Formation of Soy Protein Isolate
Cold-set Gels: Protein and Salt Effects

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ABSTRACT: The influence of protein and calcium concentration on soy protein cold-set gel formation and rheology has been investigated. Cold-set gels can be formed at soy protein concentrations from 6% to 9% and calcium concentrations from 10 to 20 mM. Gel properties can be modulated by changing the protein and/or calcium concentrations. An increase in calcium concentration from 10 to 20 mM increased gel opacity while an increase in protein concentration from 6% to 9% decreased opacity. Water-holding capacity improved with increasing protein concentration and decreasing calcium concentration. The elastic modulus (G') increased with protein and calcium chloride concentrations. Microscopy revealed an increase in the diameter of aggregates and pores as calcium concentration increased and as protein concentration decreased. Cold-set gels with a broad range of characteristics can be obtained from soy protein.

Keywords: soy protein, cold-set gel, calcium chloride, gel rheology

Introduction

Most globular proteins (albumin, whey protein, soy protein) can form heat-set gels. Heating protein dispersions causes molecular unfolding, which leads to partial aggregation of proteins and hence gelation. Gel physicochemical characteristics, such as mechanical properties, opacity, and water-holding capacity, may be modulated by modifying gelation temperature, pH, salt type, and salt concentration (Clark and Ross-Murphy 1987; Ziegler and Foegeding 1990; Lakemond and others 2003).

In fact, heat is not always required in a direct manner to induce protein gelation. Cold-induced gelation of whey protein can be achieved by adding calcium ions to a preheated protein solution (Barbut and Foegeding 1993). The preheating step induces molecular alterations (open protein structure, exposed reactive groups) necessary ultimately to obtain the formation of a network (Barbut and Foegeding 1993; Hongsprabhas and Barbut 1998). The initial pH of the solution must differ sufficiently from the isoelectric point of the gelling proteins (Bryant and McClements 1998) and the ionic strength must be low enough to avoid immediate excessive aggregation of protein molecules upon preheating. The protein concentration during preheating must also be below a critical value for forming a three-dimensional network. Adding calcium chloride to a cooled solution neutralizes electrostatic repulsion and forms salt bridges between protein aggregates, allowing these to form a space-filling network (Bryant and McClements 1998; Marangoni and others 2000).

Many studies have focused on cold gelation of whey proteins because of their importance in the food industry. They have proven useful for explaining gel structure-forming mechanisms (Hongsprabhas and Barbut 1997b; Hongsprabhas and others 1999; Mleko and others 2002; Remondetto and Subirade 2003), the influence of salt type and concentration on gel texture and microstructure (Barbut 1995a; Barbut 1995b; Bryant and McClements 2000; Remondetto and others 2002), and the effect of preheating on gel mechanical properties and water-holding capacity (Hongsprabhas and Barbut 1997a). With the exception of a recent study on the cold gelation of ovalbumin induced by lowering the pH near the isoelectric point (Alting and others 2004), little is known on the cold gelation behavior of proteins with high molecular weight and of great structural complexity, such as soy proteins.

Soy protein isolate (SPI) is extensively used as a functional ingredient in many different food products, such as baked goods and cured meats. It is composed almost exclusively of 2 globular protein fractions differentiated by sedimentation coefficient: 7S (β-conglycinin) and 11S (glycinin) (Wolf and others 1961; Saio and Watanebe 1978). Both fractions consist of several subunits, which associate and dissociate under different conditions of pH, ionic strength, and temperature (Badley and others 1975; Hermansson 1986a; Petrucelli and Añón 1995) and both have the ability to form heat-set gels (German and others 1982; Usumi and Kinsella 1985; Hermansson 1985; Hermansson 1986a; Renkema and others 2001). Adding salt to a dispersion of protein prior to heating stabilizes protein quaternary structure against dissociation and denaturation (Hermansson 1978). Ionic strength and pH play an important role in heat-set soy protein gel formation and rheological properties (Renkema and others 2000, 2002). High ionic strength causes a more aggregated structure for glycinin (Petrucelli and Añón 1995; Lakemond and others 2003), β-conglycinin (Hermansson 1985) and soy protein isolate heat-set gels (mainly composed of glycinin and β-conglycinin) (Puppo and Añón 1998a, 1998b).

Although much work has been done on heat-induced soy protein gels, nothing is known about the ability of soy proteins to form cold-set gels yet. Moreover, at this stage it is difficult to anticipate results on Cold gelation of soy proteins on the basis of literature available on the cold gelation of whey proteins, because the gelation process depends on a variety of parameters, including molecular weight (Huang and others 1994), solubility, and aggregate size (Wang and Damodaran 1991). Although the major components of soy protein...
isolates (SPI) are globular, they are characterized by molecular weights of about 200,000 for conglycinin (MW 180000 to 210000) and 350000 for glycinin, and both fractions complexes compared with whey protein isolate (WPI) or β-lactoglobulin (MW 18000). These differences may have a major impact on the ability to prepare cold-set gels and on the final properties of the gels.

The objective of this work was therefore to determine the ability of a soy protein isolate to form gel networks by adding calcium ions to preheated and cooled protein solution. Effects of protein and Ca$^{2+}$ concentrations on gel opacity, water-holding capacity, and rheological behavior ($G^*$) were deemed reproducible. The measured responses were color ($L^*$), water-holding capacity ($WHC$), and rheological behavior ($G^*$). This design allowed identification of combined effects of variables that would lead to a gel with particular physical behavior. Statgraphics Plus Professional V 4.1 (Manugistics Inc., Rockville, Md., U.S.A.) was used for all statistical analysis deem.

Materials and methods

Soy protein

Native soy protein isolate (SPI), prepared at pilot plant scale according to the method of Remondetto and others (1998), was obtained from Food Science and Technology Inst. (Facultad de Enginería Química, Santa Fe, Argentina). It contained 94% protein on a dry basis as measured by the macro-Kjeldahl method (AOAC 1984) using N-factor 6.25. Calcium chloride (anhydrous CaCl$_2$) was of reagent grade (Fisher Scientific, Springfield, N.J.)

Induction of cold gelation

Preliminary studies indicated that to prepare the pre-denatured soy proteins solution it is necessary to use pH value that is far enough from isoelectric point of the soy proteins (4.76) and to use a protein concentration lower than 10% because at higher (or similar) concentration a three-dimensional network is formed without the addition of salt. Calcium concentration below 10 or above 20 mM produced non auto-support gels. These data allowed us to define the range of the different variables used in this work.

A 9.5% (w/w) SPI solution in double-distilled water was prepared by mixing for 1 h at room temperature using a magnetic stirrer. The solution pH was then adjusted to 7.0 using 0.1 M NaOH. The sample solutions (10 mL) were introduced in tightly closed tubes to avoid evaporation and heated at 105 °C for 30 min using a 50:50 water/ethylene glycol bath and cooled for 2 h to room temperature (25 °C). A 0.5 M CaCl$_2$ solution prepared in double-distilled water were then added as required, followed immediately by vortexing, to obtain concentrations of 6%, 7%, 8%, and 9% (w/w) protein and 10, 15, and 20 mM CaCl$_2$. The samples were stored 24 h at room temperature.

Experimental design

Because no studies have been published yet on the Cold gelation of soy proteins, we used a factorial experiment to screen a large number of conditions. The effect of protein concentration (6%, 7%, 8%, and 9% w/w) and Ca$^{2+}$ concentration (10, 15, and 20 mM) on SPI gel rheological and macroscopic properties was investigated by means of a factorial model with 2 factors distributed in 4 and 3 levels, respectively.

The codes for the experimental treatment level combinations are shown in Table 1. Treatments were performed in random order within each block. Each block was a repetition of all conditions of the experiment. If no significant effect due to blocks is found, results are deemed reproducible. The measured responses were color ($L^*$), water-holding capacity (WHC), and rheological behavior ($G^*$). This design allowed identification of combined effects of variables that would lead to a gel with particular physical behavior. Statgraphics

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The experimental runs were performed in a random order.

Macroscopic textural observations

Observations of macroscopic characteristics of gels were captured as photographs using a Minolta digital camera.

Light reflectance determination

Surface reflectance of gels (20 mm thick) was measured using the standard CIELAB color system and reported as $L^*$ value (whiteness parameter) obtained by a Minolta Chroma Meter CR-200 (Minolta Camera Co. Ltd., Osaka, Japan). Calibration of the Chroma Meter was carried out using a standard white plate provided by the manufacturer. For each gel, $L^*$ values were recorded in triplicate from 5 samples.

Water-holding capacity measurements

WHC of gels was measured using the method of Kocher and Foegeding (1993) modified by Remondetto and others (2002). Samples (1 g) of protein gel were formed in ultratfiltration units (Millipore, Ultrafree-CL Filters, Yonezawa-shi, Yamagata-ken, Japan) consisting of a tube (5 mL) and an inner tube (2 mL) supporting an ultrafiltration membrane of 5000 Da nominal molecular weight limit. Samples were centrifuged in a Sorvall centrifuge (Dupont, Newtown, Pa., U.S.A.) at 5000 × g (GSA rotor of 0.0145 mm radius) for 20 min 24 h after gel induction. Released water recovered in the outer tube was weighed. WHC was calculated using Eq. 1.

$$WHC = \frac{W_f - W_r}{W_f} \times 100$$

where $W_i$ is the total quantity (g) of water in the sample, and $W_r$ is the quantity of water released from the gels. Analyses were carried out at least in duplicate.

Rheological measurements

Small-deformation oscillatory measurements of gels were performed on a controlled-stress rheometer (SR-5000; Rheometric Scientific, Piscataway, N.J., U.S.A.) using parallel plates (40 mm dia and 2 mm gap). The samples were placed on the plate immediately after addition of calcium following similar protocols (Remondetto and others 2003; Bryant and McClements 2000). Measurements
were started 5 min after calcium addition. The viscoelastic parameters were measured as a function of time within the linear region previously determined by a stress scan on gels. Analyses were performed at 1 Hz and 25 °C. An oscillating stress was applied to the gels, and the resulting strain was recorded as storage modulus (\(G'\)), loss modulus (\(G''\)), and tan δ (\(G'/G''\)) every 2 s over a period of 10 h. A humidity cover was used to prevent the gels from dehydrating during analysis. Measurements were carried out at least in triplicate.

Transmission (TEM) and scanning (SEM) electron microscopy

TEM and SEM observations were done on gels prepared under limited conditions: 6% or 9% protein in the presence of 10 or 20 mM CaCl\(_2\). Gel samples were fixed in 2.5% glutaraldehyde + 1.5% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.0 for 24 h at 4 °C, washed 4 times in cacodylate buffer, and postfixed in 1% osmium tetroxide for 1.5 h. Ethanol dehydration was then performed using a series of solutions of increasing ethanol concentration. For TEM, samples were embedded using a mixture of Epon resin and propylene oxide 1:1, thinly sliced, and stained with uranyl acetate and lead acetate. Specimens were observed at 80 kV with a JEOL 1200EX electron microscope (JEOL Ltd., Akishima, Japan). For SEM, each sample was critical-point-dried, sputter-coated with 30 nm of gold/palladium and viewed at 15 kV using a JEOL JSM 35CF electron microscope.

Results and Discussion

Statistical analysis

Table 2 shows the data obtained for \(L^*\), WHC, and \(G'\). Table 3 gives the regression analysis model coefficients. The regression coefficients obtained were fitted to a 2nd-order polynomial equation (Eq. 2) that predicts the evolution of each response as a function of the model independent variables:

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_1b_2X_1X_2 + b_2b_2X_2^2 
\]

where Y is the predicted response, \(b_0, b_1, b_2, b_1b_2, b_2b_2\) are the constant regression coefficients of the model and \(X_1\) and \(X_2\) are the independent variables protein concentration and calcium concentration, respectively.

Macroscopic textural observations

Figure 1 presents photos of gels at 6% protein with respectively 10 mM CaCl\(_2\) (Figure 1a) and 20 mM CaCl\(_2\) (Figure 1b). Gels formed at 6% protein and 10 mM CaCl\(_2\) show a translucent appearance and a soft texture. Increasing the CaCl\(_2\) concentration to 20 mM (Figure 1b) has an observable effect on gel color and texture, making it whiter, granular, and brittle. Noticeable syneresis was observed at 20 mM CaCl\(_2\) (see WHC results). At 9% protein (not shown), differences between gels formed at 10 mM calcium and 20 mM are not as obvious as for 6% protein gels. Both have a firm texture, but 20 mM calcium gave greater whiteness. It is suggested that the higher CaCl\(_2\) concentration increased aggregate size with more important protein-protein interactions and decreased protein-water interactions by occupying negative charges on the polypeptide chains, resulting in increased gel network random aggregation.

Light reflectance

Analysis of variance of the light reflectance \(L^*\) parameter, presented in Table 3, shows significant linear effects \((P \leq 0.01)\) for both protein and calcium concentration, a quadratic effect for protein concentration as well as interaction between protein and calcium concentration. Both variables are thus important determinants for the \(L^*\) parameter. Figure 2 shows the response surface for \(L^*\) parameter evolution as a function of protein and calcium concentrations within the limits studied for the model. The graph was plotted according to Eq. 2, and the regression coefficients are shown in Table 4. Surface \(L^*\) values increased as calcium concentration increased from 10 to 20 mM but decreased slightly as protein concentration increased from 6% to 9%. In fact, gels at 6% SPI had higher \(L^*\) values (Figure 2).

Surface \(L^*\) value can be used as an indicator of the aggregation level throughout the gel network. It has been demonstrated that gel whiteness is related to particle size (Doi 1993; Marangoni and others 2000; Chantharorncrai and McClements 2002). An opaque gel may have a higher \(L^*\) value, apparently because larger protein aggregates scatter more light than do smaller, more numerous particles. This indicates that increasing CaCl\(_2\) concentration at a given
protein concentration increases protein aggregate size, which leads to greater light dispersion and consequently a whiter gel appearance. This hypothesis is supported by the demonstration that high salt content leads to a denser and more aggregated structure in heat-set glycinin and β-conglycinin gels (Hermansson 1985). Similar observations have been reported for protein and gel opacity in CaCl₂-induced cold gelation of WPI as a function of CaCl₂ concentration (Barbut 1995a; Hongsprabhas and Barbut 1997a; Marangoni and others 2000).

**Water-holding capacity**

Analysis of WHC variance (Table 3) shows significant linear effects of protein concentration and calcium concentration ($P \leq 0.01$) as well as a quadratic effect of calcium concentration ($P \leq 0.05$). The significance of both the linear and quadratic terms for calcium concentration indicates that this parameter has a major impact on gel WHC. The response surface graph of WHC as a function of protein and calcium chloride concentrations (Figure 3) indicates an increase with increasing protein concentration and a decrease with increasing CaCl₂ concentration. Gel WHC may be related to the size of inner structure spaces, which determines capillary forces (Hermansson 1986b). Gels of higher protein concentration retain more water due to a denser network with greater capillary forces (Remondetto and others 2002). Conversely, increasing the CaCl₂ concentration from 10 to 20 mM induces a higher expulsion of water from the network, probably due to larger pore sizes in gels (Barbut 1995a; Roff and Foegeding 1996; Remondetto and others 2002). As salt concentration increases, electrostatic repulsion between polypeptides is increasingly screened promoting protein-protein interactions at the expense of protein-water binding. A more porous open structure results from which water can be easily expelled (Bryant and McClements 1998).

**Rheological measurements**

Figure 4 illustrates the typical evolution of the storage modulus ($G'$), the loss modulus ($G''$), and the phase angle of the gels as monitored by dynamic rheology. $G'$ indicates the evolution of the elas-
tic elements in the system and \( G' \) indicates the evolution of the viscous elements. The phase angle indicates the relative amounts of viscous and elastic elements (an elastic solid reading 0° and a viscous fluid reading 90°). Hence, the change from a viscoelastic fluid to a viscoelastic solid may be used to monitor gel evolution over time (Barbut and Fogeding 1993). Results show that \( G' \), \( G'' \), and phase angle exhibit the same tendencies for all conditions (even in the absence of calcium), displaying an increase in both \( G' \) and \( G'' \) and a decrease in the phase angle. Moreover, for all conditions tested, \( G' \) is higher than \( G'' \), indicating that the elastic component is much higher than the viscous component of the gels and confirming the solid-like character of the material. Given that the phase angle decreased to a minimum value while \( G' \) increased to a maximum, \( G' \) was considered the best indicator of gel structure formation and consolidation. It can be noticed that for the sample formed in the absence of calcium, \( G' \) was not higher than \( G'' \), as well as for the interaction between protein and calcium concentrations. The analysis of \( G' \) parameter variance (Table 3) shows significant linear effects for protein and calcium concentrations (\( P < 0.01 \)) as well as for the interaction between protein and calcium concentrations. Figure 6 displays the response surface graph of the elastic constant \( (G') \) as a function of protein and calcium chloride concentrations. \( G' \) values of gels (after 10 h of gelation) increased with both protein and CaCl\(_2\) concentrations. Increasing protein concentration increases the solid-like character of the gels, likely through gel-network-stabilizing intermolecular hydrophobic interaction and disulfide bond formation (Shimada and Cheftel 1988).

**Microstructure analysis**

Figure 7 displays TEM and SEM micrographs of gels at the 4 limit conditions studied. Figures 7a and 7b show respectively TEM and SEM of 6% protein/10 mM CaCl\(_2\) gel. The microstructure is fairly homogeneous and composed of small pores visible on the TEM micrograph, while the surface as seen by SEM is composed of small and uniform aggregates. TEM images of 6% protein/20 mM CaCl\(_2\) gel (Figure 7c) reveal a noticeably coarser microstructure than for gel with lower salt content. SEM (Figure 7d) confirms the irregular protein network with large pores and thick aggregates. At 9% protein and 10 mM CaCl\(_2\), the gel attains its highest density, as shown by TEM (Figure 7e). The pores are of uniform size and very small. A dense and homogeneous structure can be seen by SEM (Figure 7f). At 9% protein with 20 mM CaCl\(_2\) (Figures 7g and 7h), TEM reveals a finer network than at 6% protein with this amount of calcium (Figure 7g) but coarser than 9% with 10 mM CaCl\(_2\) (Figure 7f). Figure 6h shows a rough surface with smaller aggregates than at 6% protein and 20 mM CaCl\(_2\) (Figure 7d).

It should be noted that these results are consistent with those for WHC. Increasing the CaCl\(_2\) concentration from 10 to 20 mM while maintaining the protein concentration at a constant level increases both aggregate size and pore volume (observed on micrographs; Figure 7), causing the expulsion of water from the network. Conversely, for a given CaCl\(_2\) concentration, an increase in protein con-
Cold gelation of soy protein . . .

Concentration results in the formation of a denser network with smaller pores.

Conclusions

This work has allowed us to establish that a preheated soy protein isolate suspension can form a gel at room temperature by the addition of Ca\(^{2+}\). Depending on the protein and/or CaCl\(_2\) concentrations, a large range of gels can be obtained, differing by their opacity, water-holding capacity and rheological properties. Increasing the CaCl\(_2\) concentration from 10 to 20 mM resulted in increased gel opacity whereas increasing the protein concentration from 6% to 9% decreased its opacity. WHC improved with higher protein concentrations and lower CaCl\(_2\) concentrations. G’ increased with higher protein and calcium chloride concentrations. Pore size increased with CaCl\(_2\) concentration and decreased as protein concentration increased, as shown by microscopy. Even though self-supporting cold-gels have been obtained in a narrower range compared with whey protein gels and in spite of the difference in their physico-chemical properties, the data obtained with soy proteins present similarities with cation-induced cold gelling of β-lactoglobulin and WPI, suggesting that mineral-protein interactions are of similar nature, that is, mainly electrostatic.

This work represents a starting point for the development of Cold gelation of soy proteins. Different experiments are now being conducted to study the formation of gels at the molecular level using Fourier transform infrared spectroscopy. This technique has been recently used with success by our group to study molecular differences in the formation and structure of heat- and cold-set gels of β-lactoglobulin (Lefèvre and Subirade 2000; Remondetto and Subirade 2003). This technique should provide greater insight into the formation of SPI gels. It is expected that the results will allow food technologists to expand the use of soy proteins by developing new gel textures by varying either salt or protein concentrations in formulations.

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Cold gelation of soy protein...