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Effect of solution pH on solubility and some structural properties of soybean protein isolate films

Adriana N Mauri and María C Añón*

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata y Consejo Nacional de Investigaciones Científicas y Técnicas, Calle 47 y 116, 1900 La Plata, Argentina

Abstract: Changes in solubility and molecular properties of protein films obtained from soy protein isolate (SPI) solutions at different pH values (2, 8 and 11) were investigated to study protein behavior during film formation. Proteins retained their native conformation in films at pH 8, but were partially or extensively denatured at pH 11 and 2. Although film protein networks were maintained by the same type of interactions at different pH values – covalent (disulfide bonds) and non-covalent bonds (especially hydrophobic interactions and hydrogen bonds) – the intensity of each type of interaction (predicted from solubility tests in buffers with different chemical action) depended on the pH of the initial solution. Films obtained at pH 8 presented the highest solubility in all the buffers, whereas films at formed pH 2 presented the lowest, except in the buffer of pH 8 that contained urea, SDS and 2-mercaptoethanol, which totally dissolved 100% of the film proteins. Films prepared at extreme pH values had a denser microstructure than those at pH 8. SDS–PAGE patterns indicated that films were mainly formed by β-conglycinin and glycinin, which aggregated in different forms during film formation, depending on the pH of the initial solutions. Some of these proteins remained weakly bonded and/or were held by the network of films. These differences in the protein networks structure would affect the physical, mechanical and barrier properties of the films.

Keywords: protein films; soy protein isolate; microstructure; solubility; sulfhydryl group concentration

INTRODUCTION

The use of biopolymers such as polysaccharides, lipids, proteins or their composites as packaging materials could contribute to solving environmental pollution and create new markets for these agricultural products. Interest in these biodegradable materials has increased during the past decades because of their potential applications. They have been used to prepare edible films and coatings that hold promise for innovative uses in food protection and preservation. Their utility lies in their capacity to act as an adjunct for improving overall food quality, extending shelf-life, and possibly improving cost–benefit of packaging materials.[1–3]

Numerous proteins such as corn zein, wheat gluten, soy, peanut, cottonseed, sunflower, rice bran, serum albumin, egg white, collagen, gelatin, myofibrils, casein and whey proteins, and others of limited availability, have been studied as potential film-forming agents.[4–7] Biodegradable or edible films based on soy protein can be produced: on the surface of heated soymilk;[8–10] by casting from soy protein solutions;[10–13] or by the conventional processing techniques of nonbiodegradable polymers.[14,15]

During film formation, proteins may associate and dissociate in different ways depending on experimental conditions. Dispersion pH is one of many variables in film formation, and also has a considerable influence on the denaturation process. Soy protein film formation has been achieved within pH 1–3 and 6–12. No film formation occurred near the protein isoelectric point (pH 4.5). At this pH, the soy protein isolate (SPI) coagulated rather than dispersed, thereby not allowing for the casting of the protein dispersions. At pH values away from the isoelectric region, proteins denature and unfold, exposing sulfhydryl and hydrophobic groups. Such groups self-associate during film formation, forming new bonding forces. At extreme acidic and alkaline conditions, film formation is inhibited because of the strong repulsive forces of highly negative (pH > 12) or positive (pH < 1) charges along protein chains, which prevent protein molecules from associating and forming films.[12,16–18]

The three-dimensional networks of the films are largely dependent on the intra- and intermolecular interactions among proteins, but they also result from interactions between proteins and other constituents.[19] The type (covalent or non-covalent) and level of the interactions involved in film formation determine the macroscopic properties and the microstructure of the material. Research has primarily

* Correspondence to: María C Añón, Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata y Consejo Nacional de Investigaciones Científicas y Técnicas, Calle 47 y 116, 1900 La Plata, Argentina
E-mail: mca@biol.unlp.edu.ar
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focused on the development, evaluation and improvement of the physical, mechanical and barrier properties of the film. These properties may be related to the microstructural characteristics of films. Anker *et al.*[20] studied the relationship between the microstructure and the mechanical and barrier properties of whey protein films, with a focus on the effects of the protein concentration, the plasticizer and the pH. Frinault *et al.*[21] examined the variation of microstructure along the casein film thickness, such microstructure being more porous in the center than at the edges. Lent *et al.*[22] studied the topography of whey protein films, whereas Lai and Padua[23] studied the microstructure of zein films plasticized by oleic acid. Correlations between film microstructure, film macroscopic properties, and structural properties of proteins would lead to development of materials with desirable characteristics for any application.

The aim of this work was to study the structural properties and the microstructure of SPI films using the pH of film-forming dispersions as a variable.

EXPERIMENTAL

Isolate preparation

Soybean protein isolate (SPI) was prepared from defatted low-heat soybean meal produced by Bunge-Ceval SA (Brazil, PDI: $838.2 \pm 1.43\,g\,kg^{-1}$). Soy flour was dispersed ($100\,g\,kg^{-1}$) in distilled water. The dispersion was adjusted to pH 8.0 with 2 M NaOH, stirred at room temperature for 2 h, and centrifuged at $10\,000 \times g$ for 30 min at 15 °C. The supernatant was then adjusted to pH 4.5 with 2 M HCl and centrifuged at $3300 \times g$ for 20 min at 4 °C. The pellet was washed with dilute aqueous HCl at pH 4.6 and centrifuged as above. The pellet was suspended in distilled water and adjusted to pH 8. Finally SPI was frozen at −80 °C and freeze-dried.[24]

Protein content of SPI was determined by the micro-Kjeldahl technique.[25] It contained $915 \pm 20\,g\,kg^{-1}$ protein on a dry basis ($N \times 6.25$).

Isolation of β-conglycinin and glycinin

β-Conglycinin and glycinin were used to identify these bands in electrophoretic patterns and in differential scanning calorimetric peaks when using SPI. These enriched fractions were isolated from the defatted flour as described by Nagano *et al.*[26]

Film formation

Films were prepared by dispersing SPI ($50\,g\,L^{-1}$) and glycerol ($25\,g\,L^{-1}$) in distilled water. SPI solutions were magnetically stirred for 20 min at room temperature. The pH was adjusted to 2 with 2 M HCl, to 11 with 2 M NaOH or left at pH 8. Solutions were stirred for an additional 10 min an then centrifuged at $700 \times g$ for 10 min at 4 °C in order to eliminate bubbles. The film-forming solutions were then cast onto polystyrene Petri dishes and dried in an oven at 60 °C for 7 h. Films were preconditioned in a climate-controlled room at 20 °C and ambient humidity (≅75 or 80% RH), for at least 15 h prior to all testing.

Determination of protein solubility of the films

Protein solubility of the films was determined according to the method described by Hager,[27] with some modifications as far as the duration of the experiment was concerned. Pieces of films (≅100 mg) were weighed and placed into a tube containing 2 mL of water or buffer. Five different buffer systems, all at pH 8.0, were used: (a) buffer B_1 contained 0.086 M TRIZMA/HCl, 0.09 M glycine and 4 mM Na_2EDTA; (b) buffer B_2:B_1 with $5\,g\,mL^{-1}$ sodium dodecyl sulfate (SDS); (c) buffer B_3:B_1 with 8 M urea; (d) buffer B_4:B_1 with $5\,mg\,mL^{-1}$ SDS and 8 M; urea and (e) buffer B_5:B_1 with $5\,g\,mL^{-1}$ SDS, 8 M urea and $25\,mg\,mL^{-1}$ mercaptoethanol (ME). The tubes were shaken for 24 h at 20 °C. Suspensions were then centrifuged at $9000 \times g$ for 20 min and the protein content in the supernatant was determined using a bicinchoninic acid (BCA) protein assay.[28] A standard curve using bovine serum albumin was constructed for each buffer. For each type of film, at least two samples from four independent film preparations were solubilized. The soluble protein content was expressed as a percentage of the total amount of protein in the film, which was measured by the micro-Kjeldahl method.

The mercaptoethanol used in buffer B_5 interfered with the BCA assay. Proteins solubilized in this buffer were precipitated with $600\,g\,L^{-1}$ trichloroacetic acid, centrifuged at $9000 \times g$ for 20 min at room temperature and dissolved in 0.5 N NaOH before the protein assay.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein film-forming solutions and film proteins solubilized in the different buffers were analyzed by SDS–PAGE according to Laemmli,[29] and modified by Petruccelli and Añón,[30] using a linear gradient separating gel ($40–150\,g\,L^{-1}$ in polyacrylamide). A continuous dissociating buffer system, containing 0.375 M Tris-HCl, pH 8.8, and $1\,mg\,mL^{-1}$ SDS, was used for the separating gel, while the running buffer was 0.025 M Tris-HCl, 0.192 M glycine and $1\,mg\,mL^{-1}$ SDS, pH 8.3. Electrophoresis was carried out at a constant voltage of 200 V. Samples were diluted with a buffer at pH 6.8 (0.125 M Tris-HCl, $1\,mg\,mL^{-1}$ SDS, $400\,mL\,L^{-1}$ glycerol, $0.5\,mg\,mL^{-1}$ bromophenol blue). Protein molecular weights were estimated using low MW markers (Pharmacia calibration kit, Milton Keynes, UK) that included phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400). Gels were fixed, stained with R-250 Coomasie blue ($1\,mg\,mL^{-1}$) in water/methanol/acetic acid (5:5:2) overnight, and destained with $250\,mL\,L^{-1}$ methanol and $10\,mL\,L^{-1}$ acetic acid.

Differential scanning calorimetry (DSC)

A Polymer Laboratories Rheometrics Scientific DSC (Church Stretton, UK) was used for these studies. Temperature and heat flow calibration of the equipment was carried out according to ASTM Standards.[31,32] Lauric acid and indium were used as temperature standards; the latter was also used as a heat-flow standard.

Both film-forming solutions and films were studied by DSC, in order to know the enthalpy (ΔH_d) and the temperature of protein denaturation (T_d). Hermetically sealed aluminum pans containing 13–20 mg of samples were prepared. In the case of initial solutions, samples containing 200 mg mL^{-1} of SPI, β-conglycinin or glycinin and 100 mg mL^{-1} of glycerol at pH 2, 8 and 11, respectively, were used. The capsules were scanned at 10 °C min^{-1} over the range 30–150 °C. An empty double capsule was used as a reference. ΔH_d was expressed as J/g of SPI or soy fraction protein.

Determination of total sulfhydryl content (SH_T)

Synthesis of 2-nitro-5-thiosulfobenzoate (NTSB) was performed according to the method of Tannhauser *et al.*[33] as modified by Petruccelli and Añón.[24]

Determination of sulfhydryl content was carried out according to the method of Tannhauser *et al.*[33] by mixing 70 µL of the solution of SPI (10 mg mL^{-1}) or film (15 mg mL^{-1}) with 1 mL of freshly prepared NSTB test solution. Absorbance at 412 nm was determined at 20 min by using the test NTSB solution as a reference. The extinction coefficient used to transform absorbance values into concentration values was 13 600 M^{-1} cm^{-1}.

Determination of free sulfhydryl groups (SH_F)

Free SH groups were determined according to the procedure of Beveridge *et al.*[34] 50 mg isolate or 75 mg film was dissolved in 5 mL of 0.086 M Tris buffer, 0.09 M glycine, 0.004 M EDTA, and 8 M urea, pH 8.0. 40 µL of Ellman's reagent (4 mg 5,5′-dithio-bis(2-nitrobenzoic acid) mL^{-1} in methanol) was added to 1 mL aliquots. Absorbance at 412 nm was determined at different times until the maximum absorbance was reached. A molar extinction coefficient of 13 600 M^{-1} cm^{-1} was used. Protein concentration was determined by the micro-Kjeldahl method. Determinations were performed at least twice. SH groups was expressed as µmol SH g^{-1} of protein.

Scanning electron microscopy

Sample preparation was performed as described previously.[35] Film samples were immersed in 25 mg mL^{-1} glutaraldehyde with 1 mg mL^{-1} ruthenium red and 0.025 M KCl for 72 h and washed several times with 0.025 M KCl followed by 20 mg mL^{-1} OsO_4 for 2 h at 4 °C. Samples were then rinsed for 1 h in distilled water before being dehydrated with a graded acetone series, 250, 500, 700, 900, and 3 × 1000 mL L^{-1}, and dried at the critical point. Each dried sample was mounted on a bronze stub and coated with gold, and the specimens were examined with a JEOL 35 CF (Tokyo, Japan) scanning electron microscope, at an acceleration voltage of 5 kV.

Statistical analysis

Statistical analysis was carried out using Systat version 5.0 (Systat Inc., Copyright 1990–1992, Richmond, CA, USA). Tukey's test ($P < 0.05$) was used to detect significant differences in SH_T, SH_F, and solubility at different pH values for SPI and films.

RESULTS AND DISCUSSION

Soy protein isolate solutions

Film-forming solutions were first analyzed in order to study the effect of pH on the conformation of proteins before film formation. To examine the protein molecular weight distribution in the initial solutions, polypeptides were analyzed by SDS–PAGE. Patterns of SPI were compared with those of β-conglycinin and glycinin globulins at the same concentration and pH in order to identify the bands (Fig. 1) in the corresponding SDS–PAGE patterns. At pH 8, a typical profile for SPI solution was obtained. Bands corresponding to high molecular mass aggregates (>94 000 Da; some of them did not enter the gel), the α, α', and β subunits of β-conglycinin, the AB subunit and A and B polypeptides of glycinin were observed. At extreme pH values, 2 and 11, the same protein species were present, but a higher amount of soluble aggregates of high molecular weight were detected (some of them could not enter the gel). A dissociation of the AB-glycinin subunit was also observed at extreme pH values, especially at the alkaline ones. A small amount of β-conglycinin subunits was observed at all the pH values, probably due to the α and α' aggregation as was previously shown in our laboratory.[36]

Film-forming solutions were also thermally analyzed by DSC. Figure 2(a) shows the thermograms of SPI initial solutions at different pH values. Endothermic peaks were identified by comparing the denaturing temperatures (T_ds) of proteins in film-forming solutions with the T_d of purified β-conglycinin and glycinin in samples at the same concentration and pH. Table 1 shows these T_ds.

The thermograms at pH 8.0 showed the two typical endotherms, the first at 78.2 ± 0.2 °C, corresponding to β-conglycinin denaturation, and the second at 92.4 ± 0.1 °C, corresponding to glycinin. Acid treatment led to extensive denaturation of glycinin and the partial denaturation of β-conglycinin as indicated for the decrease of the denaturation enthalpy (Table 1). A shift of the peak temperature of β-conglycinin to a lower temperature (T_d 60 °C), indicating a decrease in the thermal stability, was also detected, in agreement with that reported by Puppo and Añón,[37], Hermanson[38] and Danilenko *et al.*[39] On the other hand, alkaline treatment led to

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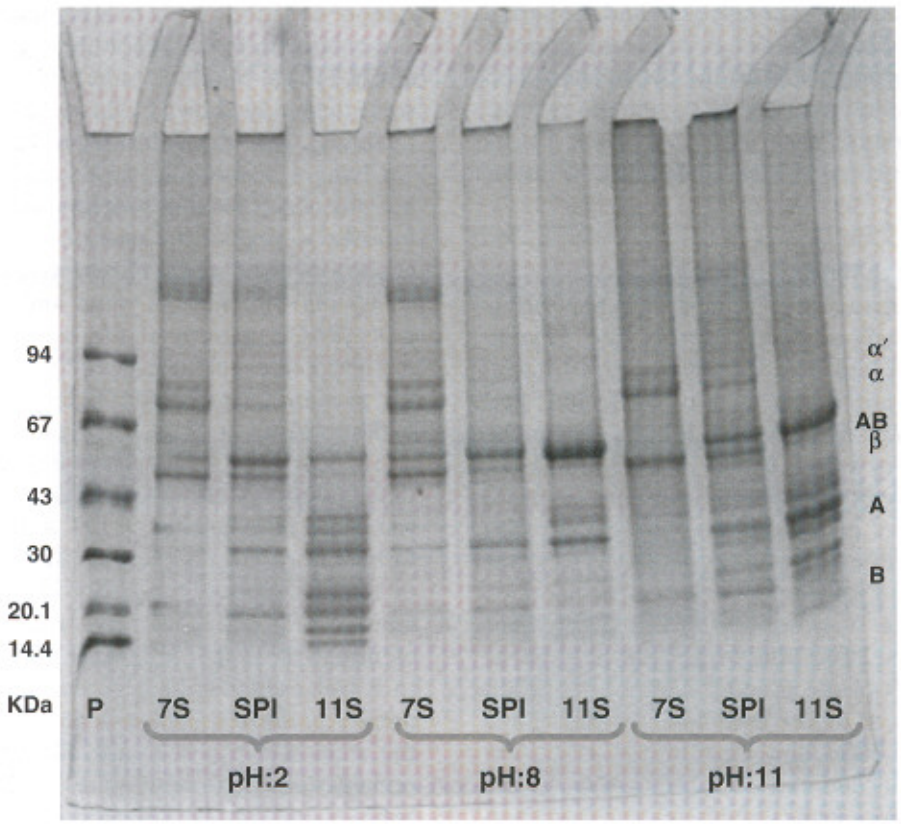

Figure 1. SDS–PAGE patterns of initial film-forming dispersions of SPI, β-conglycinin (7S), and glycinin (11S) at pH 2, 8 and 11.

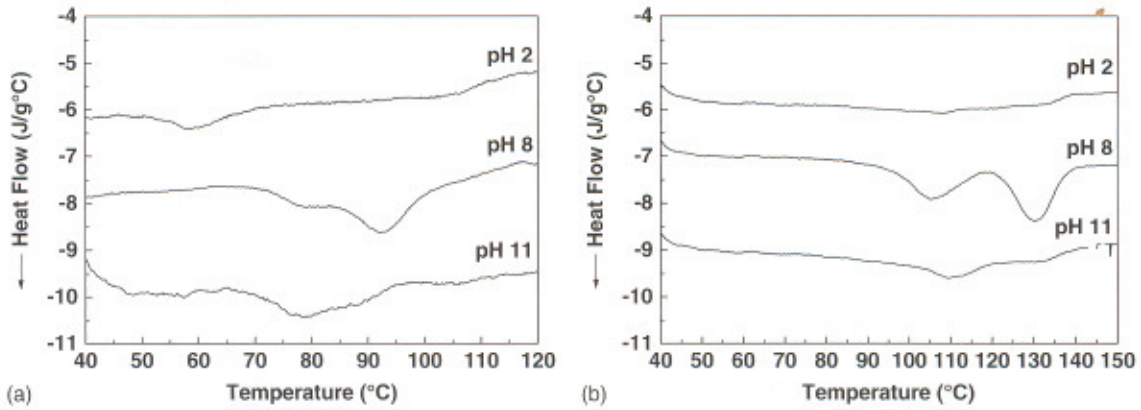

Figure 2. DSC thermograms of SPI protein film-forming solutions (a) and SPI protein films (b) at pH 2, 8 and 11.

Table 1. Denaturing temperatures (T_d) and enthalpy (ΔH_d) of SPI proteins, β-conglycinin and glycinin globulin solutions and SPI films produced at different pH values, obtained by DSC

	PH					
	2		8		11	
Sample	T_d	ΔH_d	T_d	ΔH_d	T_d	ΔH_d
β-Conglycinin solution	60.9 ± 1.2	5.74 ± 0.62	78.3 ± 0.9 91.4 ± 0.1	13.47 ± 1.30	No peak was observed	–
Glycinin solution	No peak was observed	–	94.7 ± 1.0	20.38 ± 1.00	78.3 ± 1.9 91.4 ± 0.1	13.14 ± 1.00
SPI solution	59.7 ± 1.7	3.74 ± 0.21	78.2 ± 0.2 92.4 ± 0.1	15.46 ± 1.34	78.4 ± 0.1 88.8 ± 0.5	10.00 ± 0.74
SPI films	No peak was observed	–	105.1 ± 0.5 130.2 ± 0.5	15.52 ± 1.00	109.6 ± 0.5 133.9 ± 0.5	6.48 ± 0.60

extensive denaturation of β-conglycinin and the partial denaturation and dissociation of glycinin, as suggested by the two overlapping peaks in the thermogram and enthalpy values obtained (Table 1). At pH 11 the unfolding temperature also shifted to lower values and the peak became wider, indicating a lower cooperativity.

Soy protein isolate films

According to Krochta,[4] two groups of events occur during film formation. First, during the heating phase, protein structure is disrupted, some native disulfide bonds are cleaved, and sulfhydryl and hydrophobic groups are exposed. Then, during the film drying phase, new hydrophobic interactions occur and disulfide and hydrogen bonds are formed. In order to know the effect of pH on the soy protein film formation, an analysis of protein conformation was performed. Figure 2(b) shows the DSC thermograms of soy protein films. It appears that thermal treatment at 60 °C does not affect the folding of proteins at pH 8. Only a shift of the β-conglycinin and glycinin peak temperatures to higher temperatures, due to the low water content of the film, was observed.[40–42] Film proteins were totally denatured at pH 2 (Table 1), due to the fact that, under acidic conditions, β-conglycinin is more sensitive to heat treatment and its T_d (Table 1) coincides with the temperature of film drying. In films at pH 11, glycinin maintained its original structure to some degree, as shown by the enthalpy values obtained (Table 1), with the shift of T_d to higher temperatures.

Changes in solubility and molecular weight distributions of soluble film proteins were also investigated in order to predict the type of protein–protein interactions involved in the film protein network. Figure 3 shows film solubility in water and the corresponding SDS–PAGE pattern of the soluble proteins. Water dissolved free polypeptides not strongly involved in the protein network. The highest solubility was seen in films formed at pH 8, and the lowest in films formed at pH 2. It is necessary to point out that the final pH differed among film dispersions in water: the final pH was 5 for films formed at pH 2, but was 8 for films formed at pH 8 and 11. This fact could be attributed to the lack of buffer capacity of water and a higher amount of HCl than NaOH used to modify the pH of initial film solutions. The pH increase of water to 8 will probably increase the solubility of proteins present in the pH 2 network film, but will also change the state of proteins in the film. Proteins at pH 8 remained in their native state, as shown by the DSC analysis. Proteins in films formed at pH 2 were totally

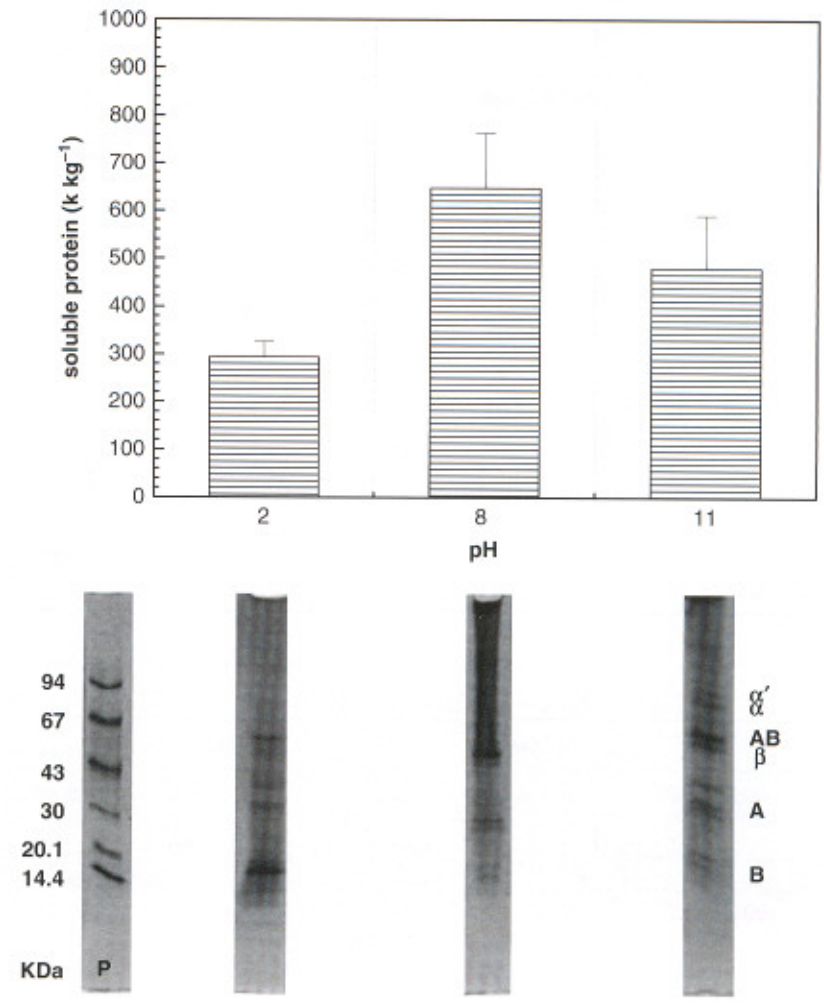

Figure 3. Solubility of the SPI films (obtained at pH 2, 8 and 11) in water and SDS–PAGE of the soluble fractions.

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denatured; their unfolded structures allowed them to easily interact intermolecularly, keeping the proteins in the film network and/or leading them to form part of insoluble aggregates of high molecular weight. Both effects could affect the film solubility.

As revealed by SDS–PAGE (Fig. 3), protein films produced at pH 8 showed a higher concentration of soluble aggregates than their corresponding film-forming solution. In films formed at pH 2 no soluble aggregates were detected; only bands corresponding to AB-glycinin, polypeptides A- and B-glycinin, and β-conglycinin subunit were observed. Films formed at pH 11 presented a behavior intermediate between those observed at pH 2 and pH 8. Their solubility in water was higher than that of films produced at pH 2, but lower than that of films formed at pH 8. The increment in solubility may be ascribed to the existence of a greater amount of soluble aggregates than that at acidic pH. The β-conglycinin and glycinin components were also detected in the SDS–PAGE patterns.

Buffer systems with specific chemical action on protein structure were used to predict the kind of aggregation forces present in soy films obtained at different pH values. Buffer B_1, containing TRISMA-HCl, glycine and EDTA, affected protein electrostatic interactions, while buffer B_2 (B_1 + SDS) disrupted hydrophobic interactions. Buffer B_3 (B_1 + urea) was especially active on hydrogen bonds and dissolved small aggregates stabilized by this type of interaction. Buffer B_4 (B_1 with SDS and urea) disrupted all the interactions mentioned above, while buffer B_5 (B_4 with mercaptoethanol) also disrupted disulfide bonds.[35,43] Figure 4 shows the solubility of films in these buffers and the SDS–PAGE patterns of the protein species that were soluble in each condition. Films obtained at pH 2 presented the lowest solubility in all the studied buffers. Solubility in buffer B_1 (ionic strength of about 0.1) was lower than in water. The low solubility in this buffer has also been observed in SPI powders and gels and has been attributed to an effect of salting-out.[36] The low solubility of film proteins in buffer B_1 indicates that electrostatic forces do not play an important role in the stabilization of the protein network, especially at pH 2. Although films formed at pH 8 showed the highest solubility, the aggregate concentration was low in the soluble fraction. For films formed at pH 2, only the polypeptides B of glycinin were present in the soluble fraction. Films produced at pH 11 presented an intermediate solubility in buffer B_1. AB-glycinin, and the respective acidic and basic

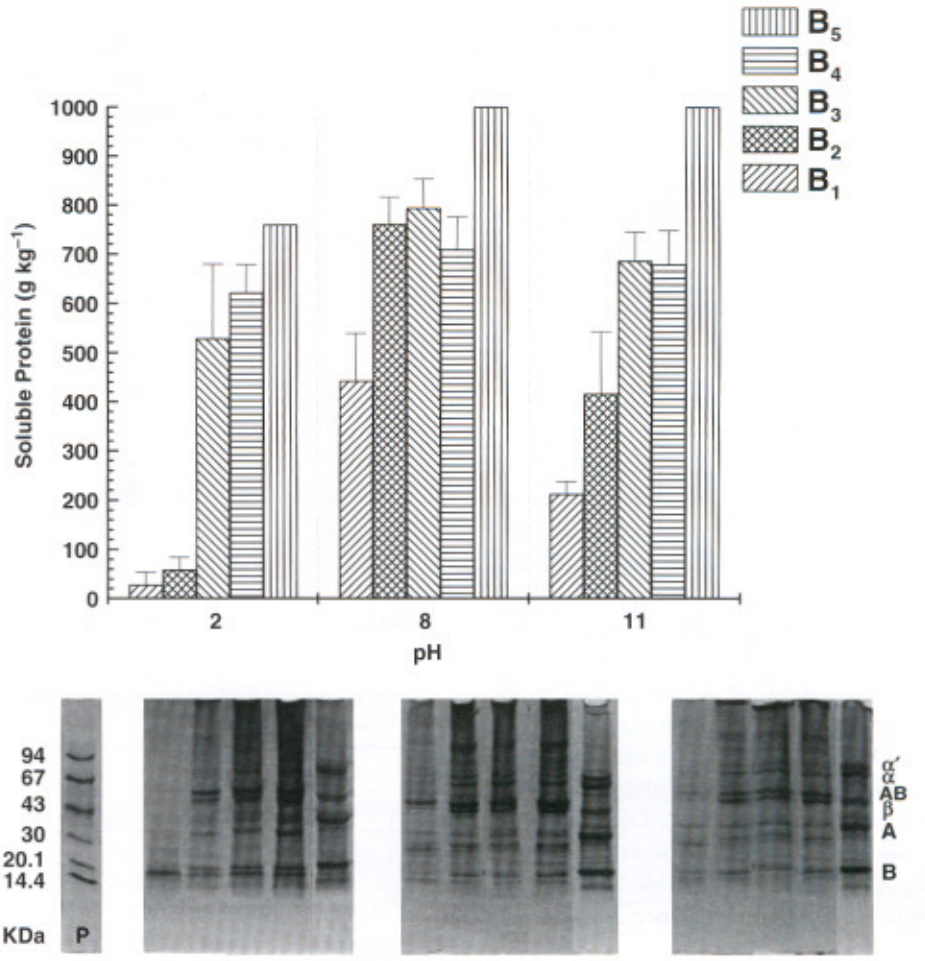

Figure 4. Solubility of SPI films in different buffers and SDS–PAGE of the soluble fractions. B_1: 0.086 M TRIZMA/HCl, 0.09 M glycine and 4 mM Na_2EDTA; B_2:B_1 with 5 g L^{-1} SDS; B_3:B_1 with 8 M urea; B_4:B_1 with 5 g L^{-1} SDS and 8 M urea; B_5:B_4 with 25 g L^{-1} mercaptoethanol; all of them at pH 8.

polypeptides, were detected in the soluble fraction together with a small amount of β-conglycinin subunit.

Solubility of protein in films prepared at pH 11 was lower in buffer B_2, containing SDS, than in buffer B_3, containing urea. Electrophoretic patterns of the corresponding soluble fraction showed bands of glycinin (A, B, and AB) and β-conglycinin (α, α and β) components, and protein aggregates of high molecular mass. Films prepared at pH 2 presented a very low solubility in buffer B_2, demonstrating that hydrophobic interactions were less important in the film network, or that under the experimental conditions this buffer did not constitute an efficient extracting solution. It appears that the presence of urea (buffer B_3) determines the solubility of these films, and that once hydrogen bonds are broken hydrophobic interactions can be disrupted. The pH of buffer dispersions of films at pH 2 was lower than those corresponding to films formed at pH 8 and 11 (pH 6–5 instead of 8), as occurred in distilled water.

In buffer B_4, containing SDS and urea, films obtained at pH 8 did not show significant differences in solubility ($P < 0.05$) with respect to buffers B_2 and B_3. All the characteristic species of β-conglycinin and glycinin, and aggregates of high molecular mass, were observed in the SDS–PAGE patterns. Film protein solubility in buffer B_4 was only about 650 mg g^{-1} for films at pH 2 and 11, and 700 mg g^{-1} for films at pH 8.

The addition of mercaptoethanol, buffer B_5, increased to 100% the solubility of films produced at pH 8 and pH 11. Films at pH 2 did not reach complete solubility in this buffer due to the final pH of the dispersion (pH 5); as said before, probably the concentration of buffer used was insufficient to completely neutralize the HCl present. However, complete solubilization was attained when the pH of the dispersions was increased to 8 (data not shown). This behavior can be attributed to the optimum pH for the SS/SH exchange. The rate of thiol–disulfide exchange depends on the extent of ionization of the nucleophilic thiol, and therefore generally increases as the pH of the reaction pH is increased as long as the pK of the nucleophilic thiol group is not exceeded. The thiols of proteins in the unfolded state generally have pK values in the range 8.7–8.9, similar to those reported for comparable monothiols, but their pK values in the folded state can vary enormously.[44] In the presence of mercaptoethanol, which facilitates the solubility of large protein aggregations maintained by disulfide bonds, SDS–PAGE patterns were identical at all pH values, the typical bands of glycinin and β-conglycinin subunits being present in all the samples.

Rhim *et al.*[45] also observed that soybean films at pH 10.0 were mainly stabilized by disulfide bonds and, to a minor extent, by hydrophobic interactions and hydrogen bonds.

The results discussed above indicate that covalent bonds (disulfide bonds) were also very important in network formation. Thus, free and total sulfhydryl group content was determined in soy protein films and SPI. As shown in Table 2, no significant differences in total sulfhydryl groups were found among the films at different pH and between them and SPI, as expected. Total and free sulfhydryl groups values are in the order of those reported in the literature.[24,43,46]

During film formation, new disulfide bonds were formed and this was reflected by the decrease of free sulfhydryl concentration in the films with respect to SPI dispersions. This effect was also observed by Rangavajhyala *et al.*[43] with heat-cured soy protein films. Films obtained at pH 2 and 11 had a lower concentration of free SH groups than the one formed at pH 8. Thus, as total sulfhydryl group concentration was the same for all samples, the concentration of disulfide bonds in the gel can be said to be higher for films obtained at extreme pH values than for films at pH 8.

Free SH group concentration correlates with the extent to which the protein is denatured. Disulfide bond formation is considered to be easier for denatured proteins, since the formation of the disulfide bond requires the two thiol groups to be brought into the correct orientation. Large values of the intramolecular rate constant for disulfide bond formation were found when the protein structure holds the two participating thiol groups in the correct orientation, and smaller values were observed in unfolded proteins.[44]

The results discussed clearly show that film protein networks formed from protein dispersions at pH 2, 8 and 11 were different. Although the same type of interactions (covalent and non-covalent) are involved in the protein networks, the level of each type of bond was different and this would be reflected in film properties and microstructure.

Figure 5 shows the surface structure of films as revealed by SEM. Films obtained at pH 2 and 11 showed denser microstructures than those formed at pH 8. At these extreme pH values, proteins were totally or partially denatured and had unfolded structures. Once extended, protein chains could interact strongly, thereby producing cohesive films. Disulfide bonds concentration also was higher for films obtained at pH 2 and 11.

For whey protein films plasticized with sorbitol or glycerol, Anker *et al.*[20] also found that when the pH increased from 7 to 9, a denser protein structure

Table 2. Free and total SH groups concentration in SPI and in SPI films at different pH

Sample	SH_F (μmol SH g^{-1} of protein)	SH_T (μmol SH g^{-1} of protein)
SPI	1.090 ± 0.099a	32.76 ± 1.86a
Films pH 2	0.177 ± 0.043b	36.13 ± 2.87a
Films pH 8	0.465 ± 0.036c	36.99 ± 4.62a
Films pH 11	0.297 ± 0.152b	32.98 ± 4.62a

Reported values for each film are means ± standard deviation. Values of SH_F and SH_T means followed by the same letter are not significantly ($P < 0.05$) different according to Tukey's test.

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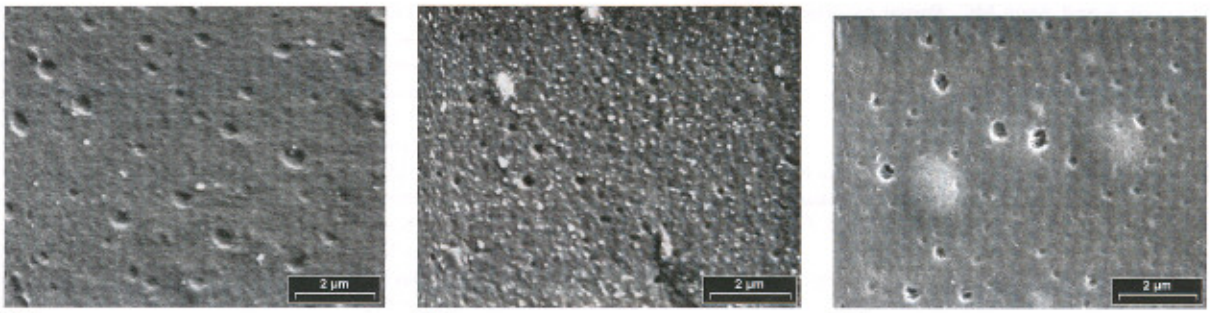

(a) (b) (c)

Figure 5. SEM micrographs of SPI films prepared at (a) pH 2; (b) pH 8; and (c) pH 11.

was formed. The denser protein structure might be due to an enhanced gel formation at extreme pH. The enhanced gelation was reported to be due to the unfolding of the protein molecule and to the increased thiol/disulfide (SH/SS) interchange reactions.

CONCLUSIONS

The studies performed allow us to correlate the structure and conformation of proteins with the solubility, microstructure and structural properties of films.

Film protein networks formed from protein solutions at pH 2, 8 and 11 differed in some aspects. The pH affects the protein charge and the degree of protein denaturation. The unfolded structures of proteins at extreme pH values allowed them to readily establish chain-to-chain associations by a combination of covalent and non-covalent interactions. This increment in protein network crosslinking was evidenced by the denser microstructure of films and lower protein solubility. At all pH values both β-conglycinin and glycinin were involved in the matrix of films; both appear to be weakly bonded and/or held by the network, especially at alkaline pH values (8 and 11).

These differences in film protein network would affect its behavior and condition its application.

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