of the gels formed. Heated samples showed higher elastic and viscous moduli and less frequency dependence than that shown in unheated samples (**Figure 5** and **Table 2**). Addition of soy proteins to casein micelles, regardless of the ratio or concentration, did not affect the overall nature of the linkages in the gel, and all systems were networks of casein micelles. Whey proteins or soy proteins

Table 1. Onset of Gelation During Acidification (defined as the time when $G' \geq 1$ Pa) and Values of G' Measured at 1 Hz with a Frequency Sweep Test on the Acid Gels[a]

sample	soy	soy	soy	whey	whey	whey	soy/whey	soy/whey	micelles
%	0.6	0.42	0.18	0.6	0.42	0.18	0.42/0.18	0.18/0.42	0.6
					unheated samples				
gelation (min)	156.83^{b}	137.8^{c,d,e}	130.13^{d,e}	177.74^{a}	133.30^{c,d,e}	145.83^{b,d}	140.11^{c,d,e}	146.82^{b,c}	141.22^{c,d,e}
G' (Pa)	14^{z}	14^{z}	14^{z}	16^{z}	12^{z}	11^{z}	22^{z}	12^{z}	21^{z}
					heated samples				
gelation (min)	106.38^{f}	99.72^{f,g}	90.44^{g,h}	61.45^{j,k}	55.97^{k}	77.57^{h,i}	74.55^{i,j}	60.26^{j,k}	126.04^{e}
G' (Pa)	73.50^{y}	76^{y}	85^{y}	354^{v}	345^{v}	227^{x}	321^{w}	362^{v}	25^{z}

[a] Averages are compared within row and between heated and unheated samples. The amount of whey proteins and/or soy proteins added is indicated. Means were determined by the general linear model at $p < 0.05$ and Duncan grouping. Different superscripts show significant differences.

Table 2. Frequency Dependence of G' and G'' for Gels Prepared with Casein Micelles with Whey Proteins, Soy Proteins, or a Mixture of Soy and Whey Proteins (amounts indicated in table legend)[a]

sample	soy	soy	soy	whey	whey	whey	soy/whey	soy/whey	micelles
%	0.6	0.42	0.18	0.6	0.42	0.18	0.42/0.18	0.18/0.42	0.6
					unheated samples				
G' (Pa)	0.20^{a,b}	0.20^{a,b}	0.19^{a,b,c}	0.20^{a,b}	0.20^{a,b}	0.20^{a}	0.20^{a}	0.20^{a,b}	0.17^{e}
G'' (Pa)	0.19^{a,b,c,d}	0.20^{a,b,c}	0.21^{a,b}	0.17^{d}	0.21^{a,b}	0.21^{a}	0.20^{a,b}	0.17^{d}	0.14^{e}
					heated samples				
G' (Pa)	0.19^{a,b,c}	0.18^{b,c,d}	0.18^{b,c}	0.19^{a,b,c}	0.17^{c,d,e}	0.19^{a,b,c}	0.18^{b,c,d}	0.18^{c,d,e}	0.16^{d,e}
G'' (Pa)	0.18^{b,c,d}	0.18^{b,c,d}	0.18^{b,c,d}	0.18^{c,d}	0.17^{d}	0.19^{a,b,c,d}	0.18^{c,d}	0.20^{a,b}	0.15^{e}

[a] Values are calculated from the slope of log G' vs log frequency and are means of three independent experiments. Differences were determined by the general linear model procedure with Duncan grouping. Different superscripts within the rows (and between heated and unheated samples) of all G' or G'' values indicate a significant difference.

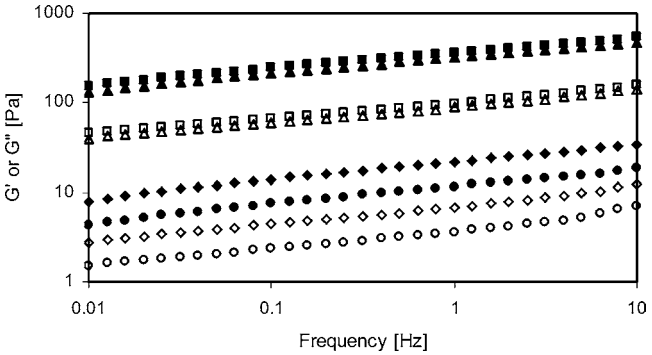

Figure 5. Average measurements of the elastic moduli G' (filled symbols) and viscous moduli G'' (empty symbols) for protein mixtures of heated (■, □) and unheated (●, ○) casein micelles with 0.18% soy proteins and 0.42% whey proteins and heated (▲,Δ) and unheated (◆, ◇) casein micelles with 0.42% soy proteins and 0.18% whey proteins.

strengthened the interactions of the acid casein gel. These results are in agreement with previous studies on acid-induced aggregation of skim milk with added soy protein (*27*), where it was also shown that regardless of the ratio of soy protein to skim milk (w/w), all gels had a similar mechanical spectrum (measured by frequency sweep). Up to a critical ratio, soy protein added to a whey protein and casein micelles system may contribute to the strength of the gel. The increase in gel strength may be attributed to interactions between soy−whey complexes as well as soy−casein complexes. Heat-induced complexes have been shown to form between soy proteins and whey proteins (*31*).

To study the effect of heating on the interactions between soy proteins and casein micelles and better understand the mechanisms related to the increase in G' of heated soy/casein micelles mixtures, size exclusion chromatography was carried out on the soluble fractions after centrifugation (19 000*g*) of the mixtures. **Figure 6** illustrates the chromatographic separation of the soluble phases of the casein samples containing soy proteins, whey proteins, or soy and whey proteins before and after heating. The soluble fraction of heated samples containing casein micelles and soy proteins showed the same elution behavior as that of unheated samples (**Figure 6A**). A peak for large aggregates eluted at 100 min for both the unheated and the heated sample. Mixtures containing whey proteins showed a peak at 180 min in the unheated samples (**Figure 6B−D**), which decreased significantly after heating. This peak corresponded to oligomers and monomers of whey proteins. When casein micelles were heated in the presence of whey proteins, size exclusion chromatography of the soluble fraction showed a large aggregated peak, eluting close to the void volume ($M_w > 10^7$ Da) at about 100 min (**Figure 6B**). These aggregates eluted earlier than in the soy protein/casein soluble fraction (**Figure 6A**). These results are in agreement with previous data on the interactions of whey proteins with casein micelles which have identified the presence of large aggregates in the soluble fraction of milk after extensive heating (*28*, *29*). These whey protein aggregates formed by disulfide exchange reactions with κ-casein (and to some extent with α_{s2}-casein) depend on heat treatment and pH and play a fundamental role in the gelling behavior of caseins during acidification (*7*, *17*, *30*, *32*). The lack of such complexes in the mixtures containing soy proteins and casein micelles suggested that a different mechanism than that attributed to whey proteins−casein mixtures is responsible for the faster onset of aggregation and higher G' values observed during rheological measurements of samples containing soy proteins.

In mixtures containing casein micelles with mixed soy and whey proteins (**Figure 6C,D**) the aggregate peak after heating was lower than that formed in samples containing caseins and whey proteins. The peak corresponding to the native whey proteins decreased significantly, indicating that whey proteins

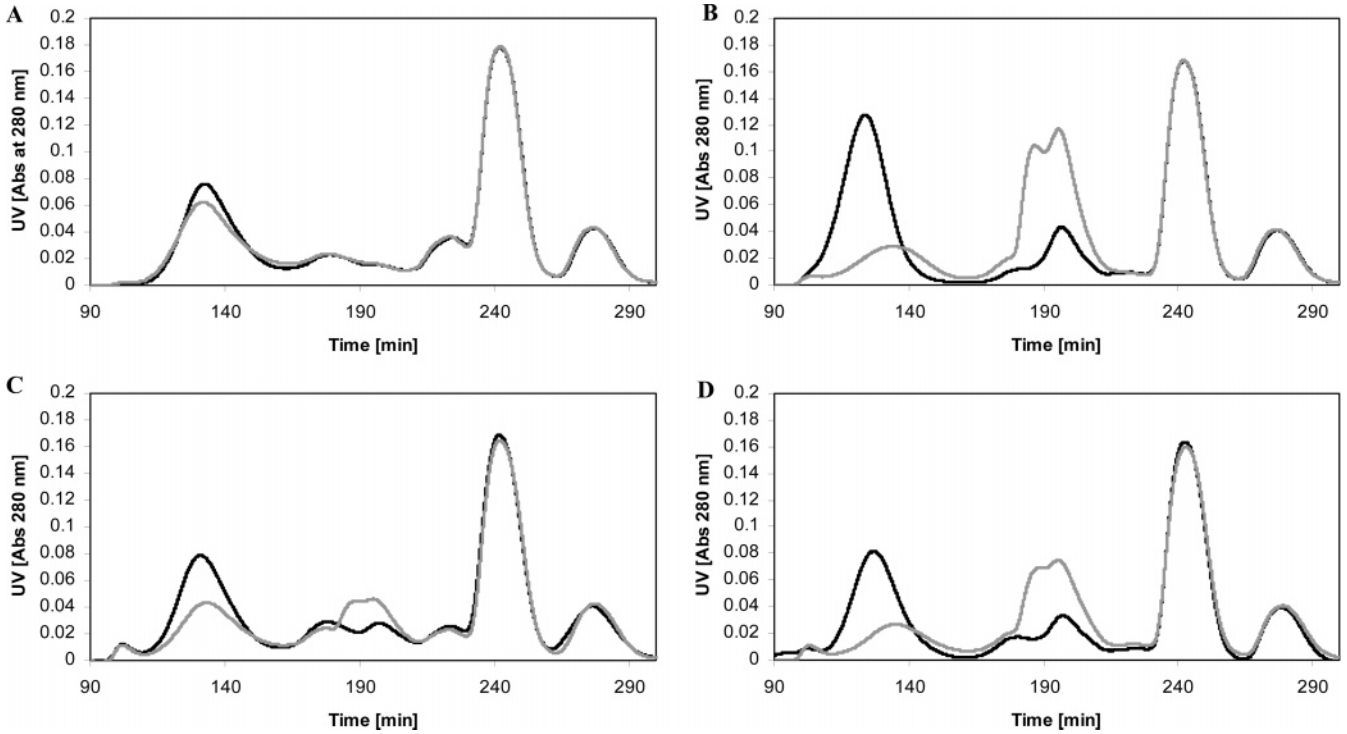

Figure 6. Protein elution profiles of soluble phases after centrifugation of unheated (gray lines) and heated at 90 °C for 10 min (black lines) casein micelles with 0.6% soy proteins (A), casein micelles with 0.6% whey proteins (B), casein micelles with 0.42% soy proteins and 0.18% whey proteins (C), casein micelles with 0.18% soy proteins and 0.42% whey proteins (D). Chromatograms are the average of two replicates.

formed complexes with heating. The absence of a significant aggregate peak in mixtures of soy proteins and whey proteins is somewhat surprising. It has been previously shown that in soy proteins/whey proteins mixtures, after heating, soluble aggregates form if whey proteins are present at low ratios (*31*). The absence of a large soluble aggregate peak in mixtures containing casein micelles, soy proteins, and whey proteins could be attributed to a preferred interaction of whey proteins with the casein micelles. It may also be hypothesized that these mixtures formed larger aggregates, which precipitated during centrifugation. A shift in the elution of the aggregated peak to an earlier time (about 100 min) was shown in the heated mixture containing whey proteins and caseins (**Figure 5B**) when compared to soy protein caseins mixtures. A similar shift, but to a lower extent, was also shown in heated mixtures containing caseins and with soy proteins and whey proteins (**Figure 6C,D**).

The aggregated peaks were collected and analyzed by SDS-PAGE electrophoresis to determine changes in the polypeptide composition of the soluble phases after heating (**Figure 7**). Samples containing only whey proteins showed the presence of κ-casein in the protein aggregate after heating (Lane 4). In samples containing soy proteins, all soy protein subunits were present in the aggregate peak as well as a κ-casein band. This κ-casein band was not as strong as in the whey protein−casein samples. Heated samples showed a decrease in the band intensity of the β-conglycinin's α and α' and the glycinin basic subunits, suggesting selective interactions between these subunits. Analysis of the composition of the aggregate peaks is just shown to indicate that specific interactions may occur between β-conglycinin and caseins.

This study differs from previous studies on soy protein−milk protein interactions because the soy protein fraction used was prepared by centrifugation; therefore, there were no insoluble soy protein aggregates in the starting mixtures. This is far from the reality of soy protein systems, where large aggregates are present in a significant amount.

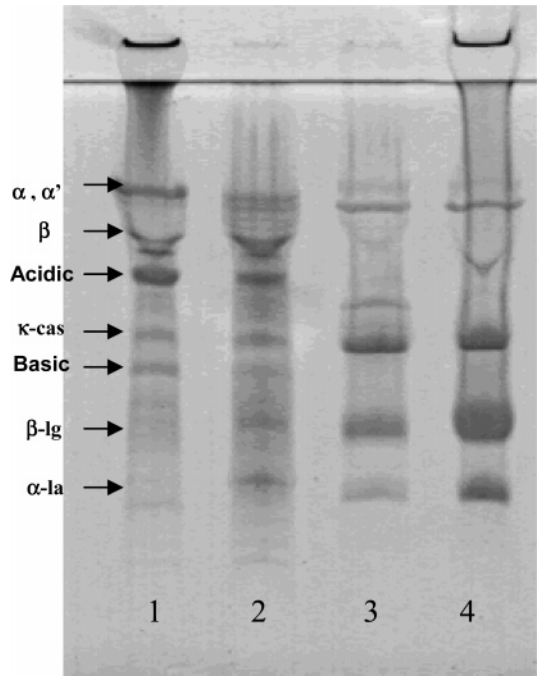

Figure 7. SDS-PAGE gel under reducing conditions of peaks collected from chromatography analysis of heated (90 °C for 10 min) and unheated protein mixtures. Lane 1: unheated casein micelles with soy proteins aggregated peak (eluting at 110 min). Lane 2: heated casein micelles with soy proteins aggregated peak (eluting at 110 min). Lane 3: unheated casein micelles with whey proteins aggregated peak (eluting at 110 min). Lane 4: heated casein micelles with whey proteins aggregated (eluting at 100 min).

The interactions occurring in these mixed systems are influenced by a combination of several factors dominated by the conditions of the protein and other insoluble materials. It is possible to hypothesize that in the presence of whey proteins,

these proteins are the main cause of the increased strength of the casein gels. Although it is understood that soy proteins interact with whey proteins during heating (*31*), in mixed systems containing casein micelles there may be a competition between the proteins and aggregate formation is not the same as for the systems in isolation. This was a first attempt at understanding how soy proteins may affect the mechanism of gelation in milk systems—looking at the soluble fraction of soy protein and how this interacts with milk proteins during acidification.

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