Effects of Calcium and Pressure Treatment on Thermal Gelation of Soybean Protein

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ABSTRACT: The effect of calcium and high-pressure (HP) treatment on the heat gelation of soybean proteins was investigated. In the presence of calcium (2 to 25 mM), the gelation of dispersions of soybean protein isolate (SPI), a β-conglycinin-enriched fraction (7SEF), and a glycinin-enriched fraction (11SEF) started with protein having a lower degree of denaturation. The gels from these dispersions had greater stiffness than the samples without added calcium. HP treatment had different effects on heat-induced gelation depending on the presence of calcium and on the nature of the proteins. In the absence of calcium, gels with low stiffness were formed after HP treatment, compared with untreated samples, and regardless of the sample type (SPI, 7SEF, 11SEF). In the presence of calcium, gel stiffness was increased after HP treatment of dispersions containing β-conglycinin (SPI and 7SEF), while the opposite effect was observed for 11SEF. In the presence of calcium, HP treatment promoted a greater contribution of hydrophobic interactions in SPI and 7SEF. In the dispersions containing β-conglycinin, these conditions also promoted the appearance of a heterogeneous distribution of molecular sizes, from enormous aggregates to dissociated species. Our results suggest that, in the presence of calcium, HP treatment has an opposite effect on the ability of glycinin and β-conglycinin to participate in the formation of a 3-dimensional network upon heating.

Keywords: calcium, high-pressure treatment, soybean protein, thermal gelation

Introduction

Soybean proteins, in addition to being a cheap source of protein, have unique functional properties and attractive nutritional characteristics, which explain their increasing use in the food industry as food ingredients. Among their functional properties, thermal gelation is vital in many food applications. Thermal gel formation of globular proteins is a complex process, involving several steps such as denaturation, dissociation–association, and aggregation (Hermansson 1978). The final characteristics of the protein gels depend on many parameters, including the nature of the proteins and their composition and concentration, as well as environmental parameters such as the presence of salts and pH, and gelation conditions (temperature, time) (Hermansson 1986; Puppo and Añón 1999; Hua and others 2005).

β-Conglycinin (7S) and glycinin (11S) are the major globulins in soybean, representing 80% of total protein in the seed. β-Conglycinin is a trimeric glycoprotein constituted by 3 subunits α, α', and β (68, 72, and 52 kDa, respectively) (Thanh and Shibasaki 1976, 1978). Glycinin is a hexameric protein constituted by 5 possible types of subunit AB (58–69 kDa), which in reducing conditions produce the polypeptides A and B by cleavage of disulfide bridges (Nielsen 1985). Soy proteins are generally added to food products as a high protein content mixture, that is as a soybean protein isolate (SPI), but glycinin and β-conglycinin have specific functional properties that might be of interest to the food industry (Rickert and others 2004). The development of simplified procedures to obtain purified glycinin and β-conglycinin (Rickert and others 2004), along with their unique functional properties, explains the interest in studying each individual protein as a food ingredient.

Calcium has a significant impact on soy protein functionality because of the protein interactions promoted by its presence (Appu Rao and Narasinga Rao 1975, 1976; Scilingo and Añón 2004). It is also an important nutritional ingredient that is present in many food products, particularly dairy analogue products. Calcium interacts with denatured soy proteins, this being the basis of tofu making, and its presence promotes the formation of complexes with soybean protein hydrolysates (Bao and others 2008). Less information is available about the mechanisms of calcium and protein interactions during heat-induced gelation of SPI, 7S or 11S dispersions.

Interest in high-pressure (HP) processing is mainly related to food preservation but this technology, which affects protein conformation and therefore modifies protein properties, can be used to obtain food products with unique attributes. It has been demonstrated that HP processing promotes soy protein aggregation and denaturation (Puppo and others 2004; Lakshmanan and others 2006). Changes in the secondary, tertiary, and quaternary structure due to pressure effects on hydrophobic and electrostatic interactions could modify the ability of soybean proteins to form 3-dimensional networks (Balny and Masson 1993). The formation of soybean protein gels under pressure has been reported for an SPI dispersion with a protein concentration equal to or higher than 17% (w/w) (Dumoulin and others 1998; Molina and others 2002). Calcium addition and HP treatment of soy proteins prior to thermal gel formation are likely to modify the properties of the gels but this has not been reported yet.

This study investigates the thermal-induced gel properties of SPI, and glycinin-enriched and β-conglycinin-enriched fractions, and how the presence of calcium and HP treatment affect these properties.

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Materials and Methods

Preparation of the soybean protein fractions

SPI was prepared from defatted flour (Nutrisoy 7B) manufactured by Archer Daniels Midland Co. (Decatur, Ill., U.S.A). An alkaline extraction (pH 8.0), followed by isoelectric precipitation at pH 4.5 was carried out according to Speroni Aguirre and others (2007). The isoelectric precipitate was dispersed into distilled water and adjusted to pH 8.0 with 2 N NaOH. The dispersion was then freeze-dried.

Preparation of 7S- and 11S-enriched fractions. Globulins 7S and 11S were partially purified according to the method of Nagano and others (1992). The defatted flour was dispersed into distilled water (1:15, w/w), adjusted to pH 8.0 with 2 N NaOH, stirred at room temperature for 90 min and then centrifuged at 10000 × *g* for 30 min at 4 °C. Dry sodium bisulfite ($NaHSO_3$) was added to the supernatant at a concentration of 0.98 g $NaHSO_3$/L, the pH was adjusted to 6.4 with 2 N HCl, and the mixture was kept overnight at 4 °C. The dispersion was centrifuged at 6500 × *g* for 20 min at 4 °C. The precipitate (11S-enriched fraction, abbreviated 11SEF) was suspended in distilled water, adjusted to pH 8.0 with 2 N NaOH, dialyzed against distilled water and freeze-dried. Solid NaCl was added to supernatant (0.25 mol/L) and the pH was adjusted to 5.0 with 2 N HCl. After 1 h, the insoluble fraction was removed by centrifugation at 10000 × *g* for 30 min at 4 °C. The supernatant was diluted 2-fold with cold water and the pH was adjusted to 4.8 with 2 N HCl. Centrifugation at 6500 × *g* 20 min at 4 °C was carried out. The washed precipitate (7S-enriched fraction, abbreviated 7SEF) was suspended into distilled water, adjusted to pH 8.0 with 2 N NaOH, and dialyzed against distilled water before freeze-drying.

Dispersions of 8% and 10% protein (w/w) were prepared with a pH 8.0 Tris-HCl 50 mM buffer. $CaCl_2$ was added at 2 and 20 mM concentration to the 8% dispersions and 2.5 and 25 mM concentration to the 10% ones.

HP treatment

HP treatment of 8% and 10% w/w dispersions (with or without calcium addition) was carried out in a 3.0 L reactor unit (ACB Pressure Systems, Nantes, France). Dispersions were sealed such that the headspace in the polyethylene bag was kept to a minimum (La Bovida, France). HP treatment was carried out at an initial temperature of 20°C at 200 and 400 MPa (±7 MPa) for a dwell time of 10 min. The level of pressure was reached at 3.4 MPa/s and released almost instantaneously. The temperature during treatment was controlled to avoid overheating the proteins. The temperature increase due to adiabatic heating was 1.5 °C/100 MPa.

Small deformation rheology

Thermal gel formation was followed by small deformation rheology measurements with an AR1000 rheometer (TA Instruments New Castle, Del., U.S.A.) equipped with a cone/plate geometry probe (40 mm dia, 2° angle). Measurements were carried out at a constant strain of 1%, which was within the linear region, and at an angular frequency of 6.28 rad/s. In order to avoid water evaporation, a layer of paraffin oil was applied around the sample. The thermal cycle consisted of a heating step from 20 to 95 °C at a heating rate of 1 °C/min, followed by an isothermal step of 30 min at 95 °C (plateau) and a cooling step to 20 °C at 1 °C/min. The stiffness of the gel was characterized by the elastic modulus (G′) at the end of the cycle (Renkema 2004). In some cases, the determination of gelation temperature (Tgel) is not simple because, for certain protein dispersions, G′ may be higher than G″ but be a liquid (Gosal and Ross-Murphy 2000). Renkema and others (2001) observed a higher G′ than G″ (before thermal treatment) for β-conglycinin. In that case, the gelation temperature was defined as the temperature at which the increase in G′ is greater than 0.5 Pa/K. In our study, the Tgel of the calcium-enriched dispersions (and its respective controls without calcium) was the temperature at which G′ was equal to G″, whereas for calcium-enriched and HP partially denatured protein dispersions (and its respective controls without calcium and not HP-treated), Tgel was the temperature at which G′ started to increase (Gosal and Ross-Murphy 2000; Renkema and others 2001).

To evaluate the fraction of structures formed during heating, a quotient (Q) was calculated as the ratio between G′ reached at the end of the plateau and G′ reached at the end of the thermal cycle. For example, if at the end of plateau at 95 °C G′ was 200 Pa and at the end of thermal cycle G′ was 1000 Pa, *Q* value was 0.2.

Differential scanning calorimetry

Differential scanning calorimetry was performed on a Micro DSC III (SETARAM, Caluire, France). Control and treated SPI, 7SEF, and 11SEF dispersions at pH 8.0 (10% w/w protein, 0.7 mL, 70 mg of dry matter) in a Tris-HCl 50 mM buffer were heated in the calorimeter from 20 to 110 °C at 1 °C/min. Samples were hermetically sealed (screw top with o-ring) in 1mL iron pans. Sample buffer was used as a reference. The enthalpy of thermal denaturation (ΔH) was estimated as the area of the peaks of the heat-flow compared to time curve. The temperature of denaturation (Td) was estimated as the temperature of maximum heat absorption (Figure 1). Enthalpy values and Td of each peak were calculated by Micro DCS III software. Values were the mean of 3 measurements of 3 independent replicates.

Size exclusion chromatography

Soy protein dispersions at an 8% (w/w) protein concentration with 20 mM and 2 mM of calcium for SPI and 7SEF, and 11SEF, respectively were diluted 8 times with pH 8.0 Tris-HCl 50 mM buffer. The samples were filtered through a 0.45 μm cellulose acetate filter (Sartorius AG, Goettingen, Germany). The column buffer was the same as the sample buffer. Samples (200 μL) were eluted on a Sephacryl S-500 column (Amersham Biosciences, U.K.) (1000 × 16 mm) at a flow rate of 1 mL/min. The absorbance was recorded at 215 nm.

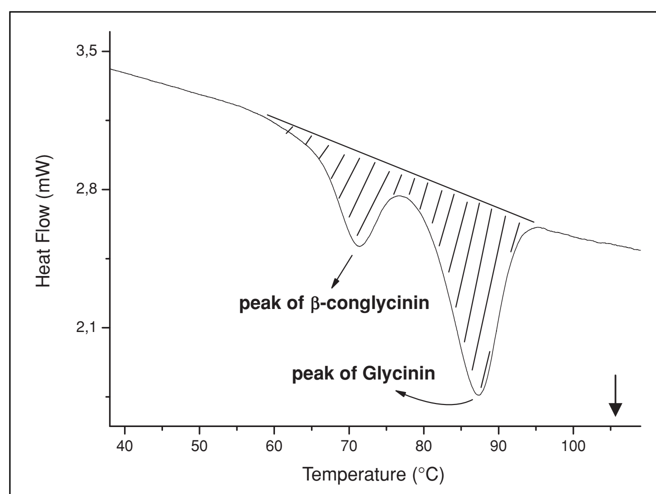

Figure 1 — DSC thermogram of control SPI. Heating rate was 1 °C/min. Striped area is proportional to ΔH. The arrow indicates endothermic heat flow.

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Electric charge of the proteins

Lasergene® v7.1.0 software (DNASTAR, Inc., Madison, Wis., U.S.A.) was used to compare the value of the electric charge of β-conglycinin and glycinin.

Statistical analysis

Each treatment was performed at least in triplicate. Statistical analysis was completed using Sigmastat software (Systat Software, Chicago, Ill., U.S.A.). Analyses of variance were conducted. Differences between the sample means were analyzed by Tukey's test at an α level of 0.05.

Results and Discussion

Protein composition of SPI, 7SEF, and 11SEF

SPI contained β-conglycinin and glycinin in a similar proportion and a small amount of lectin and Kunitz trypsin inhibitor (SDS PAGE results not shown). Its protein content determined by the Kjeldahl method was 86.6% (conversion factor of 5.8). 7SEF contained β-conglycinin in a high proportion (75%) and glycinin (25%), and its protein content was 84.5%. 11SEF was almost exclusively composed by glycinin, with a protein content of 93.0%. (SDS PAGE data not shown).

Effects of calcium addition on soybean protein gelation

(a) Effect of calcium on the native state of the proteins and gelation temperatures. Figure 1 represents a typical DSC thermogram of a control SPI showing the peaks due to denaturation (Td) of β-conglycinin and glycinin. To begin gelation, a protein dispersion must reach a temperature where the proteins undergo conformational changes that lead to the exposure of sulfhydryl and hydrophobic groups that can react together. However, the gelation process also depends on the ionic strength and protein concentration of the dispersion. Lakemond and others (2003) found that for glycinin at different pHs and ionic strengths, gelation always started at a higher temperature than the onset of denaturation. Table 1 summarizes the temperature of denaturation (Td) of β-conglycinin and glycinin, and the temperature of gel formation (Tgel) for SPI, 7SEF, and 11SEF at different calcium concentrations. For 11SEF, the maximal tested concentration was 2.5 mM, because at 5 mM, there was an instantaneous separation of the aqueous phase and the proteins that prevented the gelation assay in the rheometer. In the absence of calcium, Tgel of 7SEF had no significant difference from the value of SPI, followed by 11SEF, with values of 84.7, 87.2, and 95 °C, respectively. As illustrated in Figure 2, according to the proportion of β-conglycinin and glycinin present in each product, which is reflected by the relative areas of the peaks, both proteins exhibited a considerable degree of denaturation at their respective Tgel. For 11SEF in the absence of calcium, complete denaturation of glycinin was necessary to start gelation: the endothermal peak ended at 92.5 °C whereas gelation started after 3.3 min of plateau at 95 °C (Tgel of 95 °C and Td of 85 °C, Figure 2C). For SPI and 7SEF dispersions without calcium, Tgel coincided with the Td of glycinin, suggesting that gel formation occurred without complete denaturation of glycinin. In both cases, Tgel was well beyond the Td of β-conglycinin. Therefore, glycinin must be totally denatured to gelify while, when both proteins are present, the gelation can start at a lower temperature, which agrees with the findings of Renkema and others (2000). Some of these differences can be attributed to the polypeptide composition of the aggregates formed during gelation. When both β-conglycinin and glycinin were present, aggregates composed by β-subunit of β-conglycinin and Basic-subunit of glycinin were probably responsible for the protein network whereas aggregates composed by acidic- and basic-subunits of glycinin were involved when glycinin was alone (Utsumi and Kinsella 1985; Lakemond and others 2003; Hua and others 2005).

Addition of 25 mM of calcium significantly increased the β-conglycinin Td in SPI and 7SEF, while a concentration of 2.5 mM had no impact on the β-conglycinin Td in SPI, and slightly decreased it in 7SEF (Table 1). The Td of glycinin was increased regardless of the calcium concentration in the SPI, 7SEF, and 11SEF fractions. Adding 2.5 mM calcium decreased the Tgel values of 11SEF from 95.0 to 93.2 °C, while for SPI and 7SEF 25 mM calcium was needed to reduce them significantly. The decrease in the Tgel value was more pronounced for SPI, with a diminution of 15.5 °C, against 7 °C for 7SEF. Therefore, in the presence of calcium, soybean gel formation occurs with proteins that have maintained a great proportion of their native state (Figure 2). It is worth noting that in the 25 mM calcium SPI sample, Tgel was between the onset of denaturation and Td, suggesting that despite the high proportion of native state, rheological properties of SPI were very sensitive to thermal treatment in calcium presence.

Salt addition induces the stabilization of quaternary structure, thus increasing the Td of soybean proteins (Hermansson 1986). Moreover, when protein starts unfolding, salts diminish the electrostatic repulsion between polypeptide chains by increasing ionic strength, thus favoring the interactions between them. Besides, interactions between the imidazol group of histidine residues and calcium have been described (Appu Rao and Narasinga Rao 1976; Scilingo and Añón 2004). It is more likely that these interactions promoted protein network formation, even when proteins were not completely unfolded. As gelation started at lower temperatures, our results indicate that the balance between these effects favored the association phenomena. The soybean protein aggregation promoted by calcium would allow the gelation of proteins that were less denatured.

(b) Effect of calcium on the stiffness of gels. Values of G′ at the end of the cycle, as a measure of gel stiffness, are shown in Table 2. In the absence of calcium, the gel with the highest stiffness value (649 Pa) was obtained for the 11SEF fraction, which confirms the results of Renkema and others (2001). Utsumi and Kinsella (1985a), however, observed decreasing hardness for gels prepared from 7S,

Table 1 — Effect of calcium concentration on gelation temperature (Tgel) and temperature of denaturation (Td) of β-conglycinin and glycinin from soy protein isolate, and glycinin- and β-conglycinin-enriched fraction.

Calcium concentration (mM)	SPI[A] Td (°C) β-con[D]	SPI[A] Td (°C) Glycinin	SPI[A] Tgel (°C)	7SEF[B] Td (°C) β-con	7SEF[B] Td (°C) Glycinin	7SEF[B] Tgel (°C)	11SEF[C] Td (°C) Glycinin	11SEF[C] Tgel (°C)
0	71.2 ± 0.1^a	86.9 ± 0.2^a	87.2 ± 1.3^a	71.5 ± 0.1^a	84.0 ± 0.1^a	84.7 ± 1.9^a	84.1 ± 0.1^a	95.0 ± 0.0^a
2.5	71.2 ± 0.2^a	87.4 ± 0.2^a	87.3 ± 0.3^a	71.0 ± 0.1^b	84.6 ± 0.1^b	82.0 ± 2.4^a	87.7 ± 0.2^b	93.2 ± 0.3^b
25	73.7 ± 0.2^b	95.0 ± 0.1^b	71.7 ± 2.3^b	76.3 ± 0.1^c	97.1 ± 0.2^c	77.7 ± 0.2^b	–	–

[A]SPI = soy protein isolate, [B]7SEF = β-conglycinin-enriched fraction, [C]11SEF = glycinin-enriched fraction, [D]β-conglycinin. Protein concentration was 10% w/w. In each column, values sharing the same superscript are not significantly different ($P < 0.05$).

SPI, and 11S fractions. This discrepancy could be explained by the temperature profile applied to the samples in the study of Utsumi and Kinsella, which was an isothermal heating at 80 °C for 30 min, followed by an instantaneous cooling. These conditions might have only partially denatured the glycinin. For 11SEF, adding 2.5 mM calcium significantly increased G′ while, in the case of SPI and 7SEF, adding 25 mM calcium resulted in a dramatic G′ increase, which was 20-fold for 7SEF. Interactions between calcium and subunits of β-conglycinin, but also potentially the subunits of the contaminant glycinin, could be at the origin of this increase. The comparison of the G′ increase of 7SEF and SPI, which was much lower for SPI (only 7-fold), suggests that either glycinin contamination did not participate in the observed increased G′ value for 7SEF, and/or its concentration has a major effect.

Increased coagulation of glycinin and β-conglycinin during heating due to the presence of calcium might have contributed to the observed increase in gel stiffness (Appu Rao and Narasinga Rao 1975). Adding 25 mM calcium (11.5 mg calcium / g protein) might also have favored bridges that promoted a glycinin-calcium structure (Scilingo and Añón 1996). The exact involvement of each protein needs further investigation; Renkema and others (2000) indeed hypothesized that only the heat-precipitated polypeptides participate in the protein network.

(c) Effect of calcium on the nature of the interactions. Once denatured, interactions/associations of polypeptides between themselves and with water could result in the formation of a 3-dimensional network. Some of the interactions involved may be called "enthalpic" as they are favored by a reduction in temperature, such as hydrogen bonds. On the other hand, hydrophobic interactions are favored by an increase in temperature, and they are named "entropic." An abundant number of interactions results in a stronger gel, therefore the stiffness may be related to the number of interactions. In order to evaluate the proportions of enthalpic and entropic interactions in a gel, a quotient (Q) was calculated between the value of G′ at the end of the plateau at 95 °C and the G′ value at the end of the cooling stage. The Q value for all the gels was less than 0.27, indicating that most of the associations developed during cooling (Table 2, Figure 3), and suggesting that the main interactions involved in these gels were hydrogen bonds. It was indeed observed by Renkema and Van Vliet (2002) in similar assays that included 2 thermal cycles, that the increase achieved in G′ during cooling could be almost completely reversed on a 2nd heating,

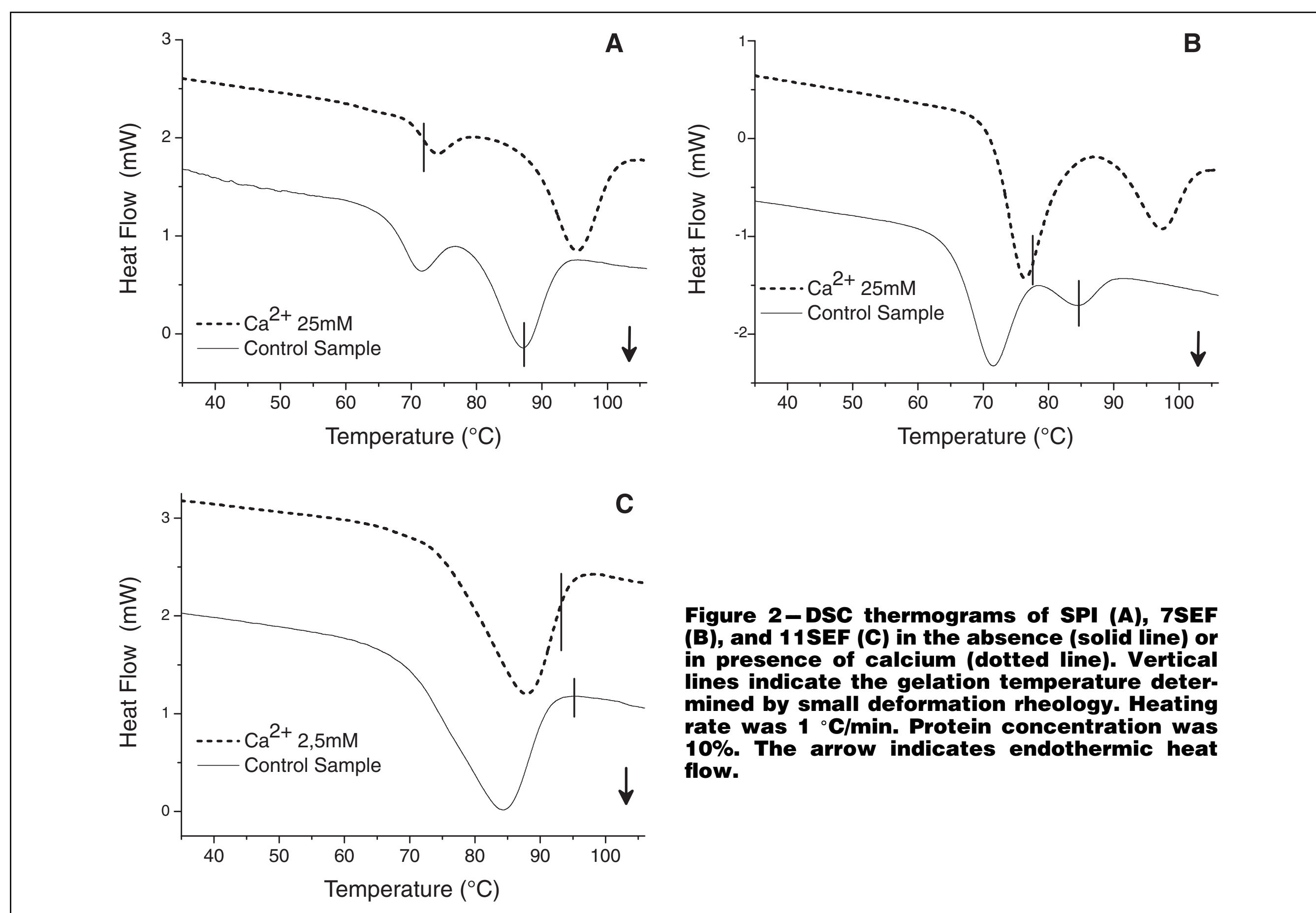

Figure 2–DSC thermograms of SPI (A), 7SEF (B), and 11SEF (C) in the absence (solid line) or in presence of calcium (dotted line). Vertical lines indicate the gelation temperature determined by small deformation rheology. Heating rate was 1 °C/min. Protein concentration was 10%. The arrow indicates endothermic heat flow.

Table 2– Effect of calcium concentration on stiffness (G′) and the nature of the interactions that establish the gel (Q).

	SPI		7SEF		11SEF	
Ca^{2+}(mM)	G′(Pa)	Q	G′(Pa)	Q	G′(Pa)	Q
0	279 ± 80^a	0.099 ± 0.017^a	213 ± 45^a	0.050 ± 0.003^a	649 ± 83^a	0.124 ± 0.012^a
2.5	337 ± 57^a	0.106 ± 0.019^a	360 ± 128^a	0.068 ± 0.007^b	1465 ± 56^b	0.155 ± 0.007^b
25	1975 ± 338^b	0.268 ± 0.008^b	4278 ± 279^b	0.211 ± 0.001^c	–	–

Protein concentration was 10% w/w. In each column, values sharing the same superscript are not significantly different ($P < 0.05$).

meaning that no irreversible process had taken place at this stage. In addition, Utsumi and Kinsella (1985b) reported that the maintenance of 7S, 11S, and SPI gels was due to hydrogen bonds with some contributions of disulfide bonds in 11S and SPI.

Without calcium addition, the 7SEF and 11SEF fractions had the lowest and highest Q value, 0.050 and 0.124, respectively. Therefore, despite the fact that the Tgel of 7SEF differed from that of 11SEF, 84.7 compared to 95.0 °C, and the denatured proteins in 7SEF had potentially more time to interact to establish a network, the greater proportion of the structure in 7SEF gels was formed upon cooling (Figure 3). This result reinforces the idea that the type of interactions that allow gel formation depends on temperature and the nature of the protein. For 11SEF, although the proteins were completely denatured at 92 °C (Figure 2) and gelation started at 95 °C, the increase in G′ during the plateau was higher than that observed in the other products. This suggests that the network present in glycinin gels exhibits a strong dependence on hydrophobic interactions.

Calcium was effective in promoting association between the protein molecules of SPI, 7SEF, and 11SEF during the heating stage and the plateau, as illustrated by the increase in the Q values. This observation suggests that calcium promoted the formation of more hydrophobic interactions and/or that calcium bridges between polypeptides were established during these steps. Scilingo and Añón (2004) also stated that calcium promoted the formation of aggregates that were stabilized by hydrophobic interactions.

Effects of both HP treatment and calcium addition on soybean protein gelation

The effects of HP treatment (200 and 400 MPa) on SPI dispersions at a protein concentration of 8% and 10% and calcium concentrations of 0, 2, 20 and 25 mM on thermal-induced gel properties are summarized in Figure 4 and Table 3. HP treatment had a significant impact on the appearance of the gelation curves (Figure 4). At the beginning of the thermal cycle, that is 20 °C, the G′ values of the pressurized SPI were higher than those obtained for the controls, this effect being more pronounced at a 10% protein concentration. This observation suggests a pressure-induced rearrangement of the protein, which increased the G′ value. The more pronounced effect at the highest concentration could be attributed to interactions that were promoted by the proximity of the proteins. Moreover, the 8% and 10% SPI treated at 400 MPa and the 10% SPI treated at 200 MPa had G′ values higher than the G″ values at 20 °C (data not shown), reflecting that HP treatment modified the rheological properties toward more elastic materials. During the initial heating stage, G′ decreased until different temperatures, and then it started to increase (Tgel - Table 3). In the presence of calcium (Figure 4C and 4D), the more intense the HP treatment, the higher the temperature at which the G′ stopped decreasing. A similar behavior was reported by Ahmed and others (2006) working with a commercial SPI containing denatured β-conglycinin. The associations between partially HP-denatured proteins were probably ruptured by a thermal treatment that caused the decrease in G′. As this behavior is

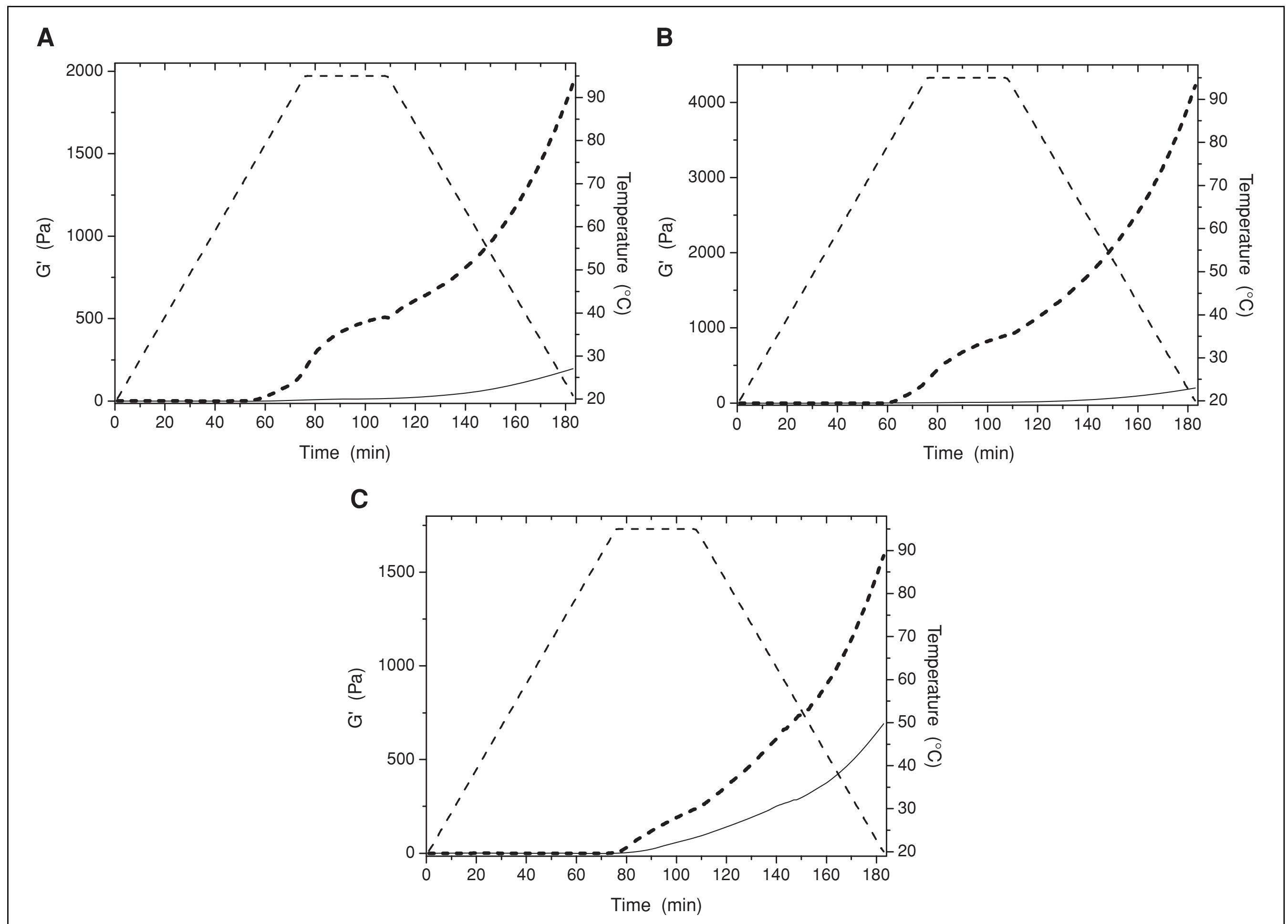

Figure 3—Typical gelation curves of 10% SPI (A), 7SEF (B), and 11SEF(C) in the absence (solid line) or presence of calcium (dotted line—25 mM for SPI and 7SEF; 2.5 mM for 11SEF). Temperature profile is indicated with a dashed line.

compatible with hydrogen bonds, and it is known that their formation is favored by HP (Boonyaratanakornkit and others 2002), these results suggest that polypeptides present in calcium-enriched/HP-treated SPI dispersions were linked via hydrogen bonds.

In the absence of calcium, the G′ value increased from 9 to 279 Pa when the SPI concentration was increased from 8% to 10%, respectively (Table 3). For 8% SPI, G′ was higher than G″ only during cooling, at 72.5 °C, revealing a great delay in gelation (rheological data not shown). The observed weakness of 8% SPI gel is in agreement with the gelation critical SPI protein concentration of 6.5% and 8% reported by Renkema and Van Vliet (2001). Torrezan and others (2007) also found that rheological properties of SPI are strongly dependent of protein concentration.

Calcium addition significantly increased the stiffness of 8% and 10% SPI, which can be explained by its participation in bridges between negatively charged residues. Therefore, the residues that were repulsed in the absence of calcium were linked in its presence thus reinforcing the protein network.

Without calcium, HP treatment of SPI, 7SEF, and 11SEF at 200 and 400 MPa decreased gel stiffness, except for 10% SPI treated at 200 MPa for which gel stiffness remained the same. Wang and others (2008) studied the effects of HP on functional properties of SPI and also found that this treatment impaired its heat-induced gelling capacity. In the presence of calcium, the stiffness of gels made from SPI and 7SEF dispersions pressurized at 200 and 400 MPa was significantly enhanced (Table 3). These results suggest that calcium modified the denaturation phenomena induced by HP, generating diverse structures with different abilities to form a gel during a later heating. HP-denatured polypeptides might have interacted with calcium ions upon heating to establish stronger structures than when proteins were only thermally denatured.

HP treatment modified the rheological behavior of soybean protein dispersions, and G′ was higher than G″ at the beginning of the thermal cycle (data not shown). Therefore, the gelation temperature in this series of experiments was defined as the temperature at which G′ started to increase. The HP treatment decreased Tgel for all samples, this effect being more pronounced for the calcium-enriched samples, for which the average decrease was 32 °C (Table 3). It is worth noting that after HP treatment, the Tgel of SPI was lower than the Td of β-conglycinin (71.2 °C), with the exception of the 200 MPa treated in the absence of calcium.

The native state of the proteins was evaluated by DSC: ΔH of control sample was 11.3 ± 0.2 J/g in absence of calcium, and

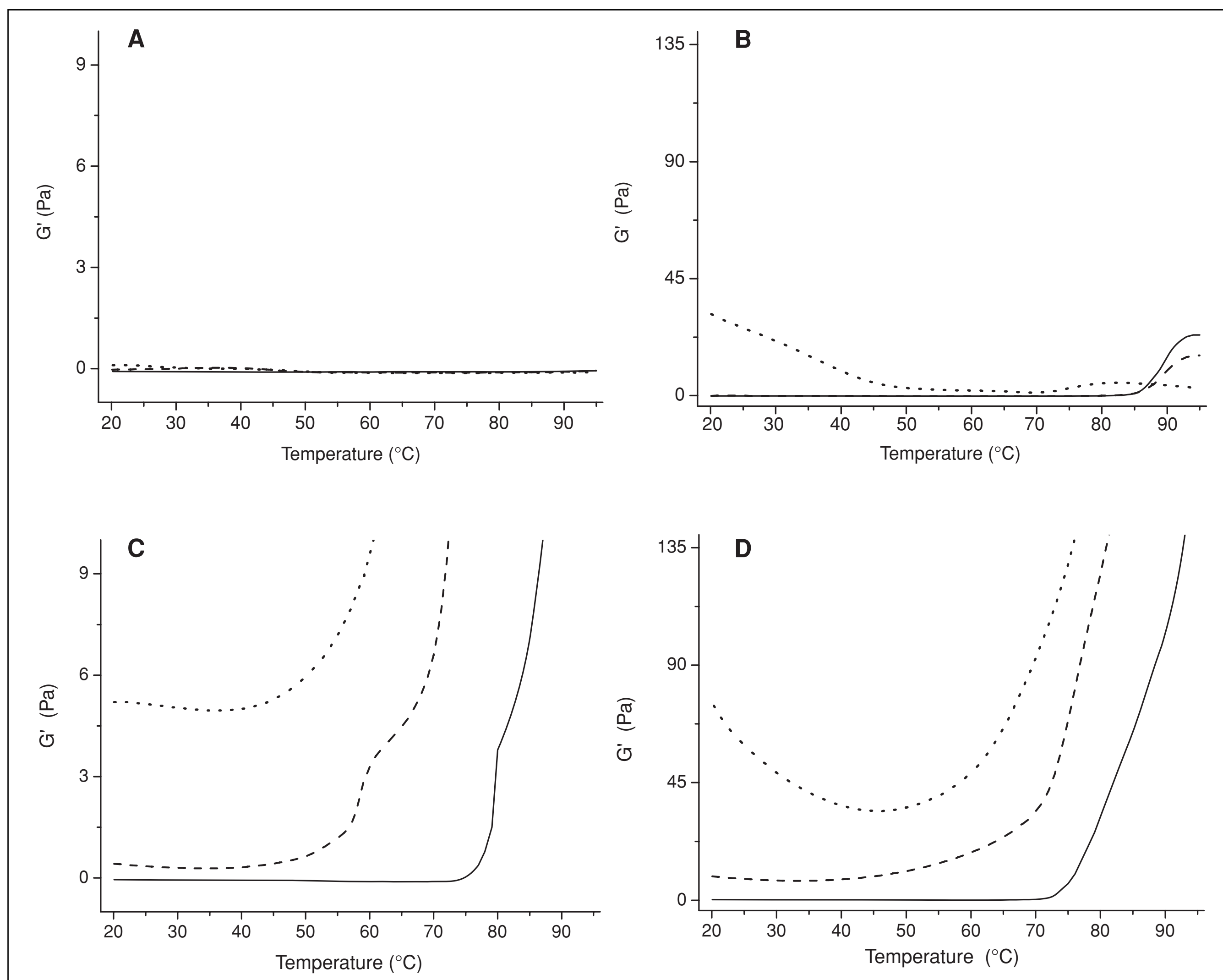

Figure 4—Effect of pressure treatment on gelation curves of SPI. Protein and calcium concentrations were respectively 8% and 0 mM (A), 10% and 0 mM (B), 8% and 20 mM (C), and 10% and 25 mM (D); control samples (solid line), 200 MPa (dashed line), 400 MPa (dotted line).

12.9 ± 0.1 J/g with 25 mM calcium. After HP treatment, these values became 8.2 ± 0.2 J/g and 11.8 ± 0.1 J/g respectively for 200 MPa, and 2.0 ± 0.1 J/g and 3.0 ± 0.3 J/g for 400 MPa. Then, at 200 MPa and 400 MPa, the percentage of denaturation of the SPI was 28% and 82%, respectively. For 25 mM calcium-added SPI, these values decreased to 8.5% and 77%, respectively. These results indicate that the unfolding that allowed the beginning of gelation was induced by HP denaturation, and suggest that the interactions between these partially denatured polypeptides were hydrophobic, as they took place during heating.

The values of Q of the calcium-enriched and HP-treated samples were higher than those of control samples (Table 3). This may reflect a different mechanism of gelation, possibly because gelation started from partially unfolded proteins. It is likely that the contribution of the hydrophobic interactions to the final G′ in calcium-enriched and HP-treated samples was higher than in the control ones.

Effects on the rheological properties of β-conglycinin and glycinin

Similarly to that observed with SPI, in the absence of calcium, the gel-forming properties of 7SEF and 11SEF were not enhanced after HP treatment. The stiffness of the calcium-enriched 7SEF increased with 400 MPa, whereas that of calcium-enriched 11SEF decreased with 200 and 400 MPa (Table 3). These opposite effects observed in 7SEF and 11SEF in the presence of calcium may rely on the differences in protein structure, ability to form disulphide bonds, and affinity and number of sites for calcium. Wang and others (2008) suggested that partial HP-denaturation promoted the formation of more compacted structures in glycinin, but more disordered ones in β-conglycinin, compared with their respective controls. Our results indicate that HP treatment on these calcium-enriched proteins resulted in structures with different properties. The presence of glycinin in SPI may decrease the improving-effect of the combination of calcium and pressure on gelation, as the magnitude of this effect was smaller in SPI than that observed for 7SEF.

The effect of HP treatment on the molecular size distribution of soybean proteins with added calcium is summarized in Figure 5. The profiles of the different fractions without HP treatment consisted of a single Gaussian peak. After HP treatment, there was an increasing quantity of 7SEF and SPI proteins that could not be filtered through the 0.45 μm filter. This indicated the formation of high-molecular weight aggregates during treatment. After treatment at 200 MPa, only a few proteins were retained on the filter but, after the 400 MPa treatment, only about half of the SPI and 7SEF dispersion was recovered. This phenomenon was not observed for 11SEF. The chromatogram of the SPI and 7SEF filtered dispersions displayed a broad distribution of molecular sizes, from enormous aggregates to dissociated species, all of them at indistinguishable abundance. This effect of HP on dispersions with added calcium may partially explain the enhanced ability of these proteins to gelify. The formation of a gel relies on the creation of a network of high- or infinite-molecular weight aggregates that extends throughout the volume of the system. The large-sized aggregates formed in dispersions containing β-conglycinin (SPI and 7SEF) would act as pre-formed units that associate with each other during the thermal cycle. For 11SEF, treatment at 200 MPa added a shoulder to the peak (arrow in Figure 5C), and slightly modified the molecular weight of the peak. After 400 MPa treatment, a peak corresponding to a higher molecular weight was observed indicating the formation of soluble aggregates, while some proteins with a molecular weight similar to that present in the control 11SEF were detected. HP treatment did not modify the quantity of filtered 11SEF proteins, therefore all the protein present in the samples was involved in the formation of the above-mentioned aggregates.

These observations demonstrate that the effect of HP treatment on aggregate formation from the glycinin dispersion containing calcium was very different from that observed with the β-conglycinin dispersion containing calcium. The aggregates

Table 3—Effect of calcium and HP treatment on gelation temperature (Tgel), stiffness (G′), and the nature of interactions that establish the gel (Q).

Fraction	Fraction concentration (%)	Calcium concentration (mM)	Pressure (MPa)	Tgel (°C)	G′(Pa)	*Q*
SPI	10	0	0.1	84.4 ± 1.8^{a}	279 ± 80^{a}	0.10 ± 0.03^{a}
			200	74.7 ± 0.4^{b}	310 ± 79^{a}	0.07 ± 0.01^{a}
			400	63.8 ± 7.8^{b}	98 ± 24^{b}	0.08 ± 0.02^{a}
		25	0.1	68.3 ± 3.0^{c}	1975 ± 338^{c}	0.27 ± 0.02^{b}
			200	30.7 ± 2.1^{d}	2837 ± 239^{d}	0.31 ± 0.01^{c}
			400	46.1 ± 2.3^{e}	2865 ± 318^{d}	0.31 ± 0.01^{c}
	8	0	0.1	84.1 ± 3.1^{f}	9 ± 2^{e}	0.00 ± 0.00^{d}
			200	70.7 ± 3.5^{g}	1 ± 2^{f}	0.00 ± 0.00^{d}
			400	65.8 ± 3.8^{g}	2 ± 0^{f}	0.00 ± 0.00^{d}
		20	0.1	70.4 ± 4.3^{h}	611 ± 177^{g}	0.24 ± 0.02^{e}
			200	34.7 ± 2.5^{i}	1253 ± 112^{h}	0.31 ± 0.00^{f}
			400	37.4 ± 1.5^{i}	1610 ± 129^{i}	0.27 ± 0.02^{f}
7 SEF	8	0	0.1	63.0 ± 4.1^{j}	20 ± 5^{j}	0.02 ± 0.02^{h}
			200	Ng	1 ± 2^{k}	0.00 ± 0.00^{h}
			400	Ng	1 ± 1^{k}	0.00 ± 0.00^{h}
		20	0.1	75.6 ± 1.0^{m}	1492 ± 66^{l}	0.21 ± 0.00^{i}
			200	67.7 ± 2.0^{n}	1350 ± 73^{l}	0.20 ± 0.01^{i}
			400	39.9 ± 1.8^{o}	3906 ± 183^{m}	0.24 ± 0.00^{j}
11 SEF	8	0	0.1	Ng	98 ± 22^{n}	0.00 ± 0.00^{k}
			200	Ng	15 ± 7°	0.00 ± 0.00^{k}
			400	Ng	12 ± 8°	0.00 ± 0.00^{k}
		2	0.1	90.6 ± 1.3^{r}	428 ± 120^{p}	0.00 ± 0.00^{k}
			200	Ng	136 ± 80^{q}	0.00 ± 0.00^{k}
			400	Ng	163 ± 51^{q}	0.00 ± 0.00^{k}

In each column, values sharing the same superscript are not significantly different ($P < 0.05$).
Ng = not gelified.

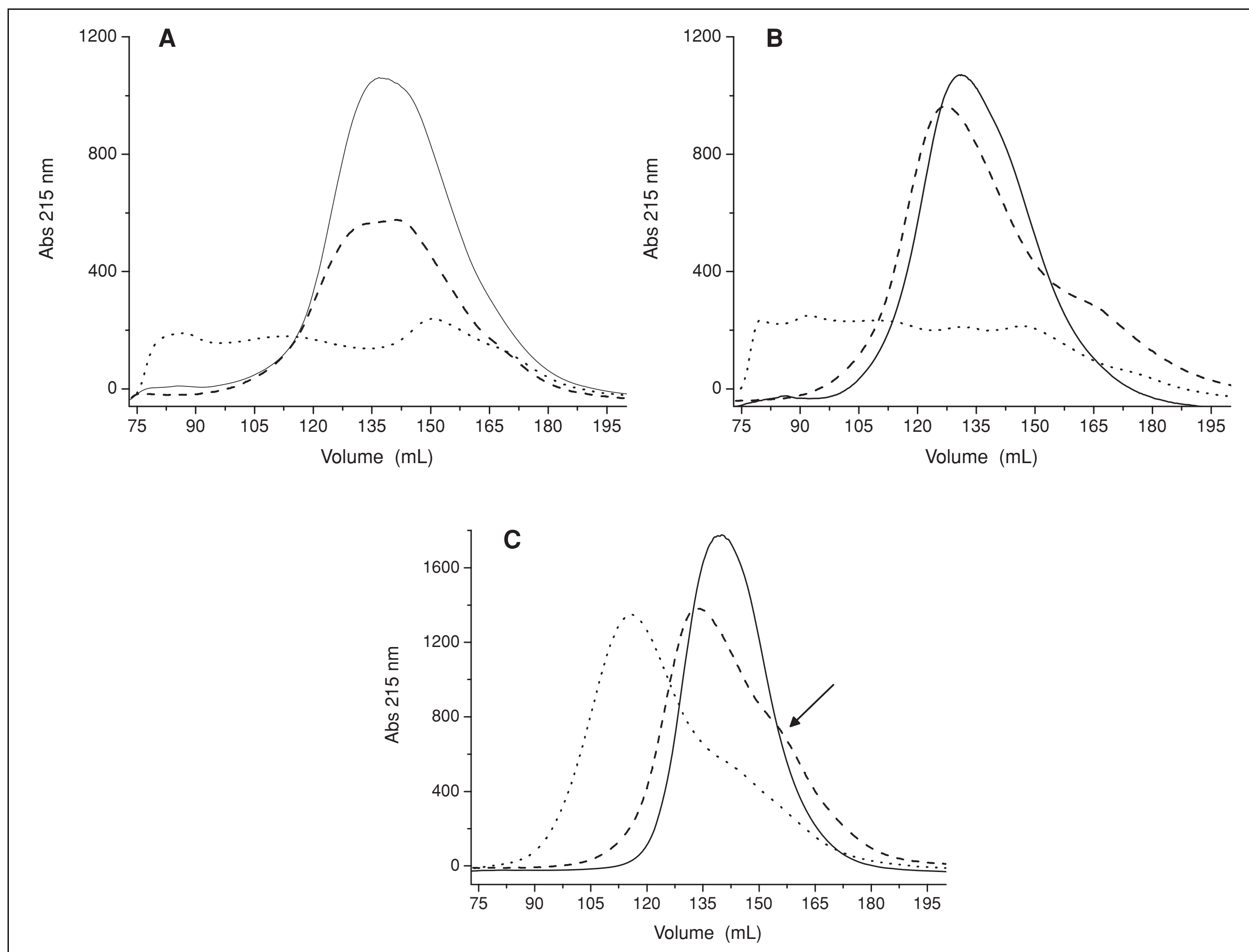

Figure 5—Effect of pressure treatment on the elution profiles of 8%-calcium added SPI (A), 7SEF (B), 11SEF (C); control samples (solid line), 200 MPa (dashed line), 400 MPa (dotted line).

formed by HP from β-conglycinin and calcium in 7SEF and SPI seemed to be intermediate species that may be rearranged by thermal treatment, while the aggregates formed from glycinin were not, explaining the different gel-forming capacity of HP-treated proteins.

Glycinin and β-conglycinin bind calcium through electrostatic attraction to negatively charged side chains, and the formation of a complex with the imidazol group of histidine. Glycinin has more accessible histidine residues and more affinity for calcium than β-conglycinin (Appu Rao and Narasinga Rao 1976), while the total negative charge of β-conglycinin is higher than that of glycinin (Lasergene software). It is well established that HP favors the disruption of electrostatic bonds (Boonyaratanakornkit and others 2002). Treatments at 200 and 400 MPa might have separated calcium from the charged side chains of soybean proteins. As a consequence, during the unfolding of the proteins, new sites became available for calcium binding, and different structures were formed. Due to the structural difference between glycinin and β-conglycinin, including a difference in the number of charged residues, pressure might have affected the 2 proteins to a different extent. Our results show that the presence of calcium modifies the mechanism of HP denaturation of β-conglycinin.

Conclusions

The presence of calcium and HP treatment both significantly affected the temperature dependence of the rheological behavior and the final characteristics of soybean protein gels. Calcium enhanced the stiffness of soybean protein gels, probably by participating in interactions that occurred when the proteins were partially denatured, while HP treatment formed weaker heat-induced gels. HP treatment in the presence of calcium promoted the formation of stronger gels upon heating of SPI and 7SEF, while the opposite effect was observed for 11SEF. Our results suggest that the diverse types of aggregates formed by glycinin and β-conglycinin with calcium after HP treatment have different abilities to participate in the formation of a 3-dimensional network upon heating.

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