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Structural and functional properties of soy protein isolate and cod gelatin blend films

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ABSTRACT

The structure-function relationship of composite films obtained from soybean-protein isolate (SPI) and cod gelatin was studied. Films with different ratios of SPI:gelatin (0, 25, 50, 75, 100% [w/w]) and plasticized by a mixture of glycerol and sorbitol were prepared by casting. Regardless of the soybean-protein concentration, the thickness and water-vapor permeability of the composite films diminished significantly as compared to pure-gelatin films. The formulation containing 25% SPI: 75% cod-skin gelatin had the maximum force at the breaking point, which was 1.8-fold and 2.8-fold greater than those of 100% gelatin and 100% SPI films, respectively. Moreover, this formulation offered high percent-deformation values lower than those of gelatin but higher than all other films containing SPI-, and the same relatively low water-vapor permeability as the 100% SPI film. While all the films exhibited high water solubility, a slight reduction in film solubility and soluble protein was observed with increasing SPI concentration. Differential-scanning calorimetry analyses revealed that gelatin was completely denatured in all films, while soy proteins largely maintained their native conformation. Analysis by fourier-transform–infrared spectroscopy revealed that the presence of 25% SPI produced gelatin conformational changes, self-aggregation of gelatin chains, and intermolecular associations via C=O bonds between gelatin and SPI proteins. All films were translucent in appearance, but the yellowish color increased with increasing proportions of the soybean proteins.

1. Introduction

The production of biodegradable materials from renewable biopolymers, such as proteins, provides an attractive alternative, at least for some applications, to synthetic polymers, whose accumulation contributes greatly to environmental pollution. The functionality of protein films is determined by their microstructure, which characteristic varies significantly depending on the initial protein structure and the preparation method (Denavi, et al., in press). All four aspects of protein structure (primary, secondary, tertiary, and quaternary) determine fundamentally the ability of protein chains to interact with each other as well as with other components of the formulation under consideration. The type and number of interactions involved in the stabilization of a proteinaceous matrix (disulfide covalent bonds, hydrogen bonding, electrostatic attractions, and hydrophobic bonding) is determined by the amino-acid composition and molecular weight of the proteins under consideration (which characteristics can vary significantly for different proteins) as well as by the experimental parameters used in film preparation. The protein–protein interactions involved in film formation determine the degree of cross-linking and the hydrophylic-hydrophobic character of the films and also correlate with the latter's physicochemical, mechanical, and barrier properties (Mauri & Añón, 2006, 2008).

The aim of preparing films from mixtures of structurally different proteins is to obtain composite materials in which each component provides a determined functional property. (Barreto, Pires, & Soldi, 2003; Cao, Fu, & He, 2007; Chambi & Grosso, 2006; Damodaran & Paraf, 1997; Güçbilmez, Yemenicioğlu, & Arslanoğlu, 2007; Longares, Monahan, O'Riordan, & O'Sullivan, 2005; Sabato et al., 2001). In particular, soybean proteins and gelatin are very different with respect to their origin, structures and amino acid composition. Soy proteins, of vegetal origin, are composed of a mixture of albumins and globulins, 90% of which are storage proteins with globular structure—consisting mainly

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in 7S (β-conglycinin) and 11S (glycinin) globulins. 7S Globulin is a trimer of 150–200 kDa formed by four subunits, of which the α (72 kDa), α' (68 kDa) and β (52 kDa) are the most important while the γ subunit (with a molecular mass similar to β) is a minor component. All of these subunits have similar amino-acid sequences and are poor in cysteine, methionine and tryptophan. By contrast, the 11S globulin, or glycinin, is a hexamer of molecular weight 300–380 kDa, whose six subunits are composed of an acidic polypeptide A (acid pI, ~35 kDa) and a basic polypeptide B (basic pI, ~20 kDa) covalently linked by a disulfide bond, with a cysteine, methionine and tryptophan content higher than that of 7S globulin (Nielsen, 1985a, 1985b; Staswick, Hermedson, & Nielsen, 1984; Thanh & Shibasaki, 1977). Gelatin, however, is a mixture of polypeptides derived from the hydrolysis of the collagen contained in bones and skins. The collagen rod, composed of three f-chains – each of molecular weight around 100 kDa –, can be solubilized in acid without altering its original triple-helix configuration. Subsequent thermal treatment, though, will cleave both the hydrogen and covalent bonds; leading to a destabilization of the triple helix as the result of a helix-to-coil transition (Djabourov, Lechaire, & Gaill, 1993) and a conversion into soluble gelatin, with this soluble product, in turn, having a definite molecular-weight–distribution pattern in accordance with the α-chains and the gelatin oligomers (Ledward, 1986). Cod-skin gelatin contains α-chains, β- and γ-components (covalently linked α-chain dimers and trimers, respectively), in addition to an appreciable amount of higher molecular weight polymers as well as species of molecular weight lower than that of α-chains. Moreover, cod-skin gelatin is mostly composed of glycine (34%), iminoacids (Pro + Hyp, 16%) and Alanine residues (10%). As for most gelatins derived from type I collagen, cysteine and tryptophan were absent, and the content of tyrosine residues was below 1% (Gómez-Guillén et al., 2002).

The structural differences between soy and gelatin proteins are reflected in their functionality, that is, in the specific characteristics of the films they form, even though both proteins may exhibit good film-forming properties by themselves. Soybean-protein–isolate (SPI) films have been reported to be rather brittle and colored, and to have relatively poor mechanical properties (Ghorpade, Li, Gennadios, & Hanna, 1995; Kim, Weller, Hanna, & Gennadios, 2002; Mauri & Añón, 2008; Rhim, Gennadios, Handa, Weller, & Hanna, 2000); whereas fish gelatin forms transparent, but weakly colored, and highly extensible films (Avena-Bustillos et al., 2006; Carvalho et al., 2008; Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007; Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006; Zhang, Wang, Herring, & Oh, 2007). The main drawback of cod-gelatin films, however, is their low water resistance and their extremely high solubility (Piotrowska, Kolodziejska, Januszewska-Jozwiak, & Wojtasz-Pajak, 2005; Pérez-Mateos, Montero, & Gómez-Guillén, 2009).

Upon considering the best characteristics of soybean and gelatin proteins separately, we speculated that their combination would lead to better films than those formed by each individual material alone. In a recent article, Cao et al. (2007) described the diverse functional properties of composite films made from soybean-protein–isolate and type-B bovine-bone gelatin, and suggested that composite films have an increased range of applications as a result of the differing mechanical and barrier properties of their components.

The goal of the present work, therefore, was to study the formation and characteristics of composite films obtained from soybean-protein isolates and cod-skin gelatin mixed together in different proportions and to analyze the structure–function relationship of such admixtures.

2. Materials and methods

2.1. Soy protein isolate (SPI) preparation

Soy protein isolate was prepared from defatted low-heat soybean meal produced by Bunge-Ceval SA (Brazil) as described by Petruccelli and Añón (1995). The protein content of the SPI, as measured by the Kjeldahl technique, was 83.82% ± 0.14% protein on a dry basis ($N \times 5.7$).

2.2. Fish skin gelatin

Cod (*Godus morhua*) – skin gelatin was obtained by means of a mild acid pretreatment followed by an overnight aqueous extraction at 45 °C (Gómez-Guillén & Montero, 2001). The protein content of the dry gelatin powder was 84.4% ± 0.23 (N × 5.4) as determined with a Nitrogen analyzer LECO FP-2000 (Leco Corporation, St Joseph, MI) calibrated with ethylenediaminetetraacetic acid (EDTA, Dumas method following A.O.A.C. 992.15; A.O.A.C., 2000).

2.3. Preparation of films

Five groups of composite films with different ratios of SPI to gelatin (0:100, 25:75, 50:50, 75:25 and 100:0) were prepared. Aqueous solutions of 4% (w/v) SPI and/or gelatin (w/v) were prepared in 250 mL Erlenmeyer flasks along with the addition of 1.5% plasticizers (*i. e.*, 0.75% glycerol + 0.75% sorbitol). The dry proteins were dissolved in distilled water with stirring, either at room temperature (soybean protein) or by heating in a water bath at 60 °C (gelatin). Upon complete dissolution of either protein, the pH was adjusted to 10.5 using 2N NaOH. The solutions were mixed at the different ratios shown in Table 1. Forty mL of each film-forming solution were poured on plexiglass plates (11.5 × 11.5 cm) and then dehydrated at 45 °C for 18–20°h in an oven with air flow and circulation (Binder FD 240, Tuttlingen, Germany). The dry films were conditioned at room temperature and 58% relative humidity in desiccators with saturated solutions of NaBr for two days before subsequently peeled from the casting surface for characterization analyses. Table 1 summarizes the nomenclature of the films.

2.4. Thickness

Film thickness was measured with a digital micrometer (Mitutoyo MDC-25M, Kanagawa, Japan). For each film, the values obtained at nine different locations were averaged.

2.5. Water solubility and protein soluble matter

Film solubility was determined in triplicate according to the method proposed by Gontard, Guilbert, and Cuq (1992). Three pieces of film (2 cm in diameter, about 0.25 g total) were immersed in 50 mL of distilled water and the system gently shaken for 24 h at room temperature (22–25 °C). The samples were then passed

Table 1
Nomenclature of simple and composite films according to the proportion (w/w) of gelatin and soybean-protein isolate (SPI).

Nomenclature	SPI (%)	Cod gelatin (%)
0S:100G	–	100
25S:75G	25	75
50S:50G	50	50
75S:25G	75	25
100S:0G	100	–

through a filter paper (Whatman 1). The unsolubilized fraction was dried in a forced-air oven (105 °C, 24 h) in order to determine the water-soluble matter as a percentage of the initial weight.

The protein content of the soluble fraction was measured with a nitrogen analyzer LECO FP-2000 (Leco Corporation, St Joseph, MI) calibrated with EDTA (the Dumas method following A.O.A.C. 992.15; A.O.A.C., 2000). The results shown are the average of three determinations and are expressed as the percent protein solubilized with respect to the amount of film originally present ($N \times 5.7$ for SPI, $N \times 5.4$ for cod gelatin, $N \times 5.5$ estimated for the mixture of SPI and cod gelatin).

2.6. Sodium-dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Aliquots of the water-soluble fractions of the films (15 μL) were mixed with 200 μL of loading buffer (12.5% SDS, 25% mercaptoethanol, 50 mM Tris–HCl, 5 mM EDTA, 10% glycerol and 0.002% bromophenol blue) to a final concentration of 2 mg/mL of protein. The samples were then heat-denatured 5 min at 90 °C and analyzed by SDS-PAGE according to Laemmli (1970) on 7.5% (w/v) gels in a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA) at 30 mA/gel. The loading volume was 20 μL in all lanes. Protein bands were stained with Coomassie brilliant blue R-250 (Merck) and destained with an aqueous solution of 30% (v/v) methanol and 10% (v/v) acetic acid. An aqueous solution of 5% (w/v) glycerol and 10% (v/v) acetic acid was used for storage. Two mixtures of known proteins covering different molecular-weight ranges (Amersham Biosciences, Buckinghamshire, England) were used as reference standards. The high-molecular-weight standard contained myosin (M_w 220 kDa), α_2-macroglobulin (M_w 170 kDa), β-galactosidase (M_w 116 kDa), transferrin (M_w 76 kDa), and glutamic-acid dehydrogenase (M_w 53 kDa) and the low-molecular-weight standard phosphorylase b (M_w 97 kDa), bovine-serum albumin (M_w 66 kDa), ovalbumin (M_w 45 kDa), carbonic anhydrase (M_w 30 kDa), trypsin inhibitor (M_w 20.1 kDa), and α-lactalbumin (M_w 14.4 kDa).

2.7. Light absorption

The light-barrier properties of films were determined by measuring their light absorption at wavelengths ranging from 280 nm to 690 nm, using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan). The transparency of the films was calculated by the equation $T = Abs_{600}/x$, where Abs_{600} is the absorbance at 600 nm and x is the film thickness (mm).

2.8. Fourier-transform-infrared spectroscopy (FTIR)

Pieces of film 2 cm in diameter were sandwiched between two KBr disks. FTIR spectra were recorded from wavenumber 400–4000 cm^{-1} in a Bruker IFS28 spectrometer (Bruker Banner Lane, Coventry, UK). Absorbance spectra were obtained with a spectrum of the KBr disk as background. In all, 50 interferograms were compiled for each spectrum.

2.9. Differential-scanning calorimeter

A Polymer Laboratories Rheometrics Scientific differential-scanning calorimeter (DSC, Church Stretton, UK) was used for these studies. Temperature and heat-flow calibration of the equipment was carried out according to ASTM Standards. Lauric acid and indium were used as temperature standards; the latter was also used as a heat-flow standard. SPI, cod gelatin and the various films were studied by DSC, in order to determine the conformational state of both proteins. Hermetically sealed aluminum pans containing 10–15 mg of samples were prepared. The capsules were scanned at 10 °C/min over the range 30–150 °C with an empty double capsule being used as a reference blank.

2.10. Water vapor permeability measurements

Water-vapor permeability was determined following the method described by Sobral, Menegalli, Hubinger, and Roques (2001). Samples cut from each film were mounted on plastic cups (permeation area = 15.9 cm^2) containing silica gel and the cups placed in desiccators with distilled water. The cups were weighed every hour for 6 h. Water-vapor permeability was calculated from the equation $WVP = w\ x\ t^{-1}\ A^{-1}\ \Delta P^{-1}$, where w is the weight gain (g), x is the film thickness (mm), t is the time of gain (h) and ΔP is the difference of partial vapor pressure of the atmosphere with silica gel and pure water (2642 Pa at 22 °C). Results were expressed as $g°mm°h^{-1}\ cm^{-2}\ Pa^{-1}$. All tests were made in triplicate.

2.11. Mechanical properties

The films were fixed in a 5.6-cm–diameter cell and perforated to the breaking point through the use of a texturometer (Instron 4501, Instron Engineering Corp., Canton, MA, U.S.A.) with a round-ended stainless-steel plunger (diameter, 3 mm). The cross-head speed was 60 mm/min and a 100 N load-cell was used. The breaking force was expressed in N and the breaking deformation as a percent, according to Sobral et al. (2001). All determinations are the means of at least five measurements.

2.12. Color

Films were placed between two steel dishes with a hole of 5.7 cm diameter. The color of films was determined with a tristimulus colorimeter (HunterLab D25 A-9, Hunter Associates Laboratory Inc., Reston, VA., USA) using the CIE Lab scale (C/2°) where L*, a* and b* are the parameters that measure lightness, redness and yellowness, respectively. A standard white plate with reflectance values of $L^* = 93.68$, $a^* = -0.69$, $b^* = -0.88$, was used as reference. The results were the average of five measurements taken at ambient temperature at different points on the sample.

2.13. Statistical analysis

The data were subjected to one-way analysis of variant (ANOVA) by means of an SPSS computer program (SPSS 14, SPSS INC., Chicago, Illinois, USA), while means were compared by the Bonferroni test with the level of significance set at $p < 0.05$. Pearson or Spearman's rho correlations ($p < 0.01$ or <0.05) were calculated to analyze the dependence of film characteristics on the ratio of SPI:gelatin.

3. Results and discussion

Table 2 shows the thickness, water content, water solubility, and soluble-protein content of the films obtained. All the films containing SPI (regardless of its proportion) had a similar thickness, which in all instances was significantly thinner than those of films made from gelatin alone. Moreover, film containing only soybean proteins exhibited significantly higher water content than that of the film containing only gelatin, while composite films had intermediate water contents without significant differences among them. It is noteworthy that in spite of the use of the same total-protein mass and drying conditions for preparing all films, those containing gelatin had a higher thickness in spite of their lower water content. This result indicates a higher degree of compaction

Table 2
Thickness, moisture content, film solubility, and percent soluble protein of films obtained with different proportions of soybean-protein isolate (S) and cod gelatin (G).

Film	Thickness (μm)	Moisture content (%)	Film solubility (%)	Soluble protein (%)
0S:100G	86 ± 11^{a}	14.53 ± 0.73^{a}	87.66 ± 0.46^{a}	58.58 ± 2.68^{a}
25S:75G	50 ± 10^{b}	15.04 ± 0.01^{ab}	85.19 ± 3.03^{ab}	58.85 ± 0.44^{a}
50S:50G	48 ± 19^{b}	15.62 ± 0.59^{ab}	84.63 ± 2.26^{ab}	57.41 ± 0.65^{a}
75S:25G	48 ± 12^{b}	15.55 ± 1.09^{ab}	81.39 ± 5.64^{ab}	53.16 ± 1.34^{b}
100S:0G	47 ± 10^{b}	17.06 ± 0.76^{b}	83.93 ± 2.44^{b}	52.92 ± 0.79^{b}

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

in the film matrix when soybean proteins were present, thus suggesting a different degree of molecular unfolding or cross-linking within the protein network of the film.

3.1. Solubility properties

All the films exhibited a water solubility above 80% (Table 2), with the gelatin films being slightly ($p \leq 0.05$) more soluble than SPI films. This observation would indicate a poor water resistance, although for some applications that property could be advantageous: for example, as a carrier of bioactive compounds. The solubility tended to decrease with an increased proportion of SPI in the composite films, although the differences were found not significant ($p \leq 0.05$). It is worth noting that the water-insoluble fraction of the composite films could not maintain the initial structural integrity, but after solubilization appeared as a viscous mass, mostly from the presence of swelled gelatin aggregates; so that this consequence might account for the considerably high variability within the results. In the case of films made from pure gelatin, the results obtained agree with those reported by Carvalho et al. (2008) for films elaborated from two differently processed Atlantic-halibut–skin gelatins, as well as with the findings of Piotrowska et al. (2005) and Pérez-Mateos et al. (2009) for cod-skin–gelatin films, but were considerably higher than those reported for bovine-gelatin films of around 30% (Bertan, Tanada-Palmu, Siani, & Grosso, 2005; Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, in press). Compared to mammalian gelatins (Montero, Borderićas, Turnay, & Leyzarbe, 1990; Norland, 1990), those from cold-water fish are characterized by a lower content of intra- and interchain covalent cross-links, mainly involving lysine and hydroxylysine residues as well as aldehyde derivatives, whose lower degree of linkage may be responsible for such a higher film solubility. Nevertheless, solubility values for pure soybean-protein films were higher than those reported by other authors (Cho & Rhee, 2004; Kunte, Gennadios, Cuppett, Hanna, & Weller, 1997; Stuchell & Krochta, 1994). Denavi et al. (in press) have shown that the solubility of films prepared by casting native SPI solutions with glycerol – as plasticizer – was significantly affected by the drying conditions used. They also reported solubilities higher than 80% when films were dried at low temperatures and low relative humidities (conditions expressly used in the present work) and attributed this finding to the fact that the proteins are not denatured under these conditions. In the native state, interactions between their chains (including thiol/disulphide—SH/SS—interchanges) would not be favored, thus minimizing the subsequent solubility of the films. The same explanation may apply to the findings here.

While the small changes in the solubility of the films represented no significant differences, the amount of protein released from the films upon water solubilization was significantly higher ($p \leq 0.05$) in the pure-gelatin film, as well as in the composite films with the SPI not exceeding 50% (Table 2). The high compaction of all the films containing soybean proteins would be expected to hamper the protein-solvent contact thus reducing the solubility of these films as compared to the 100% gelatin ones. Nevertheless, this fact did not explain the behavior of the composite films, all of which had a similar thickness that was notably lower than that of the 100% gelatin films. Rather, in agreement with the observations of Voutsinas, Cheung, and Nakai (1983), the lower protein solubility in the films with the highest SPI ratio (*i. e.*, 100S:0G and 75S:25G) was mainly attributable to the higher surface hydrophobicity of the soybean proteins as compared to those of gelatin.

3.2. Electrophoretic analysis

Fig. 1 shows the SDS-PAGE analysis of the protein fraction released upon water solubilization of the films. A clear difference in the nature of the bands as well as in their intensity was observed between the 100% SPI and the 100% G films. As expected, the soluble fractions of gelatin exhibited molecular weights notably higher than those of the soybean proteins. Furthermore, the bands corresponding to the gelatin polypeptides exhibited a considerably higher intensity than SPI bands. In agreement with solubility results, this indicated higher amount of soluble protein in gelatin films compared to SPI films. The protein released from gelatin films showed bands that could be identified mainly as α_1- and α_2-chains (~100 kDa), β-components (~200 kDa) and higher molecular weight aggregates, with a distribution similar to that of the gelatin originally used for film preparation (Gómez-Guillén et al., 2002). This finding indicates that all fractions, including those of very high-molecular weight, are soluble in water, consistent with the high water solubility of the film itself. Proteins released from SPI films upon water solubilization included lower molecular weight components (<80 kDa), possibly attributable to the polypeptides that constitute the two main globulins present in SPI, i.e. acidic polypeptide chains (A: 37–40 kDa) and basic polypeptide chains (B: 9.9–20 kDa), and the AB subunit (~60 kDa) of glycinin globulin

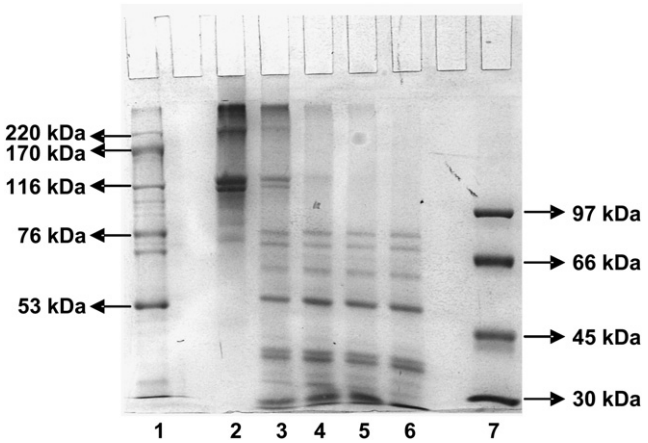

Fig. 1. SDS-PAGE gels of water-soluble fractions of the films. (Lanes 1 and 7, M_W standards; lane 2, 0S:100G; lane 3, 25S:75G; lane 4, 50S:50G; lane 5, 75S:25G; lane 6, 100S:0G).

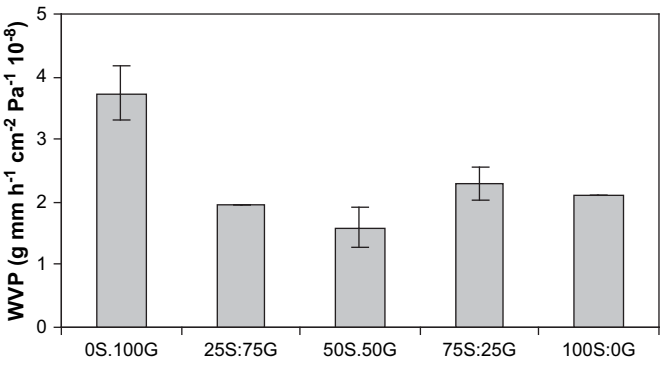

Fig. 2. Water-vapor permeability of films obtained with different soybean-protein:cod-gelatin ratios.

(11S), as well as subunits α' (57-83 kDa), α (57-76 kDa) and β (42-53 kDa) of β-conglycinin (7S). It is noteworthy that soluble aggregates of higher molecular weight, previously found in other studies (Mauri & Añón, 2006), were not observed among the soybean polypeptides in the present work. With respect to the composite films, the SPI bands exhibited a similar intensity in all formulations irrespective of the proportion of SPI in the film. On the contrary, the gelatin molecules showed a noticeable decrease in the intensity of the different soluble-protein bands when 25% of the gelatin was replaced by SPI in the formulation of the composite films (*i. e.*, 25S:75G). This observation strongly suggested that in the presence of even a low amount of SPI, gelatin aggregated and became less soluble. This fact, however, could not be clearly evidenced by determining either the water solubilities of those films (100G *vs.* 25S:75G) or their amount of soluble protein since no significant differences were found. As the SPI ratio increased further, the gelatin fractions became hardly visible, in part owing to molecular aggregation, but also as the result of a dilution effect through the reduced SPI:gelatin ratio. This change was especially apparent in the 75S:25G formulation, in agreement with the significantly lower values of soluble protein found.

3.3. Water vapor permeability

Fig. 2 shows the WVP of the films. The values for this measurement were significantly lower for the soybean-protein films than for the gelatin ones, and the presence of soybean proteins in the composite films resulted in a reduction of the WVP regardless of the amount added. This effect is clearly related to the lesser thickness of films containing soybean proteins as well as to their greater surface hydrophobicity. As reported previously for other hydrophilic films (pectin, amylose, cellulose ethers, sodium caseinate, and soybean proteins), WVP increases with film thickness (Ghorpade et al., 1995; McHugh, Avena-Bustillos, & Krochta, 1993). In the present experiments, the replacement of only 25% of the cod gelatin by SPI (*i. e.*, 25S:75G) was enough to induce a film-matrix compaction that remained unaltered even after increasing the soybean-protein level.

3.4. Mechanical properties

The puncture force and deformation of the films studied as measured in compression assays are shown in Fig. 3a and b, respectively. The gelatin films exhibited a greater deformation (at least 10-fold higher since these films did not break under the conditions assayed) and a greater breaking force (1.8-fold higher) than those respective properties of the soybean-protein films. With respect to the composite films, the deformation increased progressively with the proportion of gelatin in the mixture; nevertheless, the breaking forces exhibited by the 50S:50G and 25S:75G films were higher than those of the films made from gelatin alone. Cao et al. (2007) reported that the mechanical properties in the tensile test of composite films made from type-B–bovine-bone gelatin and SPI improved progressively with increasing proportions of gelatin, but the traction resistance, elongation, and Young module of films prepared from gelatin alone were nevertheless higher than those of all the composite films. The higher breaking force of the 50S:50G and 25S:75G films observed in the present study suggests a reinforcement of the film matrix, which was probably induced by a certain degree of cross-linking between the proteins of both gelatin and SPI.

3.5. Differential-scanning calorimetry

Fig. 4 shows film thermograms determined by DSC. The gelatin-film thermogram showed no endotherm, indicating that the gelatin of this film was totally denatured. In the thermograms pertaining to the soybean-protein film, we noted two endotherms, at 113 °C and at 137 °C, which corresponded to the denaturation of the 7S (β-conglycinin) and 11S (glycinin) globulins, respectively. These denaturation temperatures were shifted to higher values than those usually expected for dispersions of those proteins (*i. e.*, at only 78 °C and 92 °C, respectively), due to the low water content of the film (Hägerdal & Martens, 1976; Mauri & Añón, 2006). The denaturation heat values associated with these endotherms (9.0 J/g of soybean protein) were lower than those reported in literature for native proteins (15.5 J/g), but was in the range of the data reported previously for similar SPI film-forming solutions at pH 11 (10 J/g; Mauri & Añón, 2006). Thus, the drying step at 45 °C did not contribute to the denaturation of the soybean globulins. These results would indicate that the soybean proteins present in the films were denatured only partially, with a significant amount of native conformation remaining in their globular structure. All the thermograms of the composite films displayed two denaturation

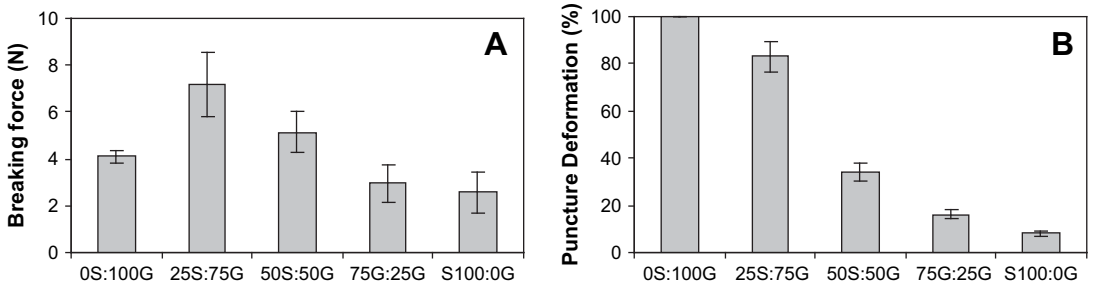

Fig. 3. Mechanical properties as determined by the puncture test of films formed with different soybean-protein:cod-gelatin ratios. a) breaking force (N); b) deformation (%).

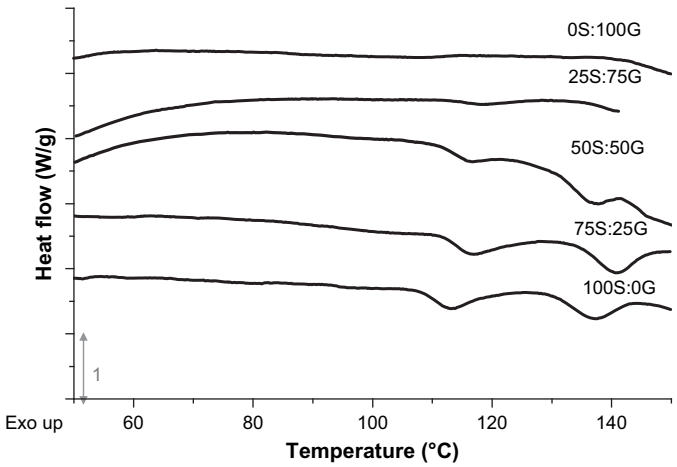

Fig. 4. DSC thermograms of films with different soybean-protein:cod-gelatin ratios.

endotherms. Even though the endotherms become smaller upon reducing the amount of soybean protein simply through the effect of dilution, the enthalpy of denaturation (as measured per g of that protein) did not change significantly. Moreover, although not pronounced, there was a tendency toward a slight upward shift of denaturation temperatures with increasing gelatin proportions partially because of the lower water content of these films. This effect was more notable in the thermograms of the 25S:75G film, which exhibited a 3 °C–7 °C increase in the denaturation temperatures for 7S and 11S subunits of 100S:0G composite, respectively. The effect of gelatin on stabilizing soy proteins was probably due to the interactions between the unfolded gelatin molecules and the soybean proteins that conserved in part their native globular structure. Such an interpretation would be supported by the previous observations on the mechanical properties and water-vapor permeabilities as well as on the solubilities of the proteins present in the films. DSC results indicated a different degree of unfolding of SPI containing samples and thus explained the reduction of film thickness of SPI containing films.

3.6. Fourier-transform-infrared spectroscopy (FTIR)

Fig. 5 shows the FTIR spectra of cod-skin–gelatin films (0S:100G), SPI films (100S:0G), and their corresponding mixtures (25S:75G and 50S:50G). The gelatin film exhibited a sharp absorption band between 3550 and 3510 cm^{-1}, which corresponded mainly to stretching vibrations of the OH groups from adsorbed water molecules as a result of the relatively high hygroscopic nature of the film (Yakimets et al., 2005). A similar band was also evident in the gelatin-SPI composite films, largely linked to the presence of gelatin. On the contrary, the SPI film showed a broad absorption ranging between 3500 and 3200 cm^{-1}; which corresponded mainly to free OH groups and amine N–H stretching of the soy proteins in the film (Guan, Qiu, Liu, Hua, & Ma, 2006; Nanda, Rao, Kar, & Nayak, 2007).

The infrared spectra of the composite films revealed notable changes occurring in the 1700–1650 cm^{-1} region, as compared to the pure gelatin and the SPI films. With respect to gelatin-containing films, changes within this frequency range are indicative of alterations in collagen or gelatin secondary structure involving the amide-I region (Muyonga, Cole, & Duodu, 2004). The spectrum of the cod-gelatin film has been described in an earlier publication, where the gelatin had been mixed with increasing proportions of sunflower oil and displayed notable lipid–protein interactions (Pérez-Mateos et al., 2009). As reported previously, the main peaks observed at 1687 cm^{-1} and 1656 cm^{-1} in the cod-gelatin film were related to the predominance of helices within aggregated collagen-like peptides and, to a lesser extent, the presence of random coils.

By contrast, the pure-SPI film showed principal peaks of relevance at 1656 cm^{-1}, 1676 cm^{-1}, and 1687 cm^{-1}. Using films made from commercial SPI, Kurose, Urman, Otaigbe, Lochhead, and Thames (2007) reported the presence of peaks at 1658 cm^{-1} and 1625 cm^{-1}, which represented the overlap signals from α-helix and random segments, and the contribution of β-sheets in the secondary structure, respectively. These peaks were reported to become enhanced with increasing glycerol concentrations—as the result of an induced activation of molecular-chain movements. On the contrary, no such absorption band at around 1650–1658 cm^{-1} could be detected in another dried commercial SPI (Nanda et al., 2007), where there had been no addition of glycerol or sorbitol reported. It is clear that, in addition to the intrinsic properties of the constituent biopolymers, the conditions used for film preparation (*e. g*, the addition of certain plasticizers) are essential in determining the prevalence of secondary structure within the film. The SPI film in the present work contained 37.5% plasticizers (glycerol plus sorbitol), which may have promoted the appearance of the peak at 1656 cm^{-1} (Kurose et al., 2007). Alternatively, the peak could have been associated with α-helix structures. Likewise, peaks

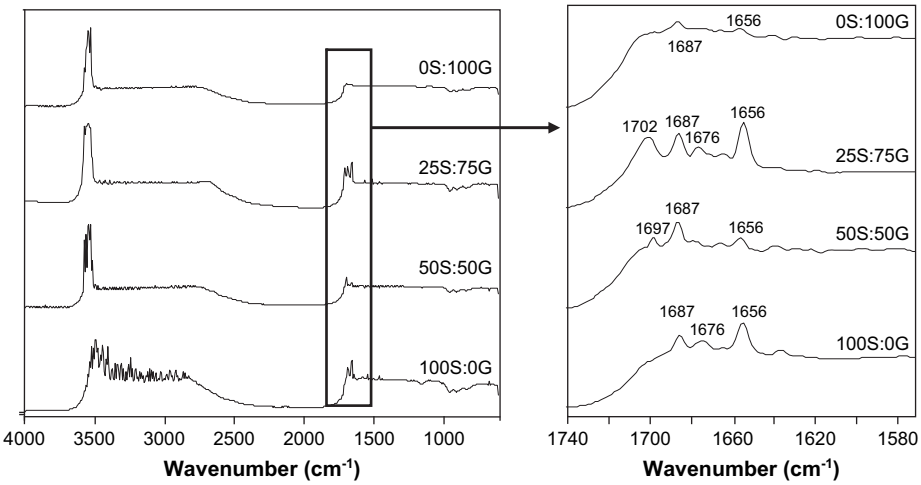

Fig. 5. FTIR spectra of the cod-skin–gelatin films (0S:100G), the soybean-protein–isolate films (100S:0G), and their corresponding composite films (25S:75G and 50S:50G).

at 1676 cm^{-1} and 1687 cm^{-1}, seen in SPI film and to a lesser extent in gelatin-film spectra, may be related to interactions with the plasticizer through C=O bonds. The extremely high pH (10.5) used for dissolving the SPI proteins for film formation, very far from their isoelectric point (≈4.5), may have induced a certain degree of soybean-protein unfolding, thus favoring the interaction between the protein and the plasticizers present, as was indicated in the DSC studies.

The gelatin-SPI mixtures, especially at 25S:75G, induced an increase in peaks that previously existed in gelatin or SPI films, as well as an appearance of new peaks. These new peaks were possibly an indication of protein–protein interactions between gelatin and SPI. The higher intensity of the peaks at 1687 cm^{-1} and 1656 cm^{-1}, especially when compared to those in the pure-gelatin film, indicated an increased aggregation of gelatin polypeptide α-chains along with a concomitant increase in the amount of disordered random coils, which were contributed from SPI. The peak at 1676 cm^{-1} was clearly visible in the 25S:75G film and, to a lesser extent, also in the 50S:50G film. Such an FTIR feature was scarcely visible in the pure-gelatin film, where it was present in the form of only a trace shoulder. Muyonga et al. (2004) reported a peak at 1675 cm^{-1} that they ascribed to the presence of β-turns in fish gelatin films. In agreement with these authors—and considering the low SPI proportion in the 25S:75G film—the increase in the intensity of this peak in the present study may be revealing gelatin conformational changes induced by the presence of the SPI. The appearance of peaks at 1702 cm^{-1} in the 25S:75G film, and to a lesser degree at 1697 cm^{-1} in the 50S:50G film, indicated an increment in intermolecular associations through C=O bond interaction, likely resulting from gelatin cross-linking as a result of esterification reactions with the SPI side-chain reactive groups. Comparable peaks have been previously reported to occur in spectra from pigskin gelatin with added lauric acid (Djagny, Wang, & Xu, 2001), or in similar cod-skin–gelatin films supplemented with different concentrations of sunflower oil (Pérez-Mateos et al., 2009). The higher intensity and frequency of appearance of the peak at 1702 cm^{-1} in the 25S:75G film, as compared to the 50S:50G film, clearly suggested that the gelatin–SPI interaction was stronger in the former.

From all the above findings, we conclude the following. The replacement of 25% gelatin by SPI in the formulation of composite films led to gelatin conformational changes that produced a twofold effect: a favoring of the self-aggregation of the gelatin polypeptide α-chains, as well as a certain degree of interaction between the gelatin and the SPI proteins. These interactions may explain why the resulting protein matrix became more compact, as manifested in its reduced thickness, and thus gave rise to stronger and less deformable films. Cao et al. (2007) reported that interactions between the amino and carboxyl groups of SPI and gelatin might determine the improved mechanical properties of composite films and proposed hydrogen bonding as being a principal mechanism responsible for those interactions. In this regard, our FTIR study revealed a stronger interaction between C=O bonds within the composite films we studied. The higher degree of compactness together with the more hydrophobic nature of the SPI proteins also caused the WVP to decrease in those films. When the ratio of SPI in the film was increased, both the gelatin cross-linking and gelatin-SPI interactions occurred to a lower extent, strongly suggesting that, in excess of a certain threshold amount, the SPI proteins may act as a simple filler in the gelatin network, causing the films to be less resistant, and especially less deformable. Low breaking force and deformability were the characteristics of 100% SPI films.

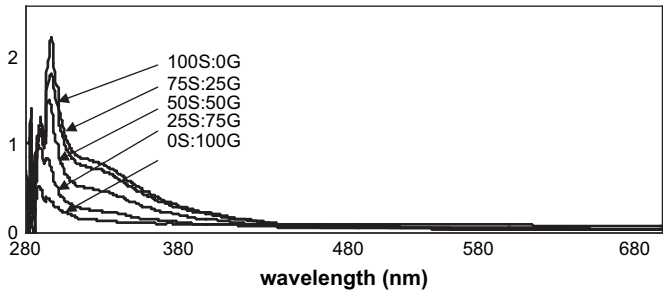

Fig. 6. Light–barrier properties of films with different ratios of soybean-protein isolate and cod gelatin.

Table 3
Color parameters (L*, a*, and b*) and transparency (T) of films formed with soybean-protein isolate and cod gelatin.

Film	L*	a*	b*	T
0S:100G	$18.82 \pm 0.30^{a,b,c}$	-2.08 ± 0.23^{a}	$2.33 \pm 0.22^{a,b}$	1.06 ± 0.21^{a}
25S:75G	$18.65 \pm 0.07^{a,c}$	-2.60 ± 0.33^{a}	2.28 ± 0.09^{a}	0.95 ± 0.05^{a}
50S:50G	$18.94 \pm 0.25^{a,b,c}$	-2.43 ± 0.20^{a}	$2.52 \pm 0.29^{a,b,c}$	0.98 ± 0.05^{a}
75S:25G	$20.12 \pm 0.62^{b,d}$	-2.97 ± 0.77^{a}	$3.22 \pm 0.50^{a,b}$	0.99 ± 0.07^{a}
100S:0G	21.22 ± 0.97^{d}	-2.59 ± 0.46^{a}	5.06 ± 0.89^{d}	1.08 ± 0.09^{a}

Reported values for each film are means ± standard deviation. Values L*, a*, b* and T means followed by the same letter are not significantly ($P < 0.05$) different according to Bonferroni's test.

3.7. Light barrier properties

Fig. 6 reveals the light–barrier properties of the films as registered in a spectroscopic scanning at wavelengths between 280 and 690 nm. All spectra exhibited maximum absorption at 290 nm and 295 nm and a minimum absorption at 280 nm. Proteins usually exhibit maximum absorbance at around 280 nm, ascribed to the hydrophobic residues tyrosine and tryptophan. The slight shift of the typical maximum absorption may be attributed to the extremely high pH (10.5), since excessive alkalinity may cause the ionization of the hydroxyl group of tyrosine (Creighton, 1993). An increase in absorption at 290 nm and 295 nm was observed when SPI was increased. This fact may be largely attributed to the exposure of the higher number of hydrophobic residues that are present in the SPI. The transparency level of the films at 600 nm was not significantly modified ($p > 0.05$) by the ratio of soybean protein:gelatin (Table 3).

Table 3 summarizes the Hunter color parameters (L*, a*, and b*) of the films. The soybean films were yellowish (b+) while those made from gelatin were colorless. The color difference among samples was a consequence of the formulation. As the proportion of SPI increased, the yellowish color did so as well (as measured by an increase in b*) and the films were clearer (indicated by the increase in L*). These changes in color parameters became significant for films formed with soybean proportions higher than 50%. Since all films showed similar a* values, the principal differences were mainly restricted to the intensity of the yellowish tone.

4. Conclusions

The polydisperse nature of gelatin molecules with the presence of unfolded proteins of high-molecular weight results in a film of considerable thickness, with extremely high deformability. When the soybean proteins (with their globular structure partially preserved) were introduced at low concentrations (25S:75G), gelatin aggregation as well as gelatin–SPI interactions were induced, leading to a notable reinforcement of the composite film. As the proportion of soybean protein increased, the gelatin matrix approached saturation, so that the consequent formation of

localized independent soybean matrices reduced the film's mechanical properties (both the breaking force and its deformation).

Furthermore, SPI, having higher surface hydrophobicity than gelatin, contributed to the higher water-vapor barrier of the composite films. Although some physical properties have been improved (mainly the breaking force and the WVP), these composite films are nevertheless highly water-sensitive, making them still far from being a real alternative to the synthetic polymers. Soybean-protein–gelatin composite films could, however, serve as an efficient carrier of active components use in food preservation or for designing functional foods.

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