

Characteristics of Heated Mixed Soy Protein Isolate and Sodium Caseinate

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ABSTRACT

This study investigated the influences of protein concentration (10-15% w/v), protein ratio, pH (3.0-5.4), salt types and concentrations (Na lactate 0-250 mM or Ca lactate 0-60 mM) on the characteristics of heated mixed soy protein isolate (SPI) and sodium caseinate (SCN). Heat treatment at 80°C for 30 min did not alter the MW profiles of the SCN or SPI-SCN mixture. However, further addition of Na lactate and Ca lactate to the heated protein suspensions resulted in a high degree of aggregation observed as turbidity results, particularly in the suspensions containing a high ratio of SCN at pH 3.0 ($p < 0.05$). Salt-mediated sulfhydryl-disulfide interchange was involved in the aggregation of heated mixed protein even with an acidic pH. The second stage heat treatment (at 80°C for 30 min) of the salt-induced aggregates, at a protein concentration below the minimum concentration required to form gel (10% w/v), resulted in the formation of a stable suspension, sedimentation, a viscous suspension or gelation depending on the protein ratio, pH and salt concentration. Such two-stage heating process applied to the SPI-SCN mixture could be used to achieve both functional properties and nutritional qualities, which are desirable characteristics of high protein diets.

Key words: aggregates, caseinate, gelation, salt-induced, soy protein isolate

INTRODUCTION

Soy proteins, namely soy protein concentrate (SPC) and soy protein isolate (SPI), as well as casein have high protein digestibility measured by a corrected amino acid score (PACAAS) (Singh *et al.*, 2008). In commercial practice, they are utilized mainly for their functional rather than their nutritional aspects. However, current consumer's needs for nutritionally formulated foods, in the form of liquid, semi-solid and solid foods containing soy and milk proteins, have prompted an increase in consumption of mixed protein ingredients.

Nevertheless, little is known on the performance of mixed commercial proteins due to their complexity generated after food processing.

The major proteins in SPI are 11S globulins (glycinin) and 7S globulins (β -conglycinin), which represent 34 and 27% of proteins in SPI, respectively. Glycinin is a heterogenous oligomer of 340-375 kDa, consisting of six subunits, which are acidic subunits (A) or basic subunits (B). The acidic polypeptides in glycinin have an MW of about 37 to 45 kDa and an isoelectric pH range of between 4.2 to 4.8, while the MWs of basic polypeptides range between 18 and 20 kDa. The isoelectric pH of basic

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polypeptides is quite high; ranging between 8.0 to 8.5. The polypeptide subunits in glycinin are known to be linked by disulfide bonds (Utsumi *et al.*, 1981). The β -conglycinin, however, is a trimeric glycoprotein with MW between 140 to 170 kDa. It is composed of three types of subunit, namely α' , α , and β , with MWs of 58, 57 and 42 kDa, respectively. The α' and α subunits have an isoelectric pH of about 5.8 to 6.2. The β -conglycinin subunits are linked mainly by ionic interactions and disulfide bonds (Petrucelli and Añón, 1995). The compact structure of soy proteins requires a temperature above 80°C to denature (Sorgentini *et al.*, 1995).

Unlike the compact structure of soy globular proteins, caseins are much more flexible since they lack ordered, secondary and tertiary structures. Four individual caseins (α_{s1} -, α_{s2} -, β - and κ -caseins) are major polypeptides in casein micelles with MWs of 22, 25, 24 and 19 kDa, respectively (Farrell *et al.*, 2004) in the proportions of 4:1:4:1 (Dalglish, 1997). The α_{s1} - and α_{s2} -caseins both contain 8-9 and 10-13 phosphoserine (ser-P) residues/mole, respectively; β -casein contains 5 ser-P residues/mole; and κ -casein has only 1-2 ser-P residues/mole. Only α_{s2} - and κ -casein contain cysteine (cys). The α_{s1} - and β -casein lack both cys and cystine (cys-cys) (Dalglish, 1997). In nature, these major caseins form the quaternary structure of the casein micelle via Ca-phosphate linkages and hydrophobic interactions. However, the sodium caseinate (SCN), which is the more commonly used form at commercial scales, is composed of phosphorylated caseins and is soluble at neutral pH. The isoelectric precipitation of micellar caseins at pH 4.6 during acidification is due to the dissolution of Ca-phosphate. This generates phosphorylated caseins in SCN, which have different physicochemical properties from those of micellar casein. The isoelectric pH of phosphorylated caseins in SCN ranges between 3.5 and 4.0, which is a much lower range than that of individual caseins (Jahaniaval *et al.*, 2000).

It is apparent that the differences in physicochemical properties of both protein sources offer opportunities to exploit their functionalities more effectively in formulated foods. The mixed proteins could yield physicochemical characteristics that differ from either of the proteins individually. Roesch *et al.* (2004) have recently shown that the presence of SPC shifted the aggregation pH of pure skimmed milk from 5.3 to 5.8 in mixed skimmed milk and SPC during acidification by glucono- δ -lactone.

This study characterized the nature of the composite aggregates influenced by heat treatment and subsequent alterations of heated protein aggregates induced by Na^+ and Ca^{2+} ions at acidic pH. Heating induces molecular alterations, which result in the formation of larger aggregates. This could lead to subsequent flocculation, aggregation or gelation. With the aid of the sulfhydryl-blocker, *N*-ethylmaleimide (NEM), Hongsprabhas and Barbut (1997a) and Alting *et al.* (2004) demonstrated that the disulfide bonds were mainly involved in the heat-induced polymerization step of the globular whey proteins and ovalbumin. The objectives of the current study were thus to investigate the aggregation and gelation characteristics of SPI and SCN under different protein concentrations, protein ratios, acidic pH levels, salt types and concentrations. The insights may help in the design of formulations of high protein diets at high ionic strengths to achieve both functional properties and nutritional qualities in commercial practices.

MATERIALS AND METHODS

Sodium caseinate (SCN) (high viscous, BBA, Lactalis Industrie USA Inc., Buffalo, NY, USA) contained 6.44% moisture, 90.49% protein (N factor = 6.25) and 0.06% fat on a dried weight basis. Soy protein isolate (SPI) (PROFAM 974, Archer Daniels Midland, Decatur, IL, USA) contained 5.09% moisture, 89.59% protein (N factor = 6.25) and 0.25% fat on a dried weight

basis (AOAC, 2000).

Commercial protein suspensions (10 % protein w/v) were prepared in distilled water adjusted to pH 3.0, 4.5 or 5.4 using 1.0 M lactic acid unless stated otherwise. The mixed protein suspensions were prepared to contain protein from SPI and SCN at the ratios of 1:0, 0.7:0.3, 0.5:0.5, 0.3:0.7 and 0:1 (w/w). Then they were analyzed for final pH using an Orion pH meter, and for apparent viscosity, microstructure and MW profiles of proteins using SDS-PAGE before and after heating at 80°C for 30 min.

Apparent viscosity

Fifty mL of mixed protein suspensions (10% protein w/v) were prepared in a 100 mL beaker, stirred at 25°C using a magnetic stirrer for 30 min and measured for apparent viscosity at different temperatures using a digital Brookfield Rheometer model RVDV III (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA) equipped with the UL-adaptor at a constant shear rate of 12.2 s⁻¹. The samples were heated from 25°C to 80°C at a heating rate of 1°C/min and recorded for apparent viscosity.

Aggregate microstructure

Five mL of mixed protein suspensions (10% protein (w/v), before and after heating at 80°C for 30 min, were prepared in a 30 mL glass vial. Rhodamine B (0.01% in 95% ethanol) was added to the protein suspensions. After incubation for 5 min, each sample was loaded onto a well slide and observed for location of fluorescent-labelled protein using the Confocal Laser Scanning Microscopy or CLSM (Axio Imager MI, Carl Zeiss Pte Ltd, Jena, Thuringen, Germany). The laser source was an He/Ne laser used at an excitation wavelength of 543 nm. The micrographs were acquired at 1024×1024 pixels using the LSM 5 PASCAL program.

Sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE)

Five mL of SCN or SPI-SCN composite (at the ratio of 0.3:0.7) suspensions containing 10% protein (w/v) in distilled water acidified to pH 5.4 were prepared in a 30 mL glass vial. Both the unheated and the heated (at 80°C for 30 min) protein suspensions were characterized for MW using SDS-PAGE as described by Laemmli (1970) by a Bio-Rad mini-Protein II cell (Bio-Rad Laboratories, Hercules, CA, USA). The separation was performed at a constant voltage (150 V) for 50 min with a stacking gel containing 4% acrylamide and a separating gel containing 15% acrylamide. A continuous buffer system, containing 0.375 M Tris-HCl, pH 8.8, and 10% SDS for the separating gel and 0.124 M Tris, 0.959 M glycine, and 0.1% SDS, pH 8.3, for the running buffer, were used. Protein samples were mixed with the dissociating sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, with or without 5% β -mercaptoethanol and 1% (w/v) bromophenol blue) to give a concentration of 4 mg protein/mL. The solutions were heated at 100°C for 3 min and centrifuged at 2153g (Spectrafuge 16M, Labnet International, Inc., Wordbridge, NJ, USA) for 5 min to remove insoluble material. To each well, 5 μ L sample solution was loaded. Gel slabs were fixed and stained simultaneously using the Bio-Rad Coomassie blue R-250 stain solution (40% methanol, 10% acetic acid, 0.1% Coomassie blue R-250) for 30 min and destained by Bio-Rad Coomassie blue R-250 destain solution for 5 h with two to three changes of destain solution. The MW of each band was determined using full-range rainbow molecular weight markers, MW~10–250 kDa (RPN 8000, Amersham Biosciences UK Limited, Buckinghamshire, UK) as the MW standards. Duplicate runs were carried out.

Effect of two-stage heating process on mixed protein suspension

Appearance

The combined effect of salt concentration and reheat treatment at 80°C for 30 min on the aggregation and gelation of heated mixed protein,

the so-called “two-stage heating process” described by Hongprabhas and Barbut (1998) was investigated. Twenty five mL of mixed proteins (15% protein w/v) was prepared using water at pH 3.0, 4.5 or 5.4 as described above. The mixed protein suspensions (SPI:SCN of 0.7:0.3, 0.5:0.5 and 0.3:0.7) were heated at 80°C for 30 min and cooled at room temperature (30°C). Either Na lactate solution or Ca lactate powder was added and mixed thoroughly to obtain final concentrations of Na lactate of 0, 100 and 200 mM and Ca lactate concentrations of 0, 10 and 50 mM. Each mixed suspension was reheated at 80°C for 30 min, poured onto a Petri-dish, cooled down and observed for sedimentation, suspension or gelation.

SH-SS interchange

Ten mL of mixed protein suspensions (10% protein w/v) were prepared in water pH 3.0, 4.5 or 5.4 in a 25 mL beaker using protein ratios between SPI and SCN of 0.7:0.3, 0.5:0.5 and 0.3:0.7 (w/w), respectively. The protein suspensions were heated at 80°C for 30 min and cooled to room temperature (30°C) as described by Hongprabhas and Barbut (1997b). A stock solution of 0.167 M of N-ethylmaleimide (NEM), an SH-blocking agent, was added to the heated mixed suspensions to obtain the final concentration of NEM at 20 mM in the solvent having the corresponding pH prior to salt addition. The heated mixed protein suspensions, both in the absence and presence of NEM, were diluted to 0.04% (protein w/v) with a stock salt solution in water adjusted to pH 3.0, 4.5 or 5.4. The final concentrations of

Na lactate were 0, 15, 45, 75, 105, 135, 165, 210 and 250 mM and those of Ca lactate were 0, 5, 15, 25, 35, 45 and 60 mM. The reaction was allowed to proceed for 30 min at 30°C and the optical density was measured as OD₅₀₀ (Spectronic 20⁺, Spectronic Instruments Inc., Rochester, NY, USA) using the method described by Hongprabhas and Barbut (1997b) and calculated as turbidity (Pearce and Kinsella, 1978) using Equation 1:

$$\text{Turbidity} = \frac{2.303 * OD_{500}}{\text{pathlength (cm)}} \quad (1)$$

Statistical analysis

The experiments were carried out in three separate trials and each trial was run in triplicate. The data were analysed by analysis of variance (ANOVA) using a significance level of $p < 0.05$. Significant differences among mean values were determined by Duncan's multiple range test. All statistical analyses were performed using the SPSS Software, Version 12.

RESULTS AND DISCUSSION

Suspending commercial SPI and SCN in water with a pH ranging from 3.0 to 5.4 resulted in mixed protein suspensions with different final pH values (Table 1). Due to the high buffering capacity of both SPI and SCN, all protein suspensions had a higher final pH, which ranged from 6.38 to 7.22. A buffer system was not used in this study to avoid an increase in ionic strength from additional buffering salts. After heating at 80°C for 30 min, the SPI suspensions were very

Table 1 Effect of solvent pH on the final pH of mixed protein suspension (10% protein w/v).

Solvent pH	Final pH of SPI:SCN mixed suspension				
	1:0	0.7:0.3	0.5:0.5	0.3:0.7	0:1
3.0	6.79 ^{bc}	6.40 ^d	6.38 ^d	6.40 ^d	6.49 ^{cd}
4.5	ND*	7.22 ^a	7.19 ^a	6.94 ^{ab}	7.06 ^{ab}
5.4	ND*	7.17 ^a	7.07 ^{ab}	7.04 ^{ab}	7.11 ^{ab}

ND = not detected since the solution was very viscous and not suitable for pH evaluation.

Means followed by different superscripts are significantly different ($p < 0.05$).

viscous. Raising the SPI concentration above 10% protein (w/v) resulted in a transition from viscous fluid to self-supporting gel at 15% protein (w/v), particularly when the solvent pH ranged between 4.5 and 5.4 (Table 2), although the final pH of these suspensions was above the isoelectric pH of soy proteins of 4.8 to 6.4 (Liu, 1997). This suggested that after heating, the pH of the solvent used also influenced the aggregation, although the final pH of the protein suspensions remained the same. Such phenomena were found in acid casein gel (Bremer *et al.*, 1990).

Although SPI at the lower concentration (10% protein (w/v)) did not form a gel upon heating, the heated protein suspension was very viscous and the viscosity could not be accurately

registered by the Brookfield viscometer. Substitution of SPI with SCN in solvent pH 3.0 at different ratios reduced viscosity to less than 100 mPa·s (Figure 1). This made a heated mixed protein suspension containing SPI:SCN with a ratio of 0.5:0.5 or 0.3:0.7 suitable for high protein drinks. Although soy proteins were present in only 30% of the mixed protein, the mixed protein aggregates (SPI-SCN) before and after heating at 80°C for 30 min adopted similar microstructures to those of SPI aggregates (Figure 2). The microstructures formed were hollow, spherical aggregates with a particle size ranging between 20 to 60 µm, being much larger than those of SCN aggregates.

Table 2 Effect of pH on aggregation and gelation of soy protein isolate after heating at 80°C for 30 min.

Solvent pH	Final pH of SPI suspension at 10% protein (w/v)	Protein concentration (% w/v)					
		10	11	12	13	14	15
3.0	6.79	+	+	+	+	+	++
4.5	ND	+	+	+	++	+++	++++
5.4	ND	+	++	++	++	+++	++++

Note: ND = not detected; + = viscous suspension; ++ = viscous suspension with observable clumping; +++ = weak gel; ++++ = self-supporting gel.

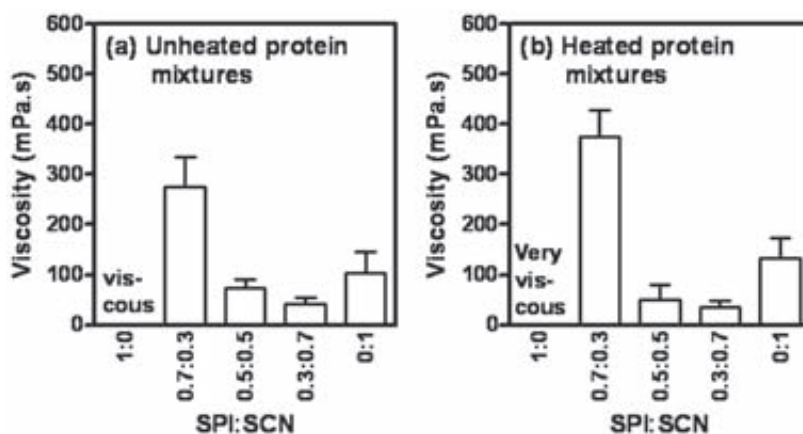


Figure 1 Apparent viscosity of mixed soy protein isolate (SPI) and sodium caseinate (SCN) suspension (10% protein w/v) in solvent pH 3.0 before and after heating at 80°C for 30 min. The final pH level of all protein suspensions was 4.2 to 4.3. Bars represent standard deviation.

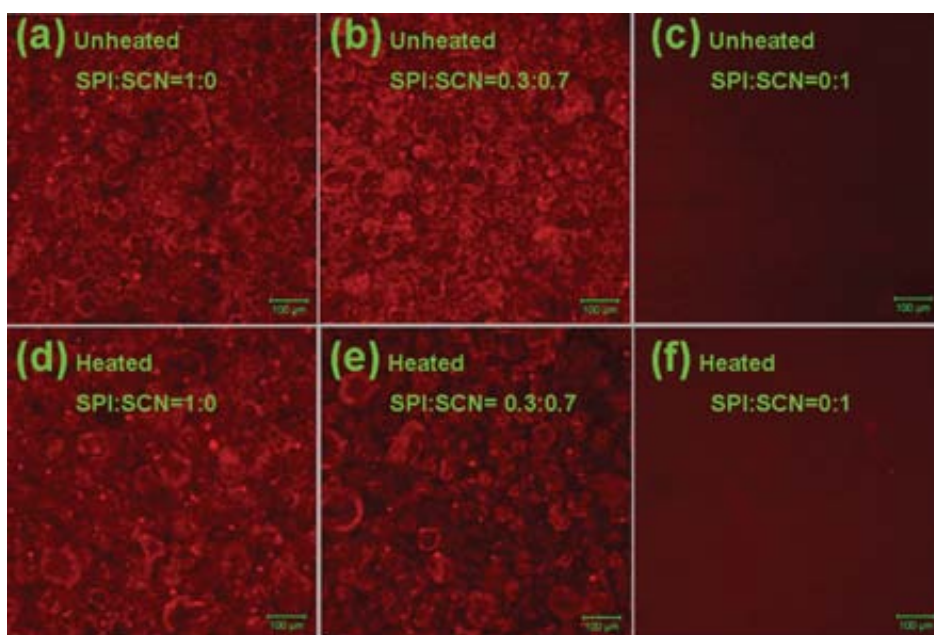


Figure 2 Microstructure of soy protein isolate (SPI), sodium caseinate (SCN) and their co-aggregates (SPI:SCN=0.3:0.7) in solvent with pH 3.0. Proteins are fluoresced in red by rhodamine B. Bar scale= 100 μ m.

However, increasing the solvent pH to 4.5 and 5.4 altered the responses of mixed proteins to Na lactate and Ca lactate addition and reheat treatment as shown in Figure 3. At a high SPI ratio (SPI:SCN=0.7:0.3), a very viscous protein suspension was formed after the reheating step, when the solvent pH was raised from 3.0 to 4.5 and 5.4 in the absence of additional salts. This suggested that reheating the heated mixed protein at a pH level above the isoelectric pH range of SPI could induce network formation although the initial heated protein suspension had low viscosity. Adding Na lactate (100 and 200 mM) or Ca lactate (10 and 50 mM) to the heated mixed protein suspension in solvents possessing a pH level of 4.5 and 5.4 and subsequent reheating, resulted in the formation of a weak- to self-supporting gel (although the protein concentration was kept at 10% protein w/v). Such a concentration was not high enough to induce the formation of heat-set gel in either SPI or SCN in the absence of additional salts.

The increase in the SCN ratio inhibited gelation of mixed protein suspensions prepared in solvents possessing a pH level of 4.5 and 5.4, although Na lactate was added up to 200 mM (Figures 3b and 3c). However, when the solvent with a pH of 3.0 was used, heavy sedimentation and water separation occurred in the presence of additional Na lactate (Figure 3a).

The mixed protein suspensions with high ratios of SCN remained fluid when Ca lactate was added up to 10 mM (Figures 3e and 3f). An increase in the Ca lactate concentration to 50 mM induced the formation of weak gel, particularly when the mixed protein suspensions were prepared in solvents with a pH level of 4.5 and 5.4. Solvents with lower pH levels generated self-supporting gels with 50 mM of added Ca lactate. Nevertheless, increasing the SCN ratio inhibited gelation, although the SPI-SCN adopted a size and shape similar to those of SPI aggregates as described previously.

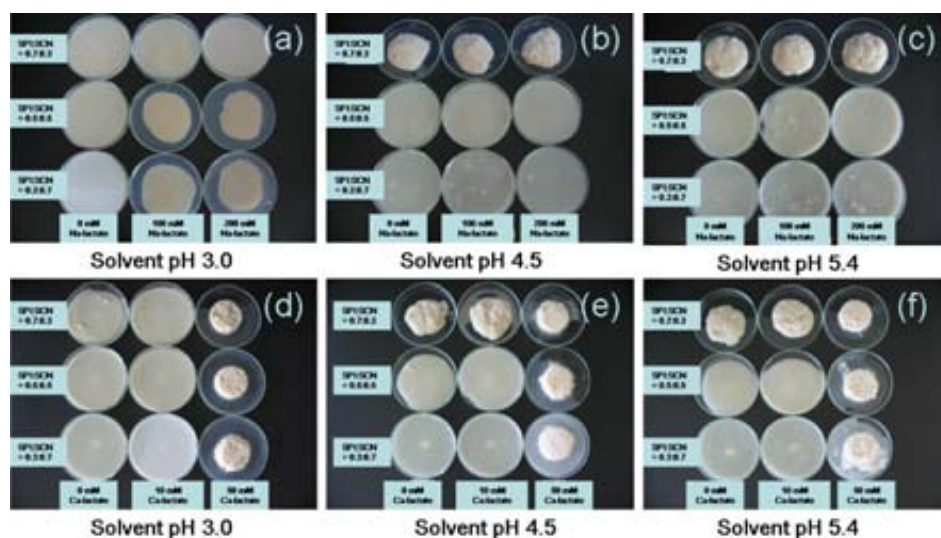


Figure 3 Combined effect of acidic pH and salt concentration on aggregation and gelation of reheated mixed soy protein isolate (SPI) and sodium caseinate (SCN) suspension (15% protein w/v).

It was apparent that pH, salt types and concentrations influenced the aggregation and gelation behavior of the heated mixed proteins at protein concentrations not high enough to induce heat-set gel. This could be crucial in the formulation and processing of, for example: diets with specific requirements, such as liquid enteral formulation or tube feeding which require complete nutrients and calories; and in high-protein diets supplemented with minerals, mixed soy and milk protein gel with high water holding capacity.

Figures 4 and 5 indicate that the pH of the suspension strongly determined the subsequent aggregation of the reheated mixed protein, compared to the salt concentration. The turbidity of the suspension was measured after the heated protein-salt reactions had proceeded for 30 min. The addition of Na lactate in heated protein suspension at a pH level of 3.0 increased the turbidity of the suspensions (Figures 4a, 4d and 4g) to the highest extent when the ratio of SCN was high (Figure 4g). At such SPI-SCN ratios, the addition of the SH-blocking agent, NEM, prior to the addition of salt lowered the aggregation. The

influence of NEM was not observed at higher pH levels and with other protein ratios of Na-induced aggregates.

Ca lactate affected the aggregation of heated mixed protein suspension differently. The presence of the phosphorylated α_{s1} -casein and β -casein, which are the major polypeptides in SCN, makes the mixed-protein suspensions prone to aggregation when ionic calcium was added above 10 mM (Dickinson and Davies, 1999; Dickinson and Golding, 1998). An increase in the Ca lactate concentration dramatically increased the aggregation in all suspensions, particularly in suspensions containing high ratios of SCN at pH 3.0 (Figure 5g). The presence of NEM could significantly block the SH-SS interchange mediated by the addition of Ca lactate to the heated mixed protein suspensions containing an SPI:SCN ratio of 0.7:0.3 at pH 5.4, 0.5:0.5 at pH 3.0 and pH 5.4 and 0.3:0.7 at pH 3.0, pH 4.5 and pH 5.4. This suggested that the SS-SH interchange was induced at a high ratio of SCN between pH 3.0 and 5.4. At a high ratio of SPI, the pH needed to be raised to 5.4 for SH-SS to be involved in the aggregation.

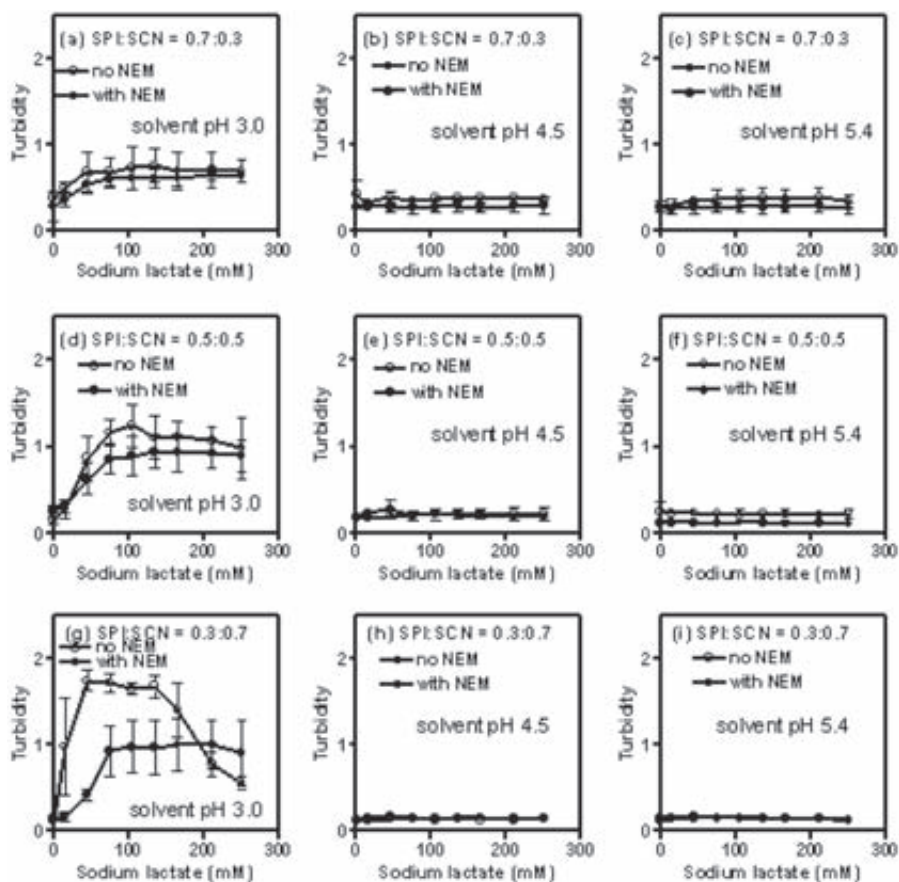


Figure 4 Effect of sodium lactate concentration on aggregation (measured as turbidity) of heated mixed soy protein isolate (SPI) and sodium caseinate (SCN) in the absence and presence of the sulfhydryl blocking agent, *N*-ethylmaleimide, prior to salt addition. Heated mixed proteins were prepared in different ratios using solvents with different pH levels. Bars represent standard deviation.

The heating step employed to induce protein suspension at 80°C for 30 min prior to salt addition was not high enough to denature all soy proteins. Figure 6 shows that the commercial SCN was mainly composed of polypeptides with their MW within the range of 25 to 30 kDa (Lane 2). This was slightly higher than the MW of caseins (19-25 kDa) due to phosphorylation and glycosylation of proteins. Heating did not induce any MW changes in commercial SCN (Lane 4). The high MW polypeptides deposited in the wells in Lane 2 were polymerized caseins due to commercial processing. They were previously

polymerized before the heat treatment at 80°C for 30 min in this study via disulfide bond formation, since they no longer existed in the presence of β -mercaptoethanol (Lane 6). Lanes 3 and 5 show additional bands of soy proteins with MWs slightly below 50 kDa, 75 kDa and slightly above 75 kDa. Heating at 80°C for 30 min did not alter these bands (Lane 5). Lane 7 shows extra bands with various MWs, indicating that some of the soy proteins were linked by a disulfide bond. The aggregates precipitated in the well in Lane 7 suggested that the heated mixed proteins contained covalent non-disulfide bonds, which could be

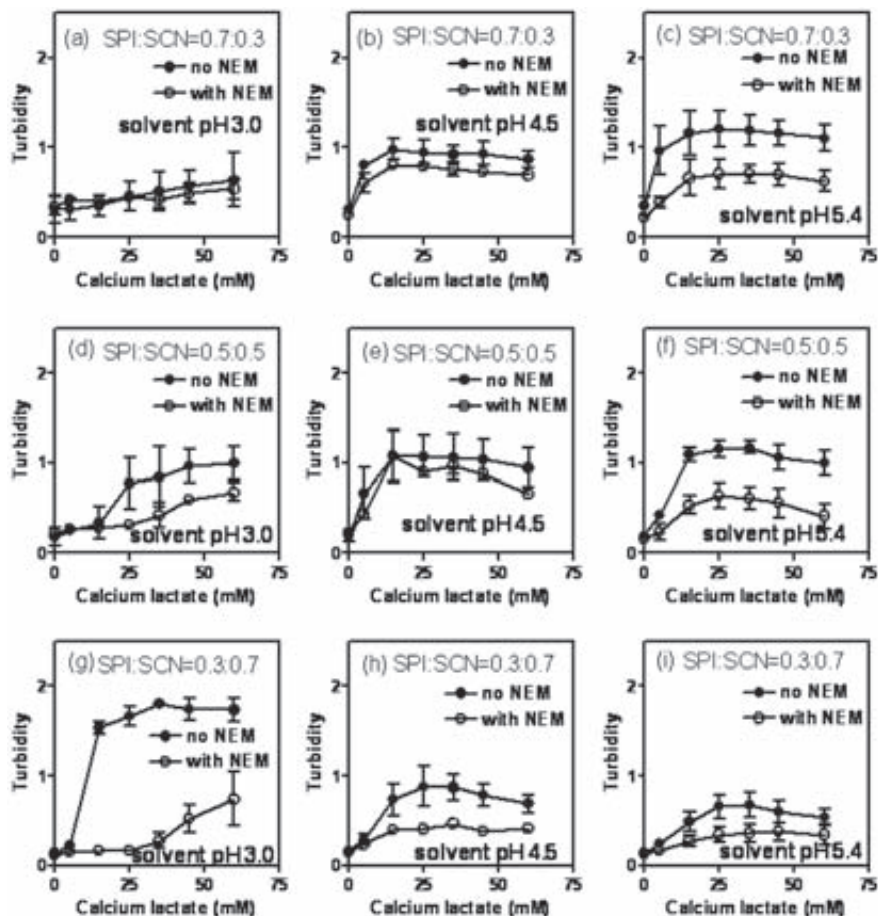


Figure 5 Effect of calcium lactate concentration on aggregation (measured as turbidity) of heated mixed soy protein isolate (SPI) and sodium caseinate (SCN) in the absence and presence of the sulfhydryl blocking agent, *N*-ethylmaleimide, prior to salt addition. Heated mixed proteins were prepared in different ratios using solvents with different pH levels. Bars represent standard deviation.

formed in the original commercial SPI and/or resulted from the heating process at 80°C for 30 min.

Although the first heating step at 80°C for 30 min was not high enough to affect protein-protein interactions via disulfide bond formation, the salt-mediated SH-SS interchange was more apparent in the composite suspensions containing high ratios of SCN to SPI ($p < 0.05$). The SH-SS interchange usually occurs at an alkaline pH; i.e., 8.3. However, Alting *et al.* (2000) and Vasbinder *et al.* (2003) have recently demonstrated that such

interaction could occur at an acidic pH level of ~4.6 to 5.0. The current study further expanded that at a pH level of 3.0, the SH-SS interchange could also occur in heated mixed proteins of SPI and SCN. The mechanism of Ca lactate-mediated SH-SS interchange even at low pH is postulated to occur via the charge dispersion of ions as described by the DLVO theory and Ca^{2+} crosslinking (Israelachvili, 1992), which induces the soluble or insoluble protein aggregates to come close enough to interact via disulfide bond formation.

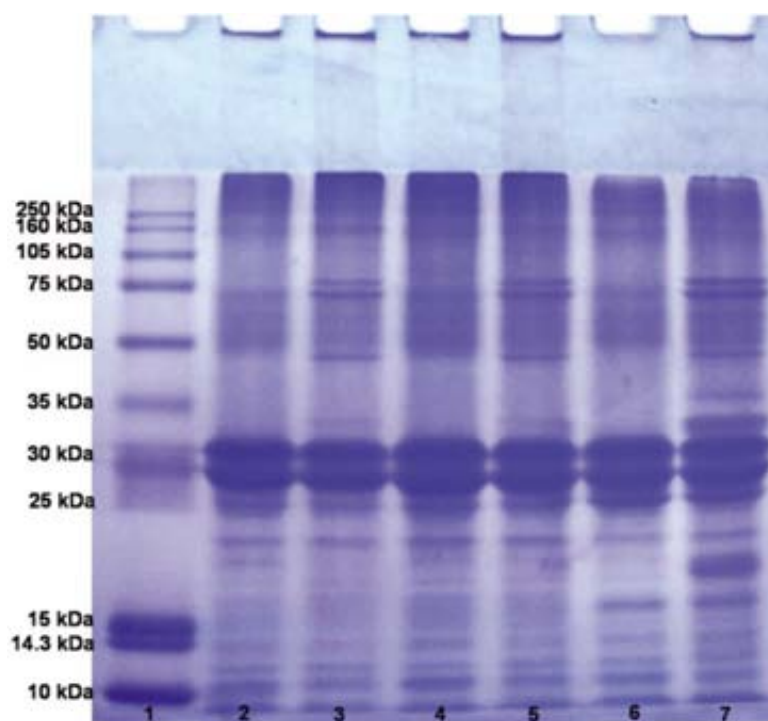


Figure 6 MW profile of sodium caseinate (SCN) and mixed soy protein isolate (SPI) and sodium caseinate (SPI-SCN) evaluated by SDS-PAGE. **Lane 1** = wide range MW standards; **Lane 2** = unheated SCN; **Lane 3** = unheated SPI-SCN (0.3:0.7); **Lane 4** = heated SCN at 80°C 30 min; **Lane 5** = heated SPI-SCN (0.3:0.7) at 80°C 30 min; **Lane 6** = heated SCN in the presence of β -ME; and **Lane 7** = heated SPI-SCN in the presence of β -ME.

CONCLUSION

This study revealed that the salt-induced aggregation of heated (80°C for 30 min) mixed protein of SPI and SCN involved charge dispersion at high ion concentration, Ca^{2+} crosslinking and Ca^{2+} -mediated SH-SS interchange even in an acidic pH range. The interactions of the aggregates depended on the ratios between both protein sources and the pH, which resulted in sensitivity to ionic Ca^{2+} in the heated mixed proteins. Further salt-induced aggregation or gelation of the heated mixed protein suspension can be generated by a second stage heating process at 80°C for 30 min. This can be used in designing the physical properties of liquid, semi-solid and solid formulations containing high

protein and mineral contents.

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