Innovative Food Science and Emerging Technologies 16 (2012) 155–162

Contents lists available at SciVerse ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Effect of high pressure on solubility and aggregability of calcium-added soybean proteins

M.C. Añón [a], M. de Lamballerie [b], F. Speroni [a,*]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos, CIDCA, CONICET-La Plata, Facultad de Ciencias Exactas, UNLP, 47 y 116, 1900 La Plata, Argentina
[b] ONIRIS, Food Process Engineering, UMR CNRS GEPEA-6144, BP 82225, F-44322 Nantes Cedex 3, France

ARTICLE INFO

Article history:
Received 10 August 2011
Accepted 18 May 2012

Editor Proof Receive Date 15 June 2012

Keywords:
Soybean proteins
High pressure
Calcium chloride
Protein solubility

ABSTRACT

The effects of High Pressure (HP) on the solubility, aggregation and sedimentation of calcium-supplemented soybean proteins present in soybean protein isolate (SPI), and protein fractions enriched in β-conglycinin and glycinin were analyzed in this work.
Calcium addition (up to 0.0075 mol/L) to soybean protein dispersions (1% w/v — pH 8.0) provoked the formation of insoluble aggregates with a very large size. HP treatments split those particles. The size of the HP-formed aggregates was a function of HP intensity, of calcium concentration and of protein composition of samples. 400 and 600 MPa improved the solubility of calcium-added soybean proteins in the three protein samples assayed. At certain calcium concentrations and HP levels, these soluble proteins were involved in the formation of macroaggregates. The velocity of sedimentation of insoluble proteins in calcium-enriched SPI dispersions decreased markedly after HP treatment. This work provides information that may be useful for the handling of complexes formed from soybean proteins and calcium, in order to obtain species with different characteristics regarding solubility and molecular weight.
Industrial relevance: The knowledge provided by this work may promote the use of high pressure in the food industry. Indeed high pressure increases the solubility of calcium-added soybean proteins and, in the case of the insoluble ones, slows down their settling. These features may allow the incorporation of calcium-added soybean proteins to different kinds of enriched drinks.

1. Introduction

Calcium addition to soybean-derived products is a very important topic. In the case of soybean milk, addition of this mineral is a useful strategy to supply the recommended daily intake of calcium, for example in vegetarian diets. In the case of tofu, calcium is utilized as coagulant due to its precipitant properties, but in some others products, such as drinks, it is important to keep the proteins in solution.

Soybean protein isolates (SPIs) are widely used as food ingredients because of their low price, and their nutritional and functional properties. The major proteins present in SPI are glycinin (an 11S globulin of about 360 kDa) and β-conglycinin (a 7S globulin of 180 kDa). Solubility and aggregability are determinants of functional properties of proteins, and are very sensitive to denaturing treatments and ion addition. A decrease in solubility greatly limits the practical application of commercial SPI in the food industry (Tang, Wang, Yang, & Li, 2009).

Yazici, Alvarez, and Hansen (1997) fortified soybean milk yogurt with calcium, in a formula that contained the same calcium concentration with that of cow-milk yogurt, but it presented higher values of syneresis, possibly owing to effects of calcium on hydration properties such as solubility. Ono, Katho, and Mothizuki (1993) reported that thermal treatment applied to raw soybean milk decreased protein solubility if calcium concentration was higher than 0.01 mol/L. In some cases the addition of sequestering agents as potassium citrate or sodium hexametaphosphate allowed to thermally stabilize calcium-enriched soybean milks. However, these agents also decreased viscosity since they eliminated the aggregating effect of calcium (Yacizi, Alvarez, Mangino, & Hansen, 1997). Heaney and Rafferty (2006) analyzed different calcium-fortified soybean drinks and reported that sedimentation is a limiting factor in potential bioavailability.

High pressure (HP) is an emerging technology that has drawn the attention of experts in soybean processing. The effects of HP on soybean protein solubility depend on the composition of the media in which proteins are dispersed. It was found that in soybean milk, an emulsion mainly composed of water, carbohydrates, proteins (45 g/L) and lipids, protein solubility decreased after treatment at 500 and 600 MPa (Lakshmanan, de Lamballerie-Anton, & Jung, 2006). No changes in solubility were found for SPI treated at up to 600 MPa in 10 g/L dispersions (Puppo et al., 2004). A weak gel was formed from

* Corresponding author.
E-mail address: franciscosperoni@gmail.com (F. Speroni).

1466-8564/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ifset.2012.05.006

calcium-enriched (0.01 mol/L) soybean milk (44 g/L protein) when HP treatments of 400, 500 or 600 MPa were applied (Zhang, Li, Tatsumi, & Isobe, 2005). Treatments with 400 or 600 MPa provoke denaturation degrees higher than 75% in glycinin and β-conglycinin. The degree of denaturation achieved at a given HP treatment is modified (increased or decreased) if calcium is present during treatment; the sign and magnitude of these effects depend on protein nature and HP level (Speroni, Añón, & de Lamballerie, 2010). These phenomena suggest that differential interactions between calcium, glycinin and β-conglycinin govern the structural changes induced by HP, and could have different consequences on solubility and aggregate formation.

Based on these data, we considered important to study the effects of HP on the solubility and aggregation state of previously calcium-added soybean proteins, in the form of SPI or partially purified glycinin and β-conglycinin. This knowledge will be interesting in order to incorporate soybean protein dispersions, supplemented with calcium, as food ingredients with tailor-made properties.

2. Materials and methods

2.1. Preparation of soybean protein isolates

Defatted soybean flour (7B Soy Flour) manufactured by ADM (Decatur, USA) was dispersed in distilled water (1:10 w/w). The pH was adjusted to 8.0 with 2 mol/L NaOH and the dispersion was stirred at room temperature for 90 min and centrifuged at 10,000×*g* for 30 min at 4 °C (Jouan GR 20-22, ThermoFisher Scientific Inc., Waltham, MA, USA). The supernatant was adjusted to pH 4.5 with 2 mol/L HCl and centrifuged at 3750×*g* for 15 min at 4 °C. The pellet was washed with distilled water at pH 4.5 and centrifuged as above. The pellet was suspended in distilled water and adjusted to pH 8.0; finally it was frozen at −20 °C and freeze-dried during 48 h at 7 Pa (FTS Systems, Stone Ridge, NY, USA). SPI contained mainly β-conglycinin and glycinin in a similar proportion, and small quantities of lectin and Kunitz trypsin inhibitor, its protein content was 86.6% (Speroni et al., 2010).

2.2. Preparation of partially purified β-conglycinin and glycinin

β-Conglycinin and glycinin globulins were obtained according to the method of Nagano, Hirotsuka, Mori, Kohiyama, & Nishimari, 1992. The same defatted flour used for SPI preparation was dispersed in distilled water (1:15 w/w), adjusted to pH 8.0 with 2 mol/L NaOH, stirred at room temperature for 2 h, and centrifuged at 10,000×*g* for 20 min at 4 °C. Dry sodium bisulfite was added to the supernatant (0.98 g $NaHSO_3$/L), the pH was adjusted to 6.4 with 2 mol/L HCl, and the mixture was kept overnight at 4 °C. The dispersion was centrifuged at 6500×*g* for 20 min at 4 °C. To obtain the glycinin enriched fraction (11SEF) the precipitate was suspended in distilled water, adjusted to pH 8.0 with 2 mol/L NaOH, dialyzed and freeze-dried. Solid NaCl was added to the supernatant (final concentration of 0.25 mol/L) and pH was adjusted to 5.0 with 2 mol/L HCl. After 1 h, the insoluble fraction was removed by centrifugation (9000×*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 mol/L HCl. Centrifugation at 6500×*g* for 20 min at 4 °C was carried out. To obtain the β-conglycinin enriched fraction (7SEF) the washed precipitate was suspended in distilled water, adjusted to pH 8.0 with 2 mol/L NaOH, and dialyzed before freeze-drying. 11SEF was almost fully composed of glycinin and its protein content was 93.0%. 7SEF contained β-conglycinin in a high proportion (75%), and a lower proportion of glycinin (25%), with a protein content of 84.5% (Speroni et al., 2010).

2.3. Protein dispersions

Dispersions of SPI, 7SEF and 11SEF were prepared at 10 g/L in a Tris–HCl 0.050 mol/L pH 8.0 buffer. $CaCl_2$ was added from a stock solution of 1.0 mol/L that was prepared from $CaCl_2$ dihydrate (Sigma, St Louis, USA). $CaCl_2$ was incorporated at three concentrations 0.0025; 0.0050 and 0.0075 mol/L, corresponding to 10, 20 and 30 mg Ca^{2+}/g protein. Calcium was added prior to HP treatment.

In order to analyze the effects of calcium incorporation after HP treatment, SPI, 7SEF and 11SEF were treated at 600 MPa and calcium (0.0025 or 0.0075 mol/L) was then added to protein dispersions. These experiments were carried out in addition to those listed in the paragraph above.

Along this work calcium content is identified as follows: Ca10 (10 mg Ca^{2+}/g protein; 0.0025 mol/L $CaCl_2$), Ca20 (20 mg Ca^{2+}/g protein; 0.0050 mol/L $CaCl_2$) and Ca30 (30 mg Ca^{2+}/g protein; 0.0075 mol/L $CaCl_2$).

2.4. High-pressure processing

HP processing was carried out in a 3 L reactor unit (ACB Pressure Systems, Nantes, France) equipped with temperature and pressure regulation. Prior to pressure processing, SPI, 11SEF, and 7SEF dispersions were vacuum-packaged in polyethylene bags (La Bovida, France). Temperature during treatment was controlled to avoid freezing or overheating of proteins. Protein dispersions were subjected to HP treatment at 200, 400, or 600 ± 7 MPa. The target pressure was reached at 3.4 MPa/s, and pressure was suddenly released after 10 min at target pressure. The pressure transmitting medium was water, and its temperature transiently increased during HP processing. Temperature raised 2–3 °C/200 MPa.

2.5. Protein solubility

Samples were centrifuged at 10,000×*g* for 20 min at 4 °C. Protein concentration was determined in the supernatants with the Sigma Bicinchoninic Acid Protein Assay Kit (Sigma Chemical Co., St. Louis, MO, USA) using bovine serum albumin as standard (Sigma Chemical Co., St. Louis, MO, USA). Solubility was expressed as the percentage ratio of protein content in the supernatant after centrifugation compared to the total protein content in the sample.

2.6. Size exclusion chromatography

Samples were filtered through a cellulose acetate filter of 0.45 μm (Sartorius AG, Goettingen, Germany). A Sephacryl S-500 (Amersham Biosciences, UK) column (1000×16 mm) was used for chromatography. Calibration was carried out with carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), beta-amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) (Molecular Weight Marker Kit for Gel Filtration, Sigma Aldrich Co., St. Louis, MO, USA). The column buffer was Tris–HCl 0.05 mol/L pH 8.0. Samples were eluted from the column at a flow rate of 1 mL/min. Chromatography was carried out with an Amersham Pharmacia system, with control software UNICORN 4.11, GE Healthcare, Chalfont, St. Giles, UK; absorbance was continuously detected at 215 nm. Elution volumes, molecular weights and area under the curve absorbance vs. time were determined. To determine the surface area of each protein fraction, deconvolution of peaks was performed with a Peak Fit software V4.0 (Jandel Scientific Software) according to the Gaussian method.

2.7. Dynamic light scattering measurements

The velocity of sedimentation of insoluble proteins was determined through the use of a vertical scan analyzer Quick Scan (Beckman-Coulter Inc., Miami, USA). The samples were loaded into a cylindrical glass measurement cell, and the transmission percentage profiles of light (λ = 850 nm) all along the tube were immediately monitored every 2 min as a function of the sample height (total height 63 mm). These measurements were used to plot the kinetics

of the mean transmission percentage in the top (61–63 mm) and the middle (30–32 mm) of the tube.

2.8. Statistical analysis

Each treatment was performed at least in triplicate. Solubility and size exclusion chromatography determinations were carried out on all samples: SPI, 7SEF and 11SEF, for the control without calcium addition and the three added calcium concentrations, and for atmospheric pressure (0.1 MPa) and the three assayed pressure levels. The statistical analysis was completed using the Sigmastat software (Systat Software, USA). Analyses of variance were conducted. Differences between the sample means were analyzed by Tukey's test at an α level of 0.05.

3. Results and discussion

3.1. Protein solubility

3.1.1. Effect of calcium addition on SPI, 7SEF and 11SEF

The addition of $CaCl_2$ to dispersions (10 g protein/L, pH 8.0) of SPI, 7SEF and 11SEF produced a decrease in solubility: for SPI and 7SEF the solubility began to diminish at Ca20 (55.8% and 38.9% reduction, respectively, compared with their controls without calcium, $p<0.01$), and at Ca30 the decrease was 71.3% and 70.6% ($p<0.01$) (Fig. 1, panels a and b). The effect of calcium on the solubility of 11SEF was even more pronounced: at Ca10 a considerable decrease was detected (87.7%, compared with its control without calcium, $p<0.01$), and at Ca30 the decrease was 93.0% ($p<0.01$) (Fig. 1, panel c). Regarding the effects on solubility and the protein composition of samples, the results indicate that, in the presence of calcium, the solubility decreased more as the proportion of glycinin increased. This effect may be due to differences between glycinin and β-conglycinin regarding their association with calcium. By measuring calcium binding to proteins using dialysis equilibrium Appu Rao and Narasinga Rao (1976) found that glycinin had higher affinity and a higher maximum number of binding sites for calcium than β-conglycinin. In the present study SPI solubility began to decrease at a higher calcium/protein ratio than that reported by Scilingo and Añón (2004) (Ca7.2). For these authors Ca7.2 corresponded approximately to 0.009 mol/L $CaCl_2$ because protein concentration was 50 g/L. It is possible that the high concentrations of calcium and protein in their study favored the formation of insoluble complexes, explaining the difference with our results. These authors suggested that the presence of calcium promoted the formation of aggregates that were soluble up to Ca4.5, whereas at higher calcium concentration they were insoluble because of their large size.

3.1.2. Effect of HP on SPI, 7SEF and 11SEF

In the absence of calcium, HP did not change the protein solubility of SPI, 7SEF or 11SEF (Fig. 1). These results agree with those of Puppo et al. (2004) who determined the solubility of HP-treated SPI dispersions. On the other hand, Lakshmanan et al. (2006) reported a decrease of up to 54% in protein solubility of soybean milk at pH 7.0 after treatment at 600 MPa; it is possible that the higher protein concentration and the presence of other compounds were involved in this behavior. These data indicate that the effect of HP on soybean protein solubility depends on the composition of the medium where proteins are dispersed.

3.1.3. Effect of HP and calcium addition on SPI, 7SEF and 11SEF

The treatments at 400 and 600 MPa increased protein solubility in dispersions in which calcium addition had decreased it (Fig. 1). At Ca30, for example, the solubility augmented from 19.5% to 48.7% for SPI ($p<0.01$) and from 19.4% to 57.6% for 7SEF ($p<0.01$) (0.1 vs. 600 MPa). This effect was dependent on calcium concentration: for

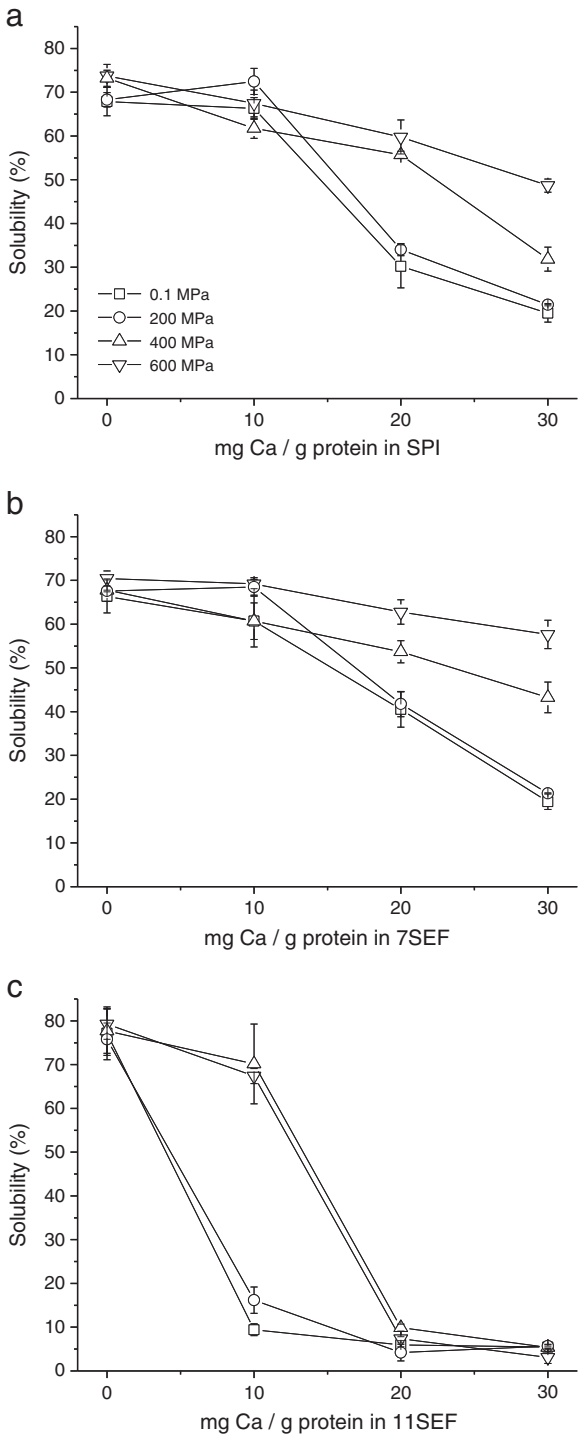

Fig. 1. Protein solubility in soybean protein isolate (SPI) (a), β-conglycinin enriched fraction (7SEF) (b) and glycinin enriched fraction (11SEF) (c), as function of calcium added concentrations and for different pressure levels: 0.1 (square), 200 (circle), 400 (up triangle) or 600 (down triangle) MPa. Values are means ± standard deviation (n = 3).

11SEF solubility was reverted at Ca10 but not at the higher concentrations, whereas for SPI and 7SEF the degree of reversion was higher at Ca20 than at Ca30. For SPI and 7SEF at Ca30 the effect depended on the HP level, so that solubility increased with increasing pressures. The solubility reverting effect of HP was more conspicuous for 7SEF, intermediate for SPI, and weaker for 11SEF. This fact seems to be related to the protein composition of samples, the effect being greater for β-conglycinin than for glycinin.

In order to evaluate the mechanism of the solubility reverting effect of HP, some samples were treated with HP in the absence of calcium, and later added with calcium before solubility measurement. Treatments at 600 MPa were applied to protein dispersions whose calcium concentration corresponded to Ca10 (for 11SEF) and Ca30 (for SPI and 7SEF). No differences were found in solubility between these samples and those in which calcium was incorporated prior to treatment (data not shown). These data suggest that the effect on solubility depends on changes in proteins, which would be independent of calcium presence during treatment. Once calcium is added, protein may remain in solution. It may be possible that structural changes induced by HP decreased the affinity of binding sites for calcium, avoiding the interaction and neutralization of charges. Another possibility is the hiding of binding sites due to conformational changes during HP.

3.2. Size exclusion chromatography

3.2.1. Chromatograms of control SPI, 7SEF and 11SEF

The chromatograms of SPI and 7SEF (Fig. 2) showed that proteins from SPI and 7SEF were involved in the formation of aggregates at pH 8.0 in Tris–HCl 0.05 mol/L buffer, since the molecular weights corresponding to the absorbance maximum were 800 and 1100 kDa respectively. On the other hand, the molecular weight corresponding to the maximum of the distribution of 11SEF was 330 kDa, suggesting that this protein was not aggregated, since its reported molecular weight is 360 kDa. These results are in accordance with those of Cole and Cousin (1994) who analyzed SPI by size exclusion chromatography and found that the SPI profile was constituted by high molecular weight complexes containing different proportions of β-conglycinin and glycinin subunits and a separated peak of glycinin. Molina Ortiz and Añón (2000) also reported that most SPI proteins were involved in 917 kDa aggregates. Ono, Choi, Ikeda, and Odagiri (1991) found that the proteins present in the filtered fraction of a homogenate of soaked beans (raw soybean milk) were involved in particles of different sizes, 40% of which were larger than 0.1 μm in diameter. Those macroaggregates coexisted with soluble non-particulated β-conglycinin and glycinin. Results from our laboratory indicated that the aggregates present in SPI and 7SEF were stabilized by non-covalent bonds since they were disassembled in the presence of 0.5 mol/L NaCl (Añón, de Lamballerie, & Speroni, 2011).

3.2.2. Effect of calcium addition on SPI, 7SEF and 11SEF

The effects of calcium addition on size exclusion chromatograms consisted mainly in a decrease in the amount of protein that entered the gel (Fig. 2). This fact was observed in SPI-Ca20, SPI-Ca30, 7SEF-Ca30, 11SEF-Ca10, 11SEF-Ca20 and 11SEF-Ca30, and indicates that calcium induced the formation of aggregates big enough to be excluded by the filter of 0.45 μm. In the case of 7SEF-Ca30 the position of the absorbance maximum did not change, but in SPI-Ca20, SPI-Ca30 and in 11SEF at the three calcium concentrations assayed, the position of the maximum shifted to an elution volume corresponding to 60 kDa, suggesting that only the free AB subunits from glycinin were not involved in the formation of high molecular weight aggregates. It is noteworthy that the decrease in the amount of filtered protein in SPI-Ca20 and 7SEF-Ca20 was small (SPI) or null (7SEF) in spite of a solubility decrease of 55.8% and 38.9%, respectively — Ca20 vs. the control without calcium addition, ($p<0.01$). These data suggest that at this calcium/protein ratio, insoluble particles were small enough to pass through the filter and had the same size distribution that the aggregates formed in the absence of calcium. It is possible that in these cases the insolubility was due to neutralization of the surface charges of protein, without the formation of new aggregates. We cannot exclude that the filtered aggregates formed in the presence of calcium were different, but once in the column they were modified by dilution of calcium and exhibited the same profiles that those formed in the absence of calcium. Since at higher calcium concentration (SPI-Ca30 and 7SEF-Ca30) the aggregates were excluded by the filter, we conclude that the size of particles was a function of calcium concentration.

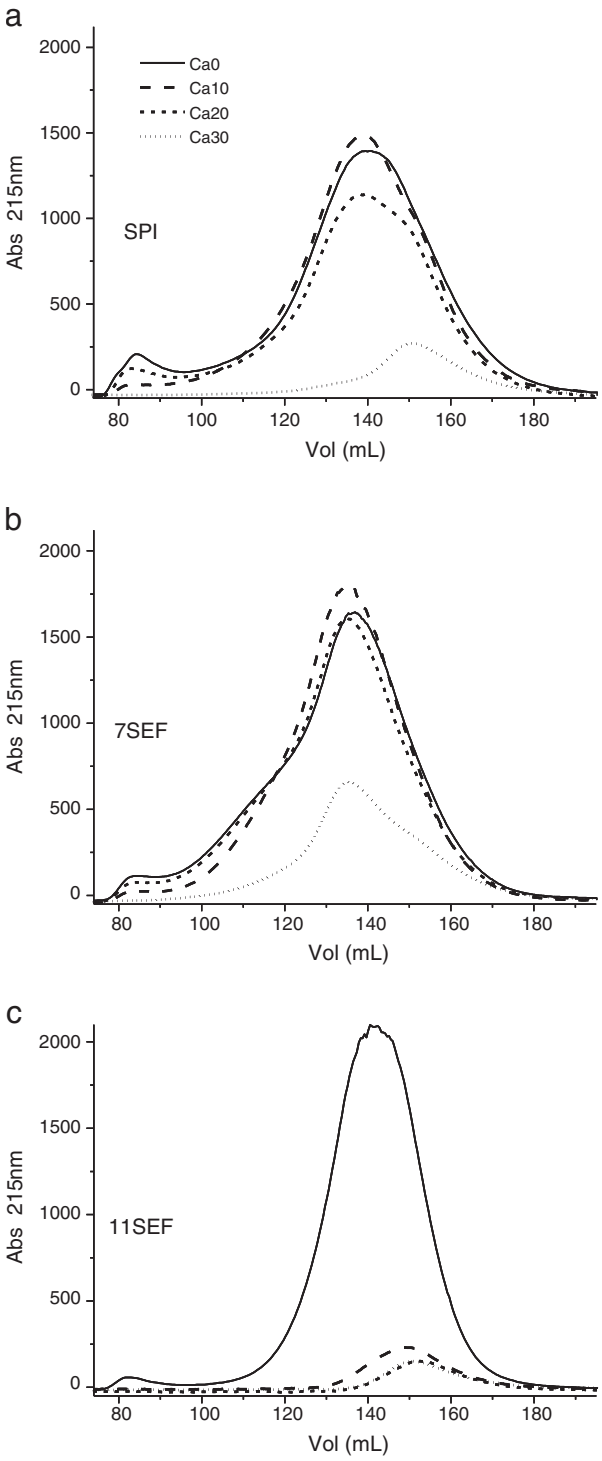

Fig. 2. Typical recordings of size exclusion chromatography of soybean protein isolate (SPI) (a), β-conglycinin enriched fraction (7SEF) (b) and glycinin enriched fraction (11SEF) (c), for different calcium added concentrations: 0 (solid line), 10 (dashed line), 20 (short dashed line) and 30 (dotted line) mg Ca^{2+}/g protein, at 0.1 MPa. Initial protein content was 10 g/L, pH 8.0, flow rate was 1 mL/min.

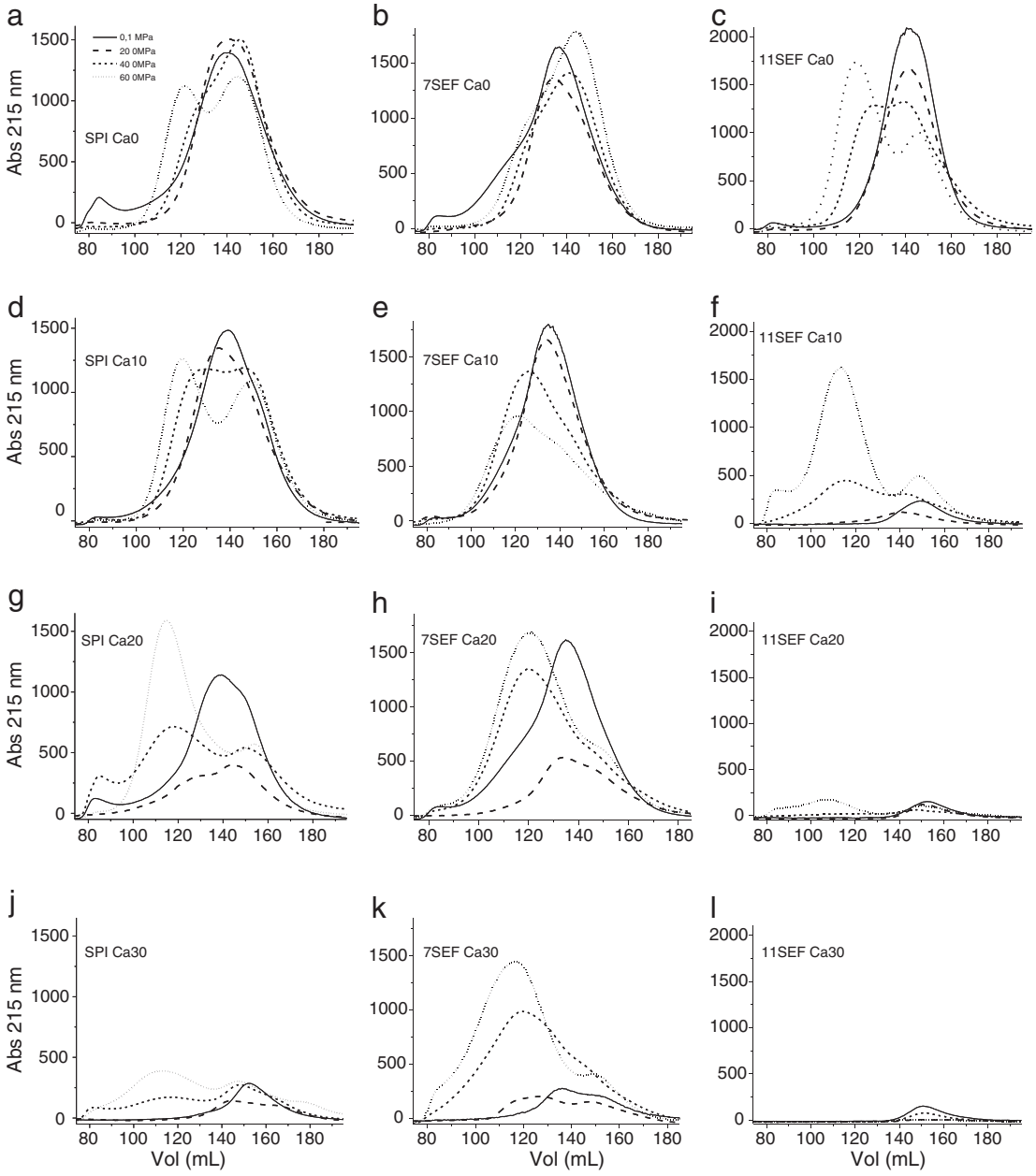

Fig. 3. Typical recordings of size exclusion chromatography of soybean protein isolate (SPI) (a, d, g and j), β-conglycinin enriched fraction (7SEF) (b, e, h and k) and glycinin enriched fraction (11SEF) (c, f, i and l), for different calcium added concentrations: 0 (a, b and c), 10 (d, e and f), 20 (g, h and i) and 30 (j, k and l) mg Ca^{2+}/g protein, for different pressure levels: 0.1 (solid line), 200 (dashed line), 400 (short dashed line) or 600 (dotted line) MPa. Initial protein content was 10 g/L, pH 8.0, flow rate was 1 mL/min.

3.3. Effect of HP on SPI, 7S and 11S

The chromatographic profiles of SPI, 7SEF and 11SEF treated with HP in the absence of calcium are shown in panels a, b and c of Fig. 3. In the case of SPI, treatments at 400 and 600 MPa promoted the dissociation of the initial aggregates and the formation of new ones, reflected by the appearance of a peak of 250 kDa and one of greater molecular weight than the initial aggregates. These findings agree with those of Puppo et al. (2004).

For 7SEF, treatment at 200 MPa promoted the narrowing of molecular weight distribution, indicating that some high molecular weight complexes disappeared from the filtered fraction. As a result of treatment at 400 MPa the initial species of 1100 kDa split into another of approximately 500 kDa with a shoulder corresponding to aggregates of molecular weight higher than 1100 kDa. At 600 MPa both dissociation and aggregation phenomena were evident, being the dissociation product smaller and the aggregation one larger than those obtained at 400 MPa. On the other hand, the effect of HP (400 and 600 MPa) on 11SEF seemed to consist only in aggregation: the more intense the HP treatment, the higher the molecular weight of the formed species.

Comparing the effects of pressure on SPI, 7SEF and 11SEF, it may be concluded that the relative amount and the molecular weight of the aggregates formed by HP treatment were higher as the proportion of glycinin increased in the samples (11SEF > SPI > 7SEF). These results suggest that, in the absence of calcium, glycinin was more prone to be involved in aggregates after HP treatment, while β-conglycinin tended to dissociate from the initial aggregates. Maruyama et al. (1998) proposed that carbohydrate moieties of β-conglycinin prevent the formation of aggregates. Although in the untreated samples β-conglycinin was indeed involved in aggregates, it is possible that after HP treatment the presence of carbohydrate

moieties affected the interactions leading to dissociation rather than aggregation.

Taking into account that the changes in solubility seem to be due to effects of HP on proteins, which did not depend on calcium presence during treatment, it is possible to hypothesize that the increased solubility of 7SEF is due to the decrease in molecular size (dissociation). On the other hand, in 11SEF it is possible that aggregation was accompanied by the hiding of binding sites for calcium. Thus, despite an increase in the molecular size of species, the resulting solubility was higher than before HP treatment.

3.4. Effect of calcium addition and HP on SPI, 7SEF and 11SEF

The profiles of SPI showed (Fig. 3, panels a, d, g and j) that the aggregating effect of HP was favored in the presence of calcium: at Ca10, the area of the peak corresponding to the HP-aggregated protein increased from 24% (a shoulder) to 50% at 400 MPa, and from 40% to 50% at 600 MPa ($p<0.05$). In SPI Ca20-400 MPa and SPI Ca30-600 MPa, the amount of protein that entered into the gel decreased more than solubility. For example, the area under the curve of absorbance vs. time in SPI-Ca30-600 MPa dropped by 55% while solubility dropped by 17% (Ca30 vs. Ca0, $p<0.05$). We conclude that part of the enormous macroaggregates, whose size brought about their exclusion by the 0.45 μm filter, were soluble. Moreover, analyzing the chromatograms obtained at Ca30 before and after HP treatment, we observe that HP produced the split of some particles, since species of high molecular weight entered the gel in SPI-Ca30-400 MPa and SPI-Ca30-600 MPa, but not in the 0.1 or 200 MPa samples.

In 7SEF samples we observed that the combination of Ca20 with 200 MPa produced the formation of aggregates or precipitates that could not be filtered by the 0.45 μm, suggesting that calcium addition determined an earlier appearance of the aggregating effect of HP. For higher HP values the aggregates were smaller than at 200 MPa, were able to pass through the filter, but were larger than the aggregates formed by HP in the absence of calcium (Fig. 3, panels b and h). The amount of protein that entered the column in 7SEF-Ca30-400 MPa and 7SEF-Ca30-600 MPa was higher than that at 0.1 or 200 MPa (Fig. 3, panel k), and it was also higher than that of SPI in the same conditions of calcium and pressure (Fig. 3, panel j). These results correlate with those of solubility, indicating that part of the HP-solubilised protein is involved in the formation of aggregates of very high molecular weight.

The chromatograms of 11SEF-Ca10 (Fig. 3, panel f) showed that treatment at 400 and 600 MPa increased the amount of protein that entered the gel. The treatment at 600 MPa was more efficient in splitting the precipitated aggregates formed by calcium than that at 400 MPa. It is remarkable that in Ca10-400 MPa and in Ca10-600 MPa the solubility was equivalent, thus a considerable fraction of soluble protein after 400 MPa treatment corresponded to aggregates large enough to be excluded by the 0.45 μm filter. In 11SEF-Ca20 and 11SEF-Ca30 the profiles only exhibited a remnant peak of 120 kDa (Fig. 3, panels i and l). Taking into account the results of solubility, that showed that for these calcium concentrations the HP was not able to revert the calcium-induced insolubility, we conclude that the insoluble protein in these cases was involved in species of very large size.

Regarding the elution volumes of the peaks corresponding to the aggregates formed by the combination of calcium and 400 or 600 MPa (Table 1), it is possible to conclude that the molecular weights of the species that entered the gel depended on calcium concentration, pressure level, and protein composition of samples. The molecular weight of aggregates increased as calcium concentration increased. At a given calcium concentration, the molecular weights were higher at 600 MPa than at 400 MPa. The largest aggregates that entered the column were formed by 11SEF protein. It is remarkable that for the three samples, conditions existed in which protein complexes were large enough to be excluded by the 0.45 μm filter, but presented very high values of solubility: SPI-Ca20-400 MPa, SPI-Ca30-600 MPa, 7SEF-Ca10-400 MPa, 7SEF-Ca10-600 MPa, and 11SEF-Ca10-400 MPa.

Table 1
Elution volumes (mL) of aggregates formed by proteins from the three assayed preparations, at different calcium concentrations and after 400 or 600 MPa.

	Ca0	Ca10	Ca20	Ca30
SPI 400 MPa	128.2 ± 0.7^{a}	129.3 ± 0.8^{a}	117.6 ± 0.5^{c}	116.7 ± 0.7^{c}
SPI 600 MPa	121.7 ± 0.6^{b}	119.6 ± 0.6^{bc}	114.5 ± 0.5^{d}	112.8 ± 0.6^{e}
7SEF 400 MPa	122.1 ± 0.9^{b}	125.8 ± 0.3^{a}	119.9 ± 0.6^{b}	119.6 ± 0.4^{b}
7SEF 600 MPa	122.9 ± 0.6^{b}	121.3 ± 0.6^{bc}	120.1 ± 0.5^{c}	115.8 ± 0.7^{d}
11SEF 400 MPa	126.4 ± 0.9^{a}	115.2 ± 0.4^{c}	–	–
11SEF 600 MPa	118.4 ± 0.6^{b}	113.3 ± 0.5^{c}	107.5 ± 0.6^{d}	–

SPI: soybean protein isolate, 7SEF: β-conglycinin-enriched fraction, 11SEF: glycinin-enriched fraction. Ca0: no calcium addition, Ca10: 10 mg Ca^{2+}/g protein, Ca20: 20 mg Ca^{2+}/g protein, Ca30: 30 mg Ca^{2+}/g protein. Values are means ± standard deviation ($n=3$). Means within a column and a row (for each protein preparation) with the same superscripts are not significantly different ($p<0.05$).

The aggregates formed by soybean protein in the presence of calcium and after HP treatment may be stabilized by different kinds of interactions. Surface hydrophobicity of SPI increase after HP (Puppo et al., 2004), and this may promote the association of polypeptides. Calcium bridges may also link some denaturated species. It is likely that during HP treatment some interactions (such as ionic ones) are disassembled and others are established (e.g., hydrogen bonds). Canabady-Rochelle and Mellema (2010) suggested that calcium–soybean protein interaction was not only coulombic, and that the release of water from the hydration shells of the calcium ion and/or dehydration of the hydrophobic core of the proteins may be the driving energy source for calcium binding to soybean proteins. As the release of water from the ionic shell is a phenomenon accompanied by an increase in the volume of the system, it would be disfavored by HP, thus avoiding or disrupting certain calcium–protein interactions. This fact may explain the splitting of certain aggregates that, before HP treatment, were stabilized by calcium bridges. The structure, size, and solubility of the resulting species depended on calcium concentration, HP level, and protein nature.

3.5. Protein sedimentation

Settling of calcium–protein complexes is an important limiting factor to stability of drinks and potential bioavailability of calcium. Heaney and Rafferty (2006) reported that the average percentage of added calcium that remained in suspension, before and after shaking of soybean drinks, was 31% and 59%, respectively. We analyzed the effect of HP on the velocity of sedimentation of calcium-enriched soybean proteins. The velocity of sedimentation of insoluble protein was analyzed by light scattering in SPI-Ca20; 7SEF-Ca30 and 11SEF-Ca10 for non-treated and 600 MPa-treated samples. In the non-treated samples, the sedimentation of insoluble protein, which confers turbidity to the dispersion, provoked a net phase separation (Fig. 4). The interface moved towards the bottom of the tube, separating a white lower phase from a more transparent upper one. The slowest sedimentation was detected in SPI-Ca20, in which a 100% transmission in the middle of the tube was reached after 150 min in the untreated samples ($p<0.05$). After 600 MPa the behavior of SPI-Ca20 changed drastically: after 14 h, the transmission in the upper 2 mm of the tube only reached to 45% ($p<0.05$) whereas under this height the transmission was 0% ($p<0.05$), indicating that the insoluble protein remained in suspension. The effect of HP on 11SEF-Ca10 consisted in a retardation of sedimentation and a decrease in the achieved percentage of transmission, suggesting that a fraction of insoluble protein remained in suspension. Treatment of 7SEF-Ca30 at 600 MPa induced the formation of macroscopic aggregates that

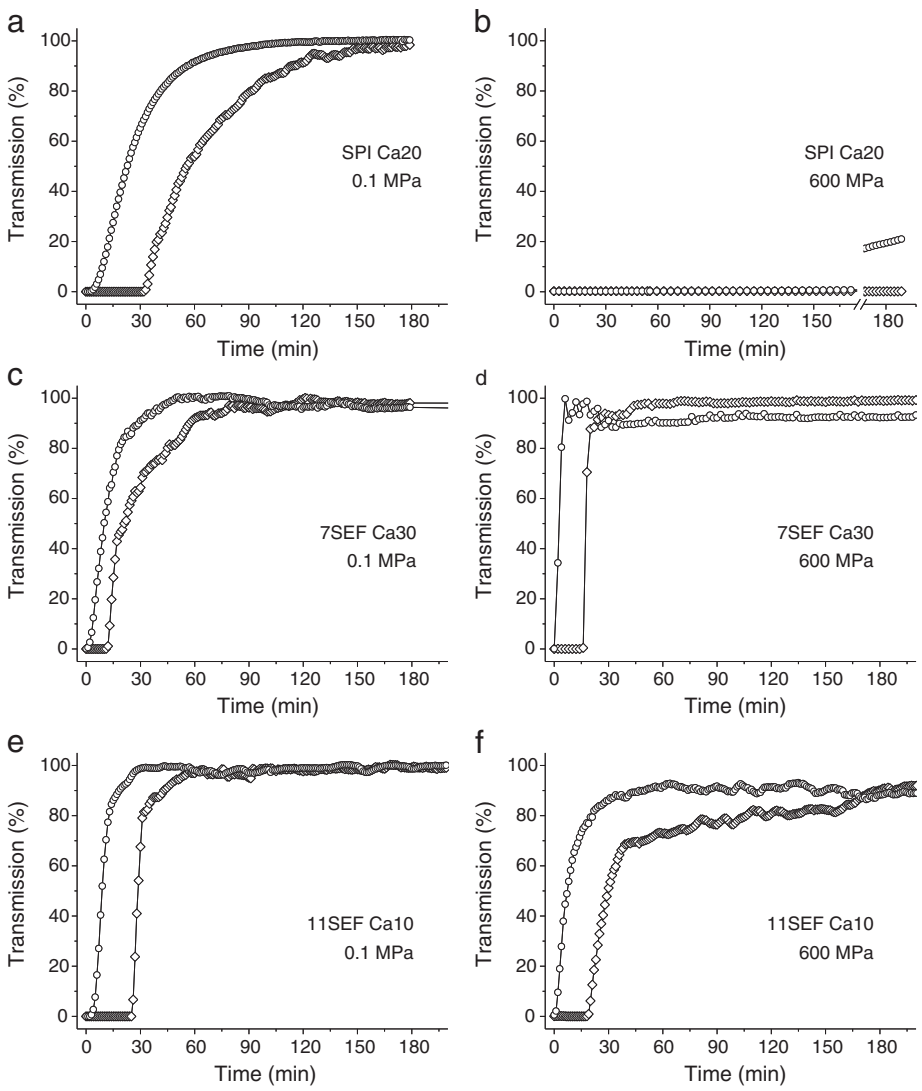

Fig. 4. Sedimentation kinetics of insoluble proteins. Typical recordings of light transmission in the top (circle) and in the middle (diamond) of the tube of calcium-enriched protein dispersions (10 g/L, pH 8.0) of soybean protein isolate (SPI Ca20) (a and b), β-conglycinin enriched fraction (7SEF Ca30) (c and d) and glycinin enriched fraction (11SEF Ca10) (e and f), at 0.1 (a, c and e) or 600 (b, d and f) MPa.

involved most of the insoluble protein, making the interface to drop faster than in the control samples. The effect observed in SPI and 11SEF might be partially explained by an increase in viscosity due to the presence of high molecular weight aggregates. In these experiments the dispersions were neither filtered (as for molecular exclusion chromatography) nor centrifuged (as for solubility), thus soluble and insoluble proteins of the whole range of molecular weights coexisted. Another explanation for the retardation in sedimentation would consist in the formation of complexes between insoluble and soluble proteins (bridged by calcium or other kind of interaction) that exhibit a low velocity of sedimentation. In the case of SPI-Ca20-600 MPa the soluble protein with high molecular weight (e.g. complexes that did not pass through the 0.45 μm filter in size exclusion chromatography assays) may be responsible for maintaining insoluble protein in suspension. It is evident that the proportion of glycinin and β-conglycinin plays an important role in this phenomenon. It is noteworthy that in 7SEF the effect of HP on solubility reversion was higher than in the other samples, while the effect on the velocity of sedimentation consisted in an increase. It is possible that in SPI and 11SEF interactions occurred between soluble and insoluble proteins whereas these did not happen in 7SEF. It is also possible that the size of soluble and insoluble species influences their ability of cross-interaction.

4. Conclusions

In the absence of calcium, glycinin was more prone to be involved in aggregates after HP treatment than β-conglycinin. After HP treatment the latter tended more to dissociate from the initial aggregates than to form aggregates.

Calcium addition to soybean proteins provoked the formation of insoluble aggregates whose size precluded their passage through a 0.45 μm filter. HP treatment split those particles allowing the passage of the resulting species through the filter. The size of the permeated aggregates was a function of HP intensity (the higher the HP level, the higher the molecular size), of calcium concentration (the higher the concentration, the higher the molecular size), and of the protein composition of samples (the largest aggregates were formed in 11SEF, in which glycinin is the major component).

Treatments with 400 and 600 MPa improved the solubility of calcium-added soybean proteins present in SPI, 7SEF and 11SEF. In SPI-Ca20-400 MPa, SPI-Ca30-600 MPa, 7SEF-Ca10-400 MPa, 7SEF-Ca10-600 MPa, and 11SEF-Ca10-400 MPa, these soluble proteins were involved in the formation of macroaggregates (their size prevented its permeation through the 0.45 μm filter).

The velocity of sedimentation of insoluble proteins in calcium-enriched SPI dispersions decreased markedly after HP treatment.

Calcium supplemented and HP-modified soybean proteins may be useful as tailor-made ingredients since they may be prepared with improved solubility and/or under insoluble form (but stable in suspension) as macro aggregates. These different features would provide different functional properties and applications.

References

Añón, M. C., de Lamballerie, M., & Speroni, F. (2011). Influence of NaCl concentration and high pressure treatment on thermal denaturation of soybean proteins. *Innovative Food Science and Emerging Technologies*, *12*, 443–450.

Appu Rao, A. G., & Narasinga Rao, M. S. (1976). Binding of Ca(II), Mg(II), and Zn(I1) by 7S fraction of soybean proteins. *Journal of Agricultural and Food Chemistry*, *24*, 490–494.

Canabady-Rochelle, L. S., & Mellema, M. (2010). Physical–chemical comparison of cow's milk proteins versus soy proteins in their calcium-binding capacities. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *366*, 110–112.

Cole, K. D., & Cousin, S. L. (1994). Size exclusion chromatography of soybean proteins and isoflavones. *Journal of Agricultural and Food Chemistry*, *42*, 2713–2720.

Heaney, R. P., & Rafferty, K. (2006). The settling problem in calcium-fortified soybean drinks. *Journal of the American Dietetic Association*, *106*, 1753–1755.

Lakshmanan, R., de Lamballerie-Anton, M., & Jung, S. (2006). Effect of soybean-to-water ratio and pH on pressurized soymilk properties. *Journal of Food Science*, *71*, E384–E391.

Maruyama, N., Katsube, T., Wada, Y., Oh, M. H., Barba De La Rosa, A. P., Okuda, E., et al. (1998). The roles of the N-linked glycans and extension regions of soybean β-conglycinin in folding, assembly and structural features. *European Journal of Biochemistry*, *258*, 854–862.

Molina Ortiz, S. E., & Añón, M. C. (2000). Analysis of products, mechanisms of reaction, and some functional properties of soy protein hydrolysates. *Journal of the American Oil Chemists' Society*, *77*, 1293–1301.

Nagano, T., Hirotsuka, M., Mori, H., Kohiyama, K., & Nishimari, K. (1992). Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. *Journal of Agricultural and Food Chemistry*, *40*, 941–944.

Ono, T., Choi, M. R., Ikeda, A., & Odagiri, S. (1991). Changes in the composition and size distribution of soymilk protein particles by heating. *Agricultural and Biological Chemistry*, *55*, 2291–2297.

Ono, T., Katho, S., & Mothizuki, K. (1993). Influence of calcium and pH on protein solubility in soybean milk. *Bioscience, Biotechnology, and Biochemistry*, *57*, 24–28.

Puppo, M. C., Chapleau, N., Speroni, F., de Lamballerie, M., Añón, M. C., & Anton, M. (2004). Physicochemical modifications of high-pressure treated soybean protein isolates. *Journal of Agricultural and Food Chemistry*, *52*, 1564–1571.

Scilingo, A. A., & Añón, M. C. (2004). Characterization of soybean protein isolates. The effect of calcium presence. *Journal of the American Oil Chemists' Society*, *81*, 63–69.

Speroni, F., Añón, M. C., & de Lamballerie, M. (2010). Effects of calcium and high pressure on soybean proteins: A calorimetric study. *Food Research International*, *43*, 1347–1355.

Tang, C. H., Wang, X. Y., Yang, X. Q., & Li, L. (2009). Formation of soluble aggregates from insoluble commercial soy protein isolate by means of ultrasonic treatment and their gelling properties. *Journal of Food Engineering*, *92*, 432–437.

Yacizi, F., Alvarez, V. B., Mangino, M. E., & Hansen, P. M. T. (1997). Formulation and processing of a heat stable calcium-fortified soy milk. *Journal of Food Science*, *62*, 535–538.

Yazici, F., Alvarez, V. B., & Hansen, P. M. T. (1997). Fermentation and properties of calcium-fortified soy milk yogurt. *Journal of Food Science*, *62*, 457–461.

Zhang, H., Li, L., Tatsumi, E., & Isobe, S. (2005). High-pressure treatment effects on proteins in soy milk. *Lebensmittel-Wissenschaft und Technologie*, *38*, 7–14.