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Gelation of soybean proteins induced by sequential high-pressure and thermal treatments

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ABSTRACT

The effect of high-pressure treatment on structural and rheological properties of soybean protein dispersions was studied. A sequential high-pressure/thermal treatment was also analyzed. Dissimilar effects on soy protein isolate (SPI) and the enriched soybean protein fractions: β-conglycinin (βCEF) and glycinin (GEF) were observed. High pressure (600 MPa) promoted βCEF gelation, but did not modify the rheological properties of GEF in spite of its complete denaturation. Pressure treatment also induced the establishment of hydrophobic interactions and disulfide bonds that allowed the formation of soluble high molecular mass aggregates from the different polypeptides of both β-conglycinin and glycinin. Protein strands formation was detected in matrix microstructure of HP-treated SPI and βCEF dispersions in accordance with their rheological behavior of weak gels. In the case of GEF modifications induced by HP in the microstructure (apparition of large granules) were not accompanied by rheological changes. Heating process after HP treatment induced protein gelation. A decrease in the temperature of onset of matrix formation of SPI and βCEF samples was observed. The magnitude of this effect was proportional to the intensity of HP treatment. Contrarily, HP provoked a delay in gelation process of GEF dispersions. During the thermal cycle, previous HP treatment diminished the ability of both soybean globulins to establish hydrophobic interactions on heating and hydrogen bonds on cooling, thus obtaining gels with small elastic modulus.

1. Introduction

Soybean protein isolates (SPI) due to their desirable functional properties, high nutritional value and associated health effects, have been included in a wide variety of formulated foods (Kinsella, 1979; Messina, 2003; Messina & Lane, 2007). Functionality of isolates depends on composition, structure, denaturation and aggregation degree of the proteins. The major protein components of soybean are glycinin and β-conglycinin. Glycinin is a hexameric protein composed of A and B polypeptides linked by disulfide bridges (Maruyama et al., 2004) while β-conglycinin is a trimer of three kinds of subunits α, α′ and β (Thanh & Shibasaki, 1978). Their differences in structure are reflected in several properties like thermal stability: temperature of denaturation is near 74 °C for β-conglycinin, whereas it is at 87 °C for glycinin (Puppo et al., 2004).

Appropriate processing treatments performed on SPI conduct to specific changes in protein structure being able to improve functional properties (Tewari, Javas, & Holley, 1999). This fact could increase the use of such isolates as ingredients in food industry. Positive structural changes may be accomplished by physical treatments such as thermal or high-pressure processing, when they are carried out in appropriate conditions: temperature or pressure levels, time processing, protein concentration, pH and ionic strength (Tewari et al., 1999).

High-pressure (HP) processing represents a possibility to satisfy the increased consumer demand for high quality, minimally processed, additive-free and microbiologically safe food (Galazka & Ledward, 1995; Gould, 1995; Knorr, 2000). High pressure can preserve small molecules (vitamins, free amino acids) and significantly modify secondary, tertiary, and quaternary protein structures, affecting non-covalent bonds (Montero, Fernández-Díaz, & Gómez-Guillén, 2002; O'Reilly, Kelly, Murphy, & Beresford, 2001).

HP treatments higher than 200 MPa produce important changes in soybean proteins at pH 8.0: secondary structure is affected leading to a more disordered one, accompanied of protein aggregation, especially of glycinin (Puppo et al., 2004). Aggregation could be due to the formation of disulfide bridges (Galazka, Dickinson, & Ledward, 1999; Galazka, Smith, Ledward, & Dickinson, 1999; Hayakawa, Linko, & Linko, 1996). The effects of HP treatment on

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soybean proteins are dependent on pH, inducing both the formation of species of higher (aggregation) and lower (dissociation) molecular mass at acidic pH than those present in the control samples (Puppo et al., 2004). These authors also analyzed the degree of denaturation induced by HP as function of the protein nature and they found that β-conglycinin partially maintained a native structure after 600 MPa, whereas glycinin is completely denatured after the same treatment.

Dumoulin, Ozawa, and Hayashi (1998) and Molina, Defaye, and Ledward (2002) reported the formation at high protein concentration (17% w/w and 20% w/v) of self-supporting soybean protein gels induced by HP at or above 300 MPa. These gels presented high water holding capacity (>80%) and their hardness (g/cm^2) was lower than that of heat-induced ones.

Combination of several techniques is a habitual procedure in food conservation and processing, e.g., for products like spore containing materials, an only HP treatment is insufficient to completely inactivate contamination, thus, pasteurization and sterilization will most probably rely on a combination of high-pressure processing and other technique. Combined pressure–temperature treatments are frequently regarded as appropriate to inactivate spores (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998).

It is known that heat induces gelation of soybean proteins at acidic, neutral or alkaline pH (Puppo & Añón, 1999; Renkema, Knabben, & van Vliet, 2001; Renkema & van Vliet, 2002). Heat-induced glycinin gels at pH 7.6 exhibit higher storage modulus values, G', and resistance to fracture than those corresponding to β-conglycinin gels at the same conditions (Renkema et al., 2001). These differences are partially explained by the formation of numerous disulfide bonds between glycinin subunits.

In the case of soybean proteins, the combination between HP and heat treatments may allow the formation of gels at a lower protein concentration exhibiting characteristics provided by HP protein denaturation.

Protein denaturation is achieved by different mechanisms when HP or heat is applied, the disrupted bonds are different and the resulting structures are also diverse. It is accepted that hydrogen bonds are destabilized by heating while they are stabilized by HP, electrostatic interactions are disrupted by HP and hydrophobic interactions are favored by moderate heating. Controversial statements exist about the stabilizing or destabilizing effects of HP on hydrophobic interactions (Balny, Masson, & Heremans, 2002; Boonyaratanakornkit, Park, & Clark, 2002). If a combination of these physical treatments were assayed, gels with desired properties would be possibly obtained. Moreover, more understanding about the combined effect of HP and heat on structural and functional changes of soy protein should be acquired.

Then, the objective of this work was to study the influence of sequential high-pressure/thermal treatments on gelation capacity and gel properties of soybean protein isolate and their main globulins, β-conglycinin and glycinin.

2. Materials and methods

2.1. Preparations of the soybean protein isolate (SPI)

Soybean protein isolates (SPI) were prepared from defatted flour manufactured by Solae S.A. (Brasil). An aqueous alkaline extraction from the flour (pH 8.0), followed by an isoelectric precipitation (pH 4.5) was carried out according to Petruccelli and Añón (1994). The isoelectric precipitate was dispersed in distilled water and adjusted to pH 8.0 with 2 N NaOH. The dispersion thus obtained was lyophilized. Protein content of SPI determined by Kjeldhal method was 90.7% w/w (w.b.) (N × 6.25).

2.2. Preparation of β-conglycinin and glycinin enriched fractions

β-conglycinin and glycinin enriched fractions (respectively βCEF and GEF) were obtained according to the method of Nagano, Hirotsuka, Mori, Kohyama, and Nishinari (1992). This method is based on differences in solubility at diverse pHs and ionic strengths. Protein content of these fractions determined by Kjeldhal method was 94.2 and 96.7% w/w (w.b.) (N × 6.25) for βCEF and GEF, respectively.

2.3. High-pressure treatment

SPI, βCEF and GEF protein dispersions (10% w/v – pH 8.0) were subjected to high-pressure treatment at 300 and 600 MPa (±7 MPa) for 10 min. The level of pressure was reached at 3.4 MPa/s and released instantaneously. Temperature of transmitting medium in the vessel was settled at 20 °C (±2 °C) during pressure processing to avoid the freezing and the overheating of proteins. High-pressure processing was carried out in a 3 L reactor unit (ACB Pressure Systems, Nantes, France) equipped with temperature and pressure regulator device. Prior to pressure processing, protein dispersions (50 mL) were vacuum conditioned in a polyethylene bag (La Bovida, France). Conditions of high-pressure processing were chosen in accordance to Puppo et al. (2004).

2.4. Rheology of gels

2.4.1. Small deformation

2.4.1.1. Frequency sweep. The samples (3 mL) were placed in an AR1000 (TA Instruments New Castle, UK) with a cone/plate geometry (40 mm ∅, 2°). Measurements were carried out at a constant strain of 1% which was within the linear region. A frequency sweep between 0.01 and 10.0 Hz was carried out at 20 °C. Storage modulus (G') and loss modulus (G'') were recorded as a function of frequency of oscillation.

2.4.1.2. Gel formation induced by thermal treatment. Assays were carried out at a laboratory scale in the Peltier system of the rheometer. Gelation was followed at an angular frequency of 1 Hz. In order to avoid water evaporation, a layer of paraffin oil was put on the surroundings of the samples. The thermal cycle consisted in a heating stage from 20 to 95 °C at a heating rate of 1 °C/min, after that an isothermal step of 30 min at 95 °C was performed and finally a cooling stage from 95 to 20 °C at 1 °C/min was applied. We have defined T_m as the temperature in which a significant increase in G' could be detected, this change in rheological behavior revealed the onset of a network formation. After the thermal cycle a second frequency sweep was carried out in the same conditions to evaluate the rheological behavior of the resulting products.

We would like to precise that our thermal treatment was conducted in laboratory conditions (Peltier heater) different to that encountered at an industrial scale.

2.5. Scanning electron microscopy (SEM)

Selected portions of gels were immersed in 2.5% glutaraldehyde and then washed with phosphate buffer of 0.5 M before dehydration process. Samples were dehydrated in a grade acetone series: 25, 50, 70, 90 and three times with 100%. Drying of samples was performed at the critical point with the intermediate CO_2 fluid. Samples were then coated with gold in a sputter coater (Pelco, Redding, USA). They were observed at 5 kV in a JEOL JSM 35 CF microscope (Tokyo, Japan) scanning electron microscope.

2.6. Protein solubility of protein dispersions

Treated and non-treated (control) SPI, βCEF and GEF dispersions were diluted 100 times with different solvents (water, 50 mM Tris–HCl pH 8.0 or 50 mM Tris–HCl pH 8.0 added with 1% SDS). Diluted dispersions were stirred during 4 h at ambient temperature and centrifuged at 15,000 g for 15 min at 15 °C. Supernatant protein content was determined by Biuret procedure. Bovine serum albumin was used as standard (Sigma Chemical Co., USA). Protein solubility was expressed as:

$$\text{Solubility}(\%) = \text{Protein in the supernatant}(\text{mg/mL}) \times 100/\text{Initial protein}(\text{mg/mL})$$

2.7. Nature of soluble HP-treated proteins

The nature of soluble proteins of supernatants obtained from the HP-treated protein dispersions was analyzed by polyacrylamide gel electrophoresis. SDS-PAGE was assayed with 50 mM Tris – 1% SDS pH 8.0 buffer extract (dissociating condition) and with the same extract, treated with 2% β-mercaptoethanol at 100 °C during 5 min (reducing condition). Resolving and stacking gels of 10% and 3.5% of acrylamide, respectively, were employed. A buffer system containing 2 M Tris-base, 0.15% SDS at pH 8.8 for the separating gel and 0.027 M Tris-base, 0.38 M glycine at pH 8.3 with the addition of 0.15% SDS, for the running buffer was used. Native-PAGE was performed with the aqueous extract using the same buffer systems but without SDS. Coomassie Brilliant Blue was used as colorant agent. Low MW markers (Biorad SDS-calibration kit) used included phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

2.8. Statistical analysis

Results were subjected to a one-way analysis of variance according to the general linear model procedure with least-square means effects. Differences between the sample means were analyzed by Tukey's test. Signification was accepted when $p < 0.05$. Statistical analysis was carried out using SYSTAT software (SYSTAT, Inc., Evanston, IL, USA).

3. Results and discussion

3.1. Rheological properties of soybean proteins

3.1.1. Effect of high-pressure treatment

Storage (G') and loss (G'') moduli of control and HP-treated dispersions were recorded at 1% strain as a function of oscillation frequency (Fig. 1). Control sample and 300 MPa treated SPI dispersions behaved as semidilute macromolecular solutions, exhibiting higher G'' than G' at low frequency and a cross over of moduli near 0.3 Hz. Treatment at 600 MPa modified the rheological behavior to one characteristic of a concentrated macromolecular solution, where G'' was smaller than G' and both parameters presented frequency dependence (Giboreau, Cuvelier, & Launay, 1994; Ross-Murphy, 1995).

At low pressures (≤300 MPa) βCEF dispersions (Fig. 1) presented a rheological spectrum corresponding to semidilute macromolecular solutions, with its cross over near 0.05 Hz. At high pressure

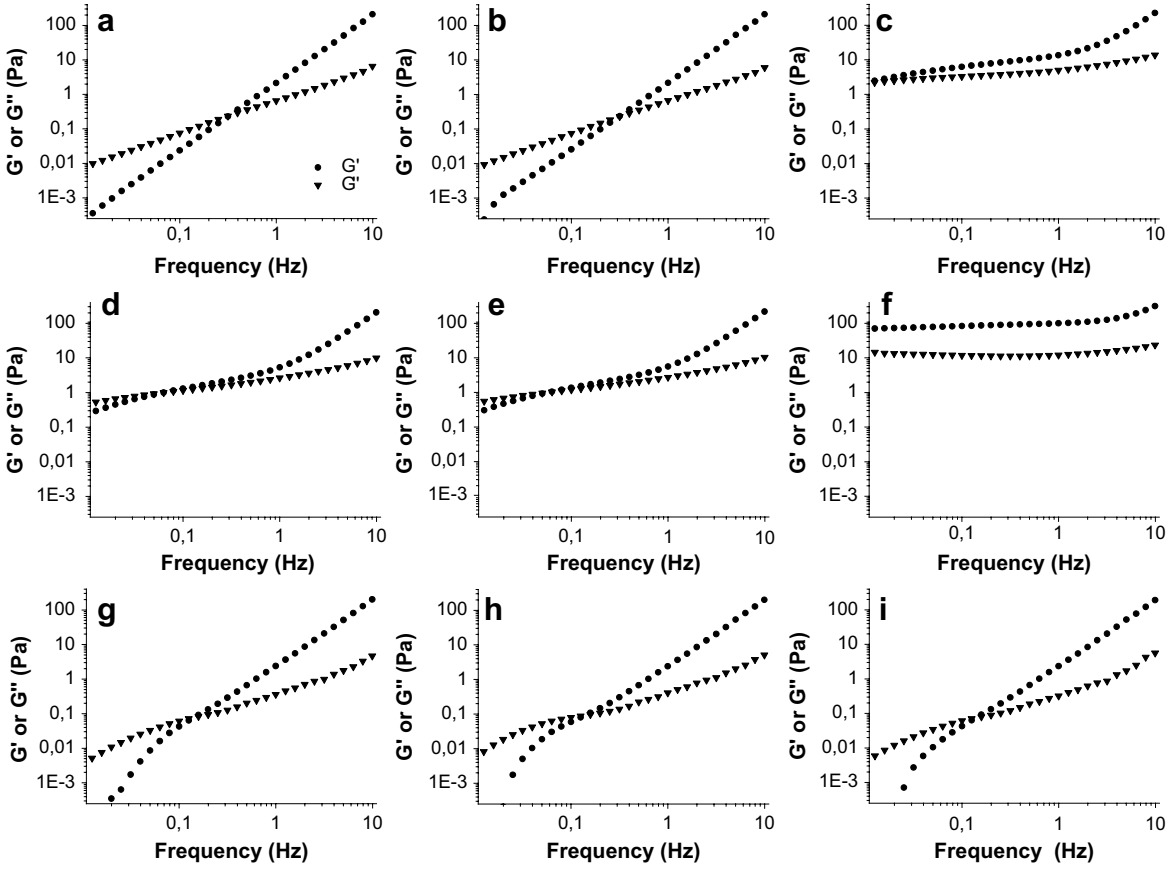

Fig. 1. Storage and loss moduli of soy protein dispersions (10% w/w) as function of oscillation frequency after HP treatment. SPI – 0.1 MPa (a), SPI – 300 MPa (b), SPI – 600 MPa (c), βCEF – 0.1 MPa (d), βCEF – 300 MPa (e), βCEF – 600 MPa (f), GEF – 0.1 MPa (g), GEF – 300 MPa (h), GEF – 600 MPa (i). (●) G', (▼) G''.

(600 MPa) a gel-like pattern was observed: G'' presented a minimum and it was smaller than G' in the whole range of frequencies. The values of G' in this range are comprised between 70 and 320 Pa, values characteristics of a weak gel. Probably stiffer gels would be obtained at higher protein concentration, as it was observed by Molina et al. (2002) and Dumoulin et al. (1998) in gels of 20% w/v and 17% w/w, respectively. Despite the non-complete denaturation (Puppo et al., 2004) of this fraction, HP treatment promoted association between polypeptides, which allowed the formation of a self-supporting gel.

On the other hand, GEF dispersions (Fig. 1) behaved as semi-dilute macromolecular solution under all experimental conditions assayed. It is noteworthy that in spite of the complete denaturation achieved by proteins at 600 MPa (Puppo et al., 2004), its rheological behavior did not change. The small degree of entanglement detected suggests that protein species would be associated through weak interactions.

The distinct HP effects on the rheological characteristics of βCEF and GEF reflect differences between the mechanisms of denaturation of HP and heat: thermal treatment induces gel formation in both βCEF and GEF (at the same protein concentration) with GEF gels stronger than those obtained with βCEF (Renkema et al., 2001), while HP treatment only leads to a gelation of βCEF.

3.1.2. Effect of thermal treatment

In order to analyze the sequential application of HP and thermal treatment, gelation was studied after the frequency sweep by a thermal cycle, composed by a heating ramp, a temperature plateau, followed by a cooling stage. The thermomechanical profiles (G' as a function of time) of the SPI samples are shown in Fig. 2. It can be observed that the main increase in G' occurred during the cooling stage and, analyzing the effect of HP, that the control SPI sample (0.1 MPa) achieved the highest value of storage modulus at the end of the cycle. Taking into account that stability of hydrogen bonds increases with temperature decrease, the observed behavior suggests that the gel matrix is mainly sustained by hydrogen bonds and that the denaturation on soybean proteins produced by HP treatment leads to a reduced ability to establish hydrogen bonds upon thermal cycle. During the heating stage of the thermal cycle a peak in the evolution of G' can be observed (inset of Fig. 2), the onset of this peak was considered the onset of the formation of a three-dimensional matrix. This event shifted to lower temperatures as HP treatment increased: 82; 76 and 68 °C for control, 300 and 600 MPa, respectively. At 600 MPa the matrix formation process started at less temperature than the temperature corresponding to β-conglycinin denaturation (74 °C, determined at a heating rate of 1 °C/min and at the same conditions of pH and ionic strength), reflecting a previous denaturation process induced by HP on the proteins present in SPI. On the other hand, the value of G' before thermal cycle for SPI treated at 600 MPa, was higher than those registered at other pressure conditions. This value diminished as temperature increased from 20 to 50 °C (Fig. 2 inset). These behaviors suggest the existence of interactions between polypeptides chains induced by HP. The interactions seemed to be enthalpic in nature, like hydrogen bonds which stability decreases with temperature increase, and their rupture would be the cause of the decrease of G' during the first part of the heating process. This hypothesis is in accordance with the statement that HP favors hydrogen bond formation (Boonyaratanakornkit et al., 2002). During the temperature plateau G' value was an increasing function of time for the three pressure conditions assayed, but it was higher for the non-treated dispersion (inset of Fig. 2). Considering that hydrophobic interactions are more stable when temperature increases, it is possible that this kind of interactions were also involved in gel structure formation, and that in HP-treated dispersions these type of interactions were weaker or less in number. Then, HP treatment will induce modifications on SPI proteins that inhibited the ulterior formation of hydrophobic interactions upon thermal denaturation.

The gelation curves for βCEF dispersions are shown in Fig. 3. It is possible to observe a peak of G' during the heating ramp and the modulus values were low during the temperature plateau. The most important increase in storage modulus was evidenced on cooling stage. The G' values at the end of the thermal cycle of HP-treated samples were lower than those of the control one. The high value of the storage modulus of βCEF-600 MPa sample at the start of thermal cycle reflects the existence of a gel structure. This gel

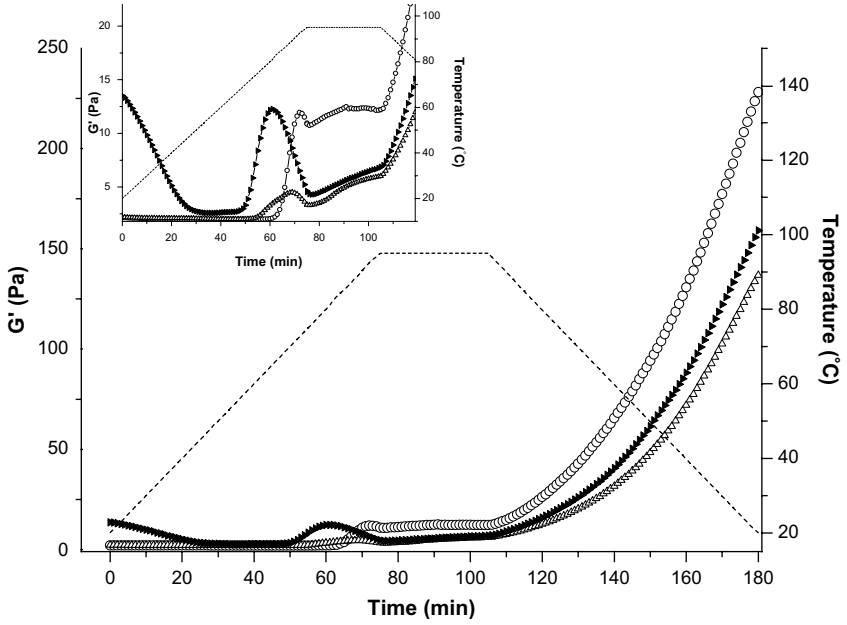

Fig. 2. Thermomechanical profiles (G' vs time) of SPI. (○) 0.1 MPa, (△) 300 MPa, (►) 600 MPa. Thermal cycle (---): 20–95 °C at 1 °C/min (stage 1), 30 min at 95 °C (stage 2), 95–20 °C at 1 °C/min (stage 3).

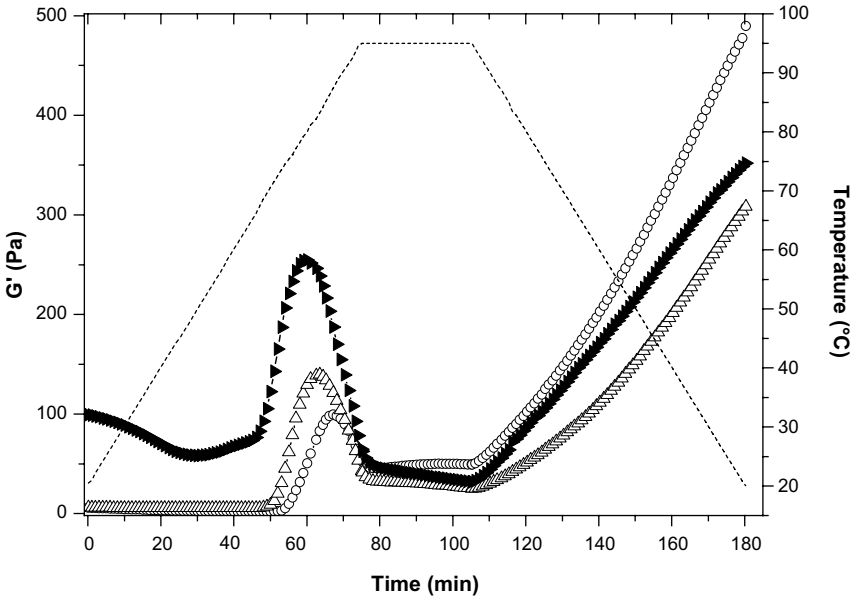

Fig. 3. Thermomechanical profiles (G' vs time) of βCEF. (○) 0.1 MPa, (△) 300 MPa, (►) 600 MPa. Thermal cycle (---): 20–95 °C at 1 °C/min (stage 1), 30 min at 95 °C (stage 2), 95–20 °C at 1 °C/min (stage 3).

seemed to be stabilized by the same type of interactions suggested for the SPI-600 MPa sample, as it also exhibited a decrease of G' value with heating (20–50 °C). The peaks in G' values detected in βCEF dispersions were more important than those corresponding to SPI, appearing at lower temperature as HP treatment increased. T_m decreased from 74 to 66 °C for control and 600 MPa treated sample, respectively. In βCEF gels G' values grew mainly on cooling stage, suggesting a great contribution of enthalpic interactions involved in gel stiffness. It was also observed for βCEF that during the temperature plateau the G' was a decreasing function of time for the HP-treated samples, while for the control one, this parameter slightly increased with time. These results suggest that HP diminished the ability of βCEF to establish hydrophobic interactions. Finally, the values of G' at the end of the cycle of the HP-treated βCEF samples were smaller than those corresponding to the control one.

Gelation curves for GEF are shown in Fig. 4. As expected considering the high denaturation temperature of this protein fraction, gelation process started later than in SPI and βCEF samples (both β-conglycinin containing products). G' started increasing during the temperature plateau at 95 °C reaching relatively important values on this step. High-pressure treatment delayed the gelation process, being the effect more important as high-pressure value increased (inset of Fig. 5), contrarily to the effect observed on SPI and βCEF. This result indicates that heat treatment produced structural changes on the previously HP modified (denaturated) proteins. The percentage of G' (G' at the end of plateau at 95 °C × 100/G' at the end of cooling) achieved at the end of plateau

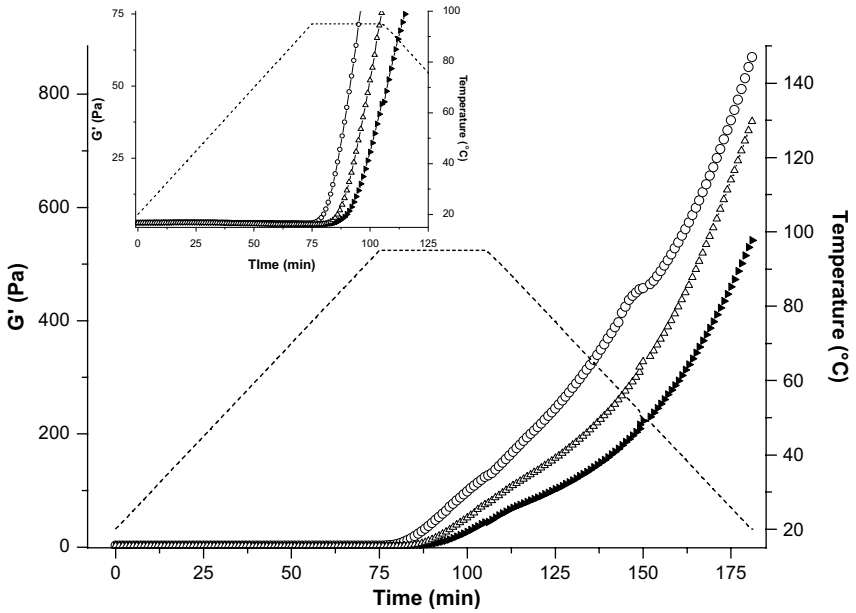

Fig. 4. Thermomechanical profiles (G' vs time) of GEF. (○) 0.1 MPa, (△) 300 MPa, (►) 600 MPa. Thermal cycle (---): 20–95 °C at 1 °C/min (stage 1), 30 min at 95 °C (stage 2), 95–20 °C at 1 °C/min (stage 3).

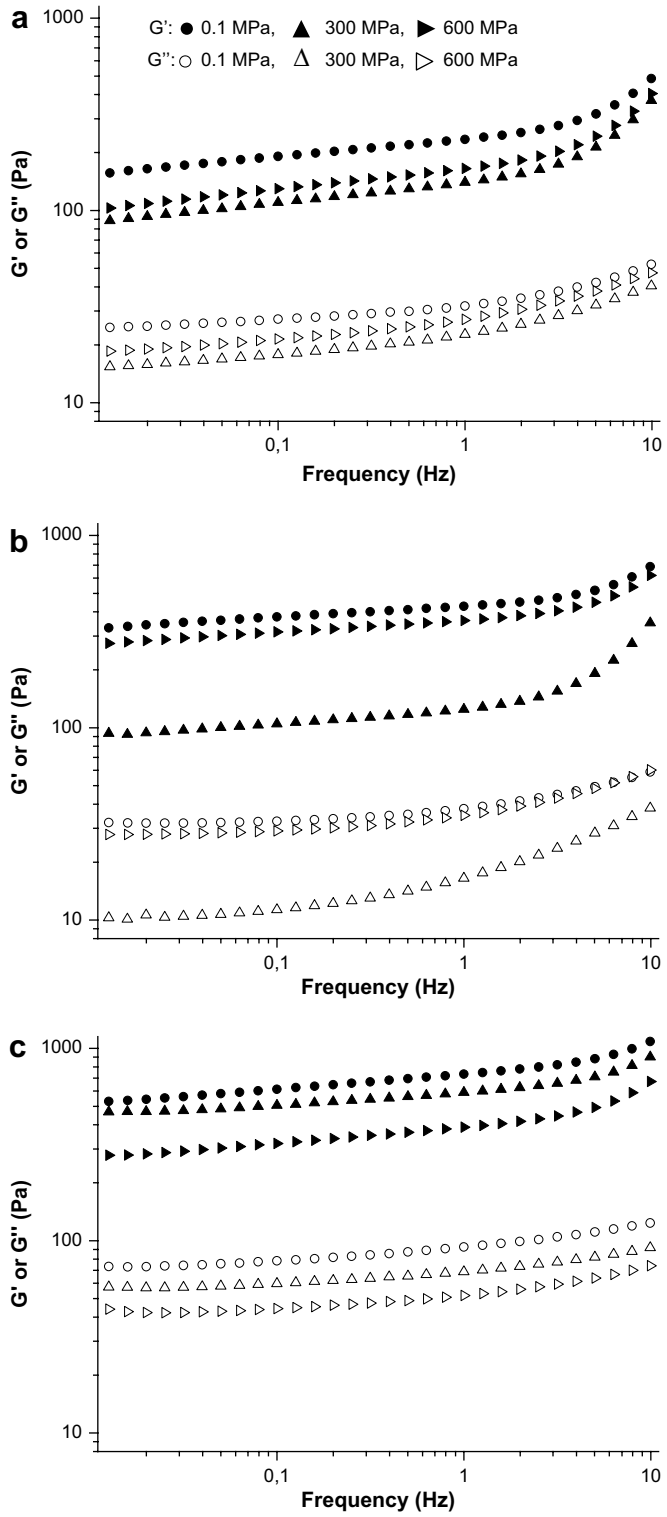

Fig. 5. Storage and loss moduli of soy protein dispersions (10% w/w) as functions of oscillation frequency after HP–thermal treatment. SPI (a), βCEF (b), GEF (c). Moduli: (●,▲,▶) G', (○,△,▷) G''. Pressure: (●,○) 0.1 MPa, (▶,△) 300 MPa, (▶,▷) 600 MPa.

for GEF dispersions was higher than that of SPI and βCEF (5.3; 9.8 and 15.0% for SPI, βCEF and GEF, respectively, at 0.1 MPa). This effect could be attributed to a greater contribution of hydrophobic interactions to gel structure in GEF. The modulus kept increasing during cooling stage until temperature reached 20 °C. The effect of HP was similar to that observed in SPI and βCEF: the highest value of G' at the end of the thermal cycle was obtained for the non-HP-treated GEF dispersion. According to the results of Renkema et al. (2001) the gels obtained from GEF by thermal treatment exhibited higher values of G' than those formed by βCEF and SPI.

On the basis of our results, all the gels obtained after the sequential HP–thermal treatment exhibited a smaller stiffness than those obtained only by heating.

Rheological spectra of sequential HP–thermal treated samples are shown in Fig. 5. A gel-like behavior was observed in all cases. A decrease in G' values through the whole frequency range was detected in HP-treated samples in comparison with untreated one. On the other hand, a smaller decrease in G'' comparing to the effect on G' was also observed. This behavior indicates that HP treatment on soy proteins previous to a thermal one induced the formation of weaker gels. These results suggest that HP interferes with the ability of soybean protein to establish inter-molecular interactions needed to thermal-induced gelation.

It is interesting to compare the effects of protein denaturation achieved by HP with those of thermal treatment regarding the rheological properties of treated dispersions (Figs. 1 and 5). The differences in treatment led to dissimilar molecular structures, thermal denaturation promotes the unfolding of glycinin, which favors hydrophobic interactions during heating, and the formation of disulfide bonds that reinforce the gel matrix (Utsumi & Kinsella, 1985). During thermal treatment hydrogen bonds are broken, being able to re-form engaging different protein groups upon the ulterior cooling stage. On the other hand, HP denaturation of glycinin did not favor interactions that could generate a three-dimensional gel-like structure. Disulfide bonds formed during HP treatment must link different positions of glycinin molecules than when they are formed by heating. In the case of βCEF, a 600 MPa pressure treatment produced a partial denaturation accompanied by a rearrangement between protein molecules through hydrogen bonds allowing the formation of a gel-like structure. Heating followed by cooling of this 600 MPa induced gel, also formed a more structured and elastic matrix gel. The different rheological properties detected reflect the different driving forces of denaturation that are engaged in these two physical treatments.

3.2. Protein solubility of HP-treated dispersions

Solubility values of HP-treated protein dispersions are shown in Table 1. HP treatment increased solubility in water for SPI, βCEF and GEF proteins. In the case of βCEF, the change in solubility was maximal after treatment at 600 MPa arose up to 99.0%. When solubility was assessed in Tris–HCl buffer, the control values were higher than those found in water, suggesting the existence of electrostatic interactions among native proteins in the three products. HP treatment also produced an increase in solubility in Tris–HCl buffer arising to 100% in all protein samples. The

Table 1
Solubility (%) of HP-treated (0.1, 300, 600 MPa) dispersions (1 mg protein/mL) of different soybean proteins (SPI, βCEF, GEF) in different solvents. (Water, Tris–HCl, SDS–Tris–HCl.)

Sample	HP (MPa)	H_2O	Tris–HCl	SDS–Tris–HCl
SPI	0.1	78.1 ± 1.3	96.5 ± 1.0	100.0 ± 0.2
	300	88.0 ± 1.4	99.6 ± 0.2	99.7 ± 0.7
	600	93.3 ± 0.4	100.1 ± 0.1	100.3 ± 0.7
βCEF	0.1	73.1 ± 1.2	97.7 ± 0.2	97.3 ± 2.7
	300	96.2 ± 1.8	101.5 ± 1.0	101.3 ± 0.2
	600	99.0 ± 0.4	99.4 ± 0.3	100.5 ± 1.2
GEF	0.1	72.5 ± 1.5	96.2 ± 1.1	100.0 ± 0.2
	300	77.5 ± 1.6	99.5 ± 0.2	99.6 ± 0.8
	600	91.9 ± 0.5	100.1 ± 0.1	100.4 ± 0.7

incorporation of SDS to Tris–HCl solvent allowed the solubilization of all protein in all HP assayed conditions.

It was reported that HP treatment promotes the aggregation of soybean proteins at low and high concentrations (1 and 10% w/v). This treatment also induces gelation of these proteins at 17% w/w and 20% w/v of protein concentration (Dumoulin et al., 1998; Molina et al., 2002; Puppo et al., 2004). Therefore, a decrease in solubility would be expected after HP treatments due to aggregation and/or gelation processes. Our experimental data showed that solubility not only decreased but also was enhanced after HP treatment. Furthermore, the highest relative effect on solubility was observed in βCEF – 600 MPa sample, in which a gel matrix was formed. This fact could be attributed to an increase in the ability of protein to interact with water, due to HP treatment, that in our experimental conditions (protein concentration = 0.1% w/v) may be reflected as an enhanced solubility. Supporting this hypothesis, Molina et al. (2002) reported that water holding capacity of βCEF HP-induced gels was very high (more than 95%), suggesting that HP-treated β-conglycinin interacts more effectively with water. Another possibility to explain this increased solubility could be the formation of nanometric scale aggregates or particles (100–200 nm) that would be in thermodynamic stability (no precipitation) that could be linked by weak interactions to form a matrix gel. This assumption should be confirmed by dynamic light scattering.

Puppo et al. (2004) studied the free sulfhydryl content in SPI and reported a decrease in this value after HP treatment. Molina, Papadopoulou, and Ledward (2001) suggested that glycinin aggregates through disulfide bridges formation as consequence of HP processing. Taking into account this data and our results of solubility in SDS–Tris–HCl buffer (almost 100% for every condition), it is possible to postulate that the protein aggregates stabilized by disulfide bonds remain soluble in the operational conditions used to determine solubility.

It was reported by Puppo et al. (2004) that the HP treatment on 1% w/v SPI dispersions conducted to protein dissociation and aggregation. At the same protein concentration and pH 8.0 they also found that aggregation did not decreased the protein solubility being constant for every pressure assayed, whereas at acid pH (pH 3.0) an increase in solubility was observed. In our case (10% w/w of protein and pH 8.0), after the HP treatment, a great proportion of proteins present in SPI was involved in the formation of macro-aggregates with a size higher than 0.45 μm (they did not pass trough a filter of that cut-off) that remain soluble (data not shown).

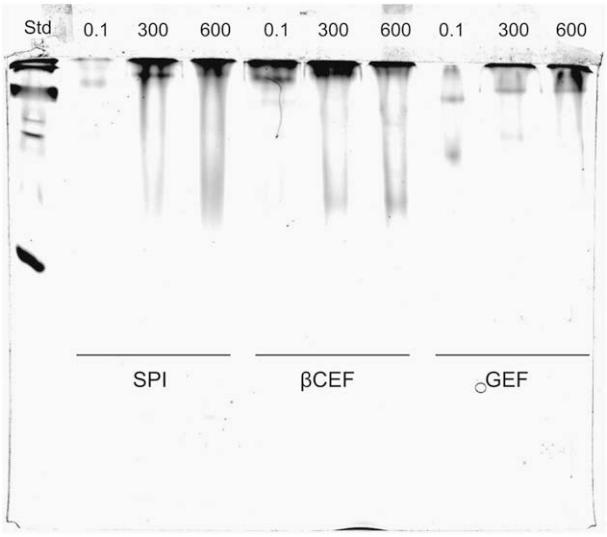

Fig. 6. Native-PAGE of soluble protein extracted with water from SPI, βECF and GEF dispersions. Pressures: 0.1, 300 and 600 MPa.

3.3. Composition of soluble proteins extracted from HP-treated dispersions or gels

The electrophoresis analysis of soluble proteins extracted from dispersions or gels obtained allows the identification of polypeptides species involved in the gel network and/or aggregates formed as consequence of HP treatment as well as the kind of interaction established between protein molecules.

3.4. Native-PAGE

Electrophoretic patterns of SPI and βCEF revealed that HP treatment promoted the appearance of polypeptide species with enhanced mobility (Fig. 6). These species could be generated by dissociation of electrostatic bonds due to the electrostriction favored by HP treatment (Boonyaratanakornkit et al., 2002). Concerning GEF, it is possible to observe that the more the HP intensity, the more the material that did not enter into the gel, certainly due to the formation of high molecular weight aggregates. Despite the aggregation phenomena observed in GEF dispersions, the GEF-HP-

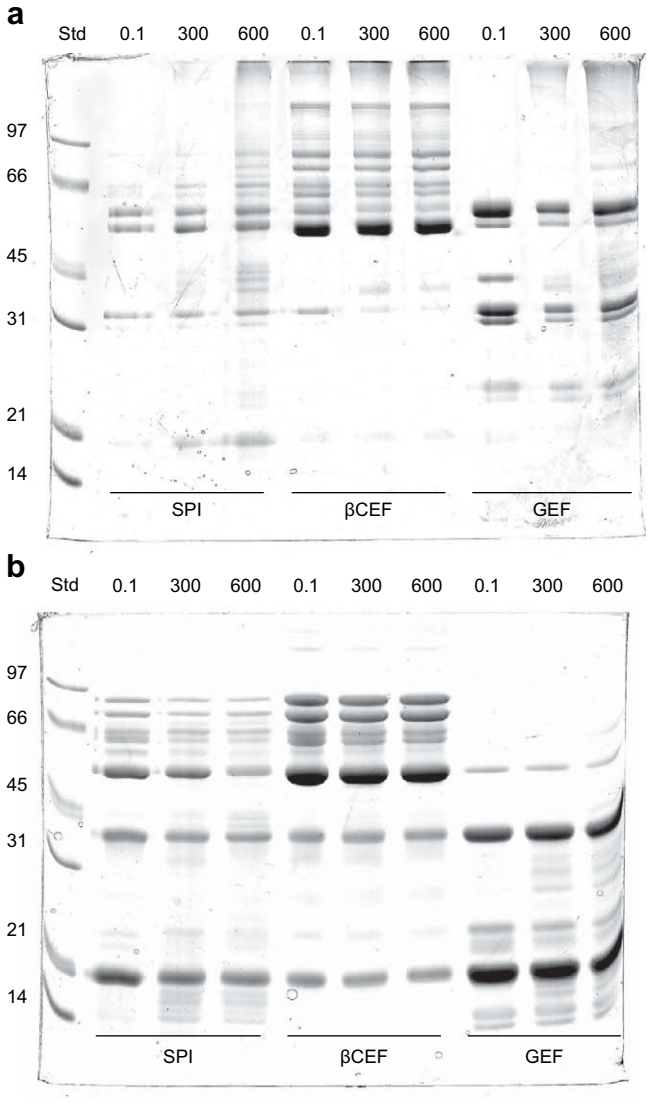

Fig. 7. SDS-PAGE of soluble protein extracted with 50 mM Tris – 1% SDS pH 8 buffer from SPI, βECF and GEF dispersions. Pressures: 0.1, 300 and 600 MPa. (a) dissociating condition (SDS), (b) reducing condition (SDS + β-mercaptoethanol).

induced aggregates would be unable to form a three-dimensional network, fact that their rheological behavior evidenced.

3.5. SDS-PAGE

Denaturing electrophoretic patterns of the different protein dispersions assayed (SPI, βCEF and GEF) are shown in Fig. 7. Under dissociating conditions (Fig. 7a) SPI presented mainly bands corresponding to β-7S, AB-11S and A-11S polypeptides. Few bands between 31 and 45 kDa appeared after HP treatment which were more evident in 600 MPa treated samples. These polypeptide species may be originated from the dissociation of aggregates detected in native electrophoresis (Fig. 6). Moreover, after 600 MPa, polypeptides species of high molecular mass were scattered in the gel, suggesting the existence of a contemporaneous aggregation phenomena.

The electrophoretic analysis of βCEF revealed that in absence of reducing agent a variety of protein species of high molecular weight was present (Fig. 7a). These protein aggregates may be formed by β-conglycinin subunits (α′,α,β) or by a combination of these proteins with the A- and/or B-glycinin polypeptides that in low extent contaminate the sample. The HP treatment produced the decrease of intensity of the free A-glycinin polypeptide suggesting that it was involved in the formation of other aggregated species. A polypeptide band between 31 and 45 kDa also appeared, probably as consequence of a dissociation process.

In the case of GEF, the control sample (0.1 MPa) presented only the AB-11S and A-11S polypeptides. It was observed that polypeptides bands of high molecular mass were scattered in the gel after HP treatment. On the other hand, the absence of HP-induced macro-aggregates that could not enter in the native-PAGE gel indicates that those high molecular mass specimens observed in native conditions were not stabilized by disulfide bonds, but probably by hydrophobic bonds.

Proteins derived from different samples were analyzed after a reducing treatment with β-mercaptoethanol (Fig. 7b). In non-treated SPI, α′,α-7S, A-11S polypeptide and in a greater proportion β-7S and B-11S were observed. A decrease in the intensity of β-7S

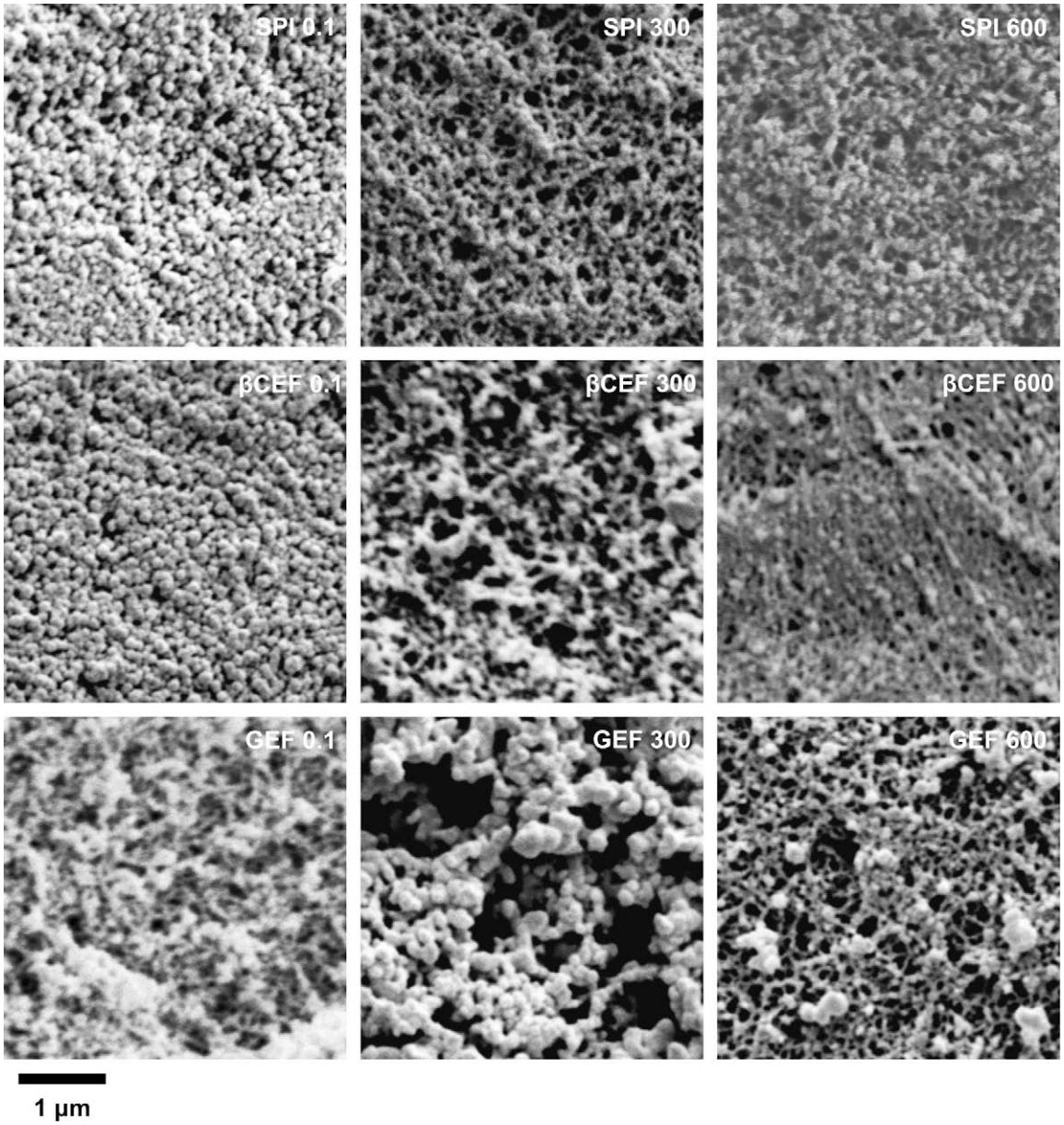

Fig. 8. SEM micrographs of SPI, βCEF and GEF 10% w/w dispersions at different pressure conditions (0.1, 300 or 600 MPa).

and B-11S bands was detected after HP treatment. It was observed that the species of high molecular mass that appeared in βCEF lanes in the absence of β-mercaptoethanol, did not appear in this electrophoretic pattern; suggesting that they were stabilized by disulfide bonds. No differences between pressure treated and control samples were observed.

In the case of GEF, the typical electrophoretic pattern of A and B-11S polypeptides was detected. A band between 66 and 97 kDa appeared after 600 MPa treatment. From another side, bands of very high molecular mass observed in the absence of β-mercaptoethanol in the HP-treated samples did not appear in this electrophoretic gel, suggesting that they were stabilized by disulfide bonds. This data confirms that HP-induced aggregation of glycinin is, at least partially, mediated by disulfide bond formation.

3.6. SEM structure

Scanning electronic microscopy structures of different samples are shown in Fig. 8. In the case of SPI and βCEF granules of sub-micrometric size, without any regular order were observed in the control samples. After the HP treatment a network was observed, being more conspicuous in the 600 MPa treated βCEF dispersion, in which small pores were scattered between protein granules and strands. These changes in matrix structure correlated with the rheological behavior detected (Fig. 1).

It was observed that control GEF dispersions presented an uneven structure. After 300 MPa of pressure treatment, protein strands acquired the form of large granules that seemed to be random aggregated forming a network with low compactness. The 600 MPa produced a slightly ordered structure, but without granule agglomeration. It is noticeable that the changes observed in glycinin molecule arrangement after HP processing were not reflected in any alteration of rheological properties. It is likely that the aggregates randomly formed by HP and stabilized by disulfide bonds, do not interact between them nor interact in a strong manner with water, justifying the constant rheological behavior.

4. Conclusions

Denaturation induced by HP treatment was reflected as changes in the rheological behavior of β-conglycinin-containing protein dispersions (SPI and βCEF). The elasticity of dispersions was increased, even achieving gel formation in βCEF. Nevertheless the HP-induced denaturation of glycinin in GEF was not followed by viscoelasticity changes.

The previous HP treatment on SPI and βCEF promoted a decrease in the temperature of heat-induced gelation, while provoked retardation on gelation of GEF. The sequential HP/thermal treatments on soy proteins induced the formation of weak gels, suggesting that HP inhibited the formation of hydrophobic interactions on heating and the establishment of hydrogen bonds during cooling.

Protein solubility of all dispersions (SPI, βCEF, GEF) increased after HP treatment. High-pressure treatment, especially at 600 MPa, also induced the establishment of hydrophobic interactions and disulfide bonds that allowed the formation of high molecular mass aggregates of different polypeptide composition. Soybean protein isolate aggregates would be mainly formed by β-7S subunits and B-11S polypeptides, while 600 MPa induced aggregation of A and B polypeptides of GEF, both aggregates stabilized throughout disulfide bonds. High pressure also promoted dissociation of aggregates. Species of molecular mass between 31 and 45 kDa were detected in SPI. In the case of GEF a band between 66 and 97 kDa appeared after a 600 MPa treatment.

A network formation in SPI and βCEF at 600 MPa, in accordance with the rheological behavior changes, was observed. Nevertheless changes detected in microstructure of βCEF did not correlate with its constant rheological behavior.

It seems that the structure of soybean species denatured by HP (dissociated subunits and aggregates) was the limiting factor to unfolding and re-association during the heat treatment, avoiding the molecular weight increase needed to form a strong network. This sequential combination of pressure and heat treatments would be avoided if strong gels are desired to be obtained.

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