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Properties of Cast Films from Hemp (*Cannabis sativa* L.) and Soy Protein Isolates. A Comparative Study

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The properties of cast films from hemp protein isolate (HPI) including moisture content (MC) and total soluble mass (TSM), tensile strength (TS) and elongation at the break (EAB), and surface hydrophobicity were investigated and compared to those from soy protein isolate (SPI). The plasticizer (glycerol) level effect on these properties and the interactive force pattern for the film network formation were also evaluated. At some specific glycerol levels, HPI films had similar MC, much less TSM and EAB, and higher TS and surface hydrophobicity (support matrix side), as compared to SPI films. The TS of HPI and SPI films as a function of plasticizer level (in the range of 0.3−0.6 g/g of protein) were well fitted with the exponential equation with coefficient factors of 0.991 and 0.969, respectively. Unexpectedly, the surface hydrophobicity of HPI films (including air and support matrix sides) increased with increasing the glycerol level (from 0.3 to 0.6 g/g of protein). The analyses of protein solubility of film in various solvents and free sulfydryl group content showed that the disulfide bonds are the prominent interactive force in the HPI film network formation, while in the SPI case, besides the disulfide bonds, hydrogen bonds and hydrophobic interactions are also to a similar extent involved. The results suggest that hemp protein isolates have good potential to be applied to prepare protein film with some superior characteristics, e.g., low solubility and high surface hydrophobicity.

KEYWORDS: Hemp (*Cannabis sativa* L.) protein isolate; protein film; mechanical property; disulfide bond

INTRODUCTION

There has been an increasing interest in edible packaging films due to concerns about the environment, as well as consumer demand for higher-quality food products. Biopolymer films and coatings are generally designed using biological materials such as polysaccharides, proteins, lipids, and derivatives (*1*). The ability of proteins to form a continuous matrix has been known for a long time, and a wide range of proteins such as wheat gluten (*2*, *3*), maize zein (*4*), soy proteins (*5*−*7*), gelatin (*8*, *9*), collagen (*10*, *11*), pea proteins (*12*), egg and milk proteins (*13*−*16*) and fish myofibrillar proteins (*17*, *18*) have been widely investigated.

Cannabis sativa L., commonly referred to as hemp, is a widely cultivated plant of industrial importance, as an important source of food, fiber, and medicine. As the byproduct during commercial utilization (i.e., for the valuable fiber), hemp protein, accounting for about 25% of hempseed, attracted increasing interest due to its superior essential amino acid composition and good digestibility (*19*, *20*). Edestin representing about 82% of total protein is the major protein component in hemp protein isolates (HPI) (*20*). Like the hexamer of soy glycinin, the edestin is also composed of six identical AB subunits, and each AB subunit consists of an acid subunit (AS) and a basic subunit (BS) with molecular weight (MW) of about 33.0 and 20.0 kDa, respectively (*21*).

The films cast with 11*S* globulins (glycinin) have much higher tensile strength (TS) as compared to those with 7*S* globulins (*22*). This phenomenon was attributed to the higher tendency of 11*S* protein (glycinin) to form disulfide bonds compared to 7*S* protein (*23*, *24*). The cluster−cluster aggregation of protein by low-energy bonds (e.g., hydrogen bonds, hydrophobic interactions) and covalent linkages may in part account for the network formation of glycinin-based gel (*25*−*28*). Thus, it is reasonable to expect that hemp protein isolates (HPI) rich in 11*S* globulins (edestin) have good film-forming ability.

The objective of this work was to investigate the properties of cast films from HPI, as compared to that of soy protein isolates (SPI). The effects of plasticizer level on the properties of cast films were also investigated. Additionally, protein solubility of films and free sulfydryl content of protein were determined, to further reveal related formation mechanism of film.

MATERIALS AND METHODS

Materials. Defatted hempseed protein meal, a byproduct during the utilization of the valuable hempseed oil and fiber, was kindly supplied by YUNNAN Industry Hemp Co. Ltd. (Yunnan province, China). This meal had been on a large scale obtained from hemp (*Cannabis sativa* L.) seeds, through dehulling, disintegrating, and defatting with super-

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critical liquid (CO_2) at low temperatures (less than 40 °C). Soy protein flour was provided by Wonderfu Technol. Co. (Shangdong Province, China). 5,5′-Dithio-bis 2-nitrobenzoic acid (DTNB) reagent was purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Fitzgerald Industries International Inc. (Concord, MA). All other chemicals used in this study were of analytical or better grade.

Preparation of Protein Isolates. HPI was produced from the defatted hempseed meal according to Tang and others (*20*), with slight modifications. Defatted hempseed meal was dispersed in deionized water (1:20, w/v), and the pH of the dispersion was adjusted to 10.0 with 2 N NaOH. The resultant dispersion was gently stirred at 37 °C for 2 h, and then centrifuged at 8000*g* at 20 °C for 30 min. The pellet was discarded, and the supernatant was adjusted to pH 5.0 with 2 N HCl and then centrifuged at 5000*g* at 20 °C for 20 min. The obtained precipitate was redispersed in deionized water. The dispersion was homogenized and adjusted to pH 7.0 with 2 N NaOH, then followed by freeze−drying to produce HPI product. SPI was prepared from defatted soybean meal (Wonderfu Technol. Co., Shandong Province, China), according to the method described by Tang and others (*25*). The protein content of HPI and SPI was 91.2 and 92.0%, respectively (determined by Kjeldahl method, $N \times 6.25$, wet basis).

Film Preparation. The film-forming solutions were prepared by dispersing HPI or SPI (5%, w/w) and 0.3, 0.4, 0.5, and 0.6 g of glycerol/g of protein in deionized water. The dispersions were magnetically stirred for 30 min at room temperature. The pH of the dispersions was adjusted to 9.0 with 2 N NaOH. The resultant solutions were incubated at 90 °C for 30 min in a shaking water bath. Following degassing under vacuum, the film-forming solution was cast onto rimmed, leveled glass plates coated with polyethylene films (Clorox China Co. Ltd., Guangzhou, China). The film thickness was controlled by casting the same solids (2.8 g) on each plate (18 × 20 cm). The castings were air-dried at room conditions [25 ± 1 °C, 50 ± 5% relative humidity (RH)] for 48 h, then the films were peeled off the plates, and various specimens for physical property testing were cut. Specimens of 2.5 × 10 cm rectangular strips were for tensile testing, and 2 × 2 cm squares for MC and TSM measurements. Furthermore, films cast with 50 g of glycerol/100 g of protein were used to analyze solubilization of films in different solvent systems.

Tensile Strength (TS) and Elongation at Break (EAB). TS and EAB were measured using a TA-XT2i texture analyzer (Stable Micro Systems, London, U.K.). Samples were preconditioned at 25 °C and 50 ± 3% RH in a desiccator containing magnesium nitrate saturated solution [$Mg(NO_3)_2 \cdot 6H_2O$] for at least 2 days prior to analysis. Initial gap separation and cross-head speed were set at 50 mm and 1 mm/s, respectively. TS was calculated by dividing the maximum load at break by initial specimen cross-sectional area (*29*). EAB was calculated by dividing the extension at break of the specimen by the initial gage length of the specimen (50 mm) and multiplying by 100 (*29*). Each trial was replicated at least eight times, and the averages were taken as the data.

Moisture Content and Total Soluble Mass (MC and TSM). The MC of films was determined by oven-drying at 105 ± 2 °C for 24 h, and expressed as the percentage of initial film weight lost during drying. The TSM of each film was determined as the percentage of film dry matter solubilized after 24 h immersion in deionized water (*30*). Three randomly selected 2 × 2 cm samples from each type of film were first dried at 105 °C for 24 h to determine the weight of the initial dry matter. Then the dry film specimens were placed in a 50 mL cuvette containing 30 mL of deionized water. The cuvettes were covered with polyethylene films (Clorox China Co. Ltd., Guangzhou, China) and incubated in a shaking water bath at 25 °C for 24 h with gentle vibrating. Undissolved dry matter was determined by removing the film pieces from the beakers, gently rinsing them with deionized water, and then oven-drying the rinsed films (105 °C, 24 h).

Protein Solubility of Film. The protein solubility of film in different solvents was determined according to the method described by Lupano and colleagues (*31*−*33*), with some modifications. Samples (5 mg/mL) were dispersed in various solvents as follows: DW, deionized water at pH 8.0; B, Tris-Glycine buffer (0.086 M Tris, 0.09 M glycine, and 4 mM Na_2EDTA, pH 8.0); BSU, B containing 0.5% sodium dodecyl sulfate (SDS) and 8 M urea; BSUM, BSU plus 1% (v/v) β-mercaptoethanol (2-ME). The mixtures were incubated for 24 h at 25 °C in a shaking water bath. The resultant suspensions were centrifuged at 20000*g* for 20 min at 25 °C, and the protein concentration in the supernatants was determined by the Lowry method (*34*) using bovine serum albumin (BSA) as the standard. To prevent interference of glycine, Na_2EDTA, and 2-ME in protein determination, the supernatants were dialyzed. The colorimetry was performed at 750 nm in a T6 spectrophotometer (Beijing Purkinje General Instrument Co. Ltd, Beijing, China). All determinations were conducted in triplicate.

Free Sulfydryl (SH_f) Content. The SH_f content of protein isolates and films was determined according to the method of Ellman (*35*) as modified by Beveridge and others (*36*), with some modifications. Ellman's reagent was prepared by dissolving 4 mg of DTNB reagent in 1 mL of Tris-Glycine buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA, pH 8.0). Ground samples of 12.5 mg of protein or 25.0 mg of film were suspended in 5.0 mL of reaction buffer consisting of Tris-Glycine buffer with 8 M urea and 0.5% SDS, and 50 μL of the Ellman's reagent were added. The resultant suspensions were incubated for 1 h at room temperature (25 ± 1 °C), with occasional vibration, and then centrifuged at 13600*g* for 10 min. The absorption of the supernatant was determined at 412 nm against reagent blank, or against buffer blank solution. The protein content in isolates or films was determined by the Kjeldahl method. The SH_f contents were calculated using an extinction coefficient of 13,600 M^{-1} cm^{-1}, and the sulfydryl content was expressed as μmol/g of protein.

The SH_f content in film-forming solutions was determined according to the same method as mentioned above. Briefly, samples of the solutions were diluted 1:10 with reaction buffer consisting of Tris-Glycine buffer with 8 M urea and 0.5% SDS. Aliquots (5 mL) of the diluted samples were mixed with 50 μL of 4 mg/mL DTNB, and the absorbance of the mixtures was determined at 412 nm. In this case, the protein concentration was determined by the Lowry method with BSA as the standard.

Surface Hydrophobicity. The sessile drop method, based on optical contact angle method, was used to estimate surface hydrophobicity of the test films. Contact angle measurements were carried out with an OCA 20 AMP (Dataphiscis Instruments GmbH, Germany). A droplet of deionized water (4 μL) was deposited on the film surface with a precision syringe. The drop image was recorded by a video camera, and the profile of the droplet was numerically solved and fitted to La Place−Young equation. The specimens were preconditioned in an environment chamber containing saturated magnesium nitrate solution for at least 48 h prior to analysis. Ten parallel measurements were performed for each film. The surface in contact with LDPE support during drying will be referred as the "support side" in this study, and the other side in contact with the air during drying will be referred as the "air side".

Film Thickness Determination. Film thickness was measured with a digital micrometer (TAIHAI apparatus Co. Ltd., Shanghai, China) to the nearest 0.001 cm. Measurements were taken along the length of the specimen five times, and the mean values were used to calculate film tensile strength.

Statistics. An analysis of variance (ANOVA) of the data was performed using the SPSS 13.0 statistical analysis system, and a least significant difference (LSD) or Tamhane's with a confidence interval of 95 or 99% was used to compare the means.

RESULTS AND DISCUSSION

MC and TSM. MC and TSM of HPI- and SPI-based films cast with various levels of glycerol are shown in **Table 1**. In both cases, the MC values significantly (at $P < 0.05$) increased with increasing glycerol level from 0.3 to 0.6 g/g of protein. Similar results have been observed in other protein films cast from gelatin (*37*) and wheat gluten (*38*). This is due to the hygroscopicity of glycerol, or partially due to the increase in interspacing of film network induced by glycerol molecule. HPI-based films had similar MC with SPI-based films, except at higher glycerol level (0.6 g/g of protein). At this glycerol level,

Table 1. Moisture Content and Total Soluble Mass for HPI- and SPI-Based Films Cast with Different Glycerol Contents[a]

glycerol content (g/g of protein)	HPI		SPI	
	MC	TSM	MC	TSM
0.3	13.40 ± 0.66^{ce}	22.09 ± 0.38^{ah}	13.56 ± 1.80^{de}	32.07 ± 0.49^{ag}
0.4	20.49 ± 1.85^{be}	21.16 ± 1.15^{ah}	19.43 ± 2.37^{ce}	32.64 ± 3.03^{ag}
0.5	24.76 ± 1.92^{ae}	21.91 ± 0.58^{ah}	25.09 ± 1.85^{be}	31.93 ± 0.67^{ag}
0.6	26.32 ± 0.80^{af}	21.77 ± 0.46^{ah}	32.64 ± 1.40^{ae}	32.65 ± 2.05^{ag}

[a] Values are the means and standard deviations of six measurements. Different superscript characters (a–d) indicate significant ($P < 0.05$) difference within the same column. Different superscript characters (e, f) indicate significant ($P < 0.05$) difference between MC within the same row. Different superscript characters (g, h) indicate significant ($P < 0.05$) difference between TSM within the same row.

the MC of HPI films was significantly ($P < 0.05$) lower than that of SPI films.

Solubility of film in water is an important property of edible films, and water insolubility and water resistance are usually required for potentially commercial film. After 24 h incubation in deionized water, the HPI films were found to still maintain their integrity, while most of SPI films would change in shape. In each film case, TSM was nearly unchanged with the glycerol concentration (**Table 1**). However, at any glycerol level, the TSM of HPI films was significantly ($P < 0.05$) lower than that of SPI films. The difference in TSM may be attributed to the difference of interactive forces maintaining the film network. The insolubility in water of whey protein films has been attributed to the presence of high intermolecular disulfide bonds (*39*, *40*).

Mechanical Properties. The mechanical properties (including TS and EAB) of HPI- and SPI-based films prepared using various levels of glycerol are shown in **Figure 1**. Decreased TS and increased EAB with increasing glycerol concentration are typical behaviors of protein-based cast films (*38*, *41*). As expected, the TS of both films significantly decreased with increasing glycerol level, while the EAB gradually increased. In the HPI case, the increase in glycerol level from 0.3 to 0.6 g/g of protein led to the decrease in TS from 4.39 to 1.66 MPa. At more than 0.4 g glycerol/g of protein, the TS of HPI films was significantly ($P < 0.05$) higher than that of SPI films. As for EAB, the increase in the glycerol concentration in the range of 0.3−0.5 g/g of protein led to gradual and significant increase in the EAB for SPI-based films. However, in the HPI case, the EAB significantly increased only when the glycerol concentration was increased from 0.3 to 0.4 g/g of protein. Meanwhile, the significantly lower EAB for HPI-based films was observed when compared with that of SPI films ($P < 0.05$) (**Figure 1**). The more resistant and less ductile characteristics may be attributed to the strong cohesion of HPI-based films. In the present study, we used an exponential equation, proposed by Ghorpade and others (*42*), to fit the TS and EAB data with the glycerol concentration:

$$\text{TS or EAB} = a \exp(bC_{\text{gly}})$$

where C_{gly} is the concentration of glycerol (g/100 g of protein), and a and b are empirical parameters. The empirical parameters and the related coefficient for TS and EAB of both films are listed in **Table 2**. As shown in this table, the exponential equation can be well applied to describe the relationship between the TS of both HPI and SPI films and the C_{gly}, and the coefficient of correlation was higher than 0.96. As for EAB, only SPI films had a good coefficient of correlation between EAB and the C_{gly} (0.922). Similar results about the relationship between mechanical strength and plasticizer concentration have been reported on soy protein and gelatin films (*42*, *43*).

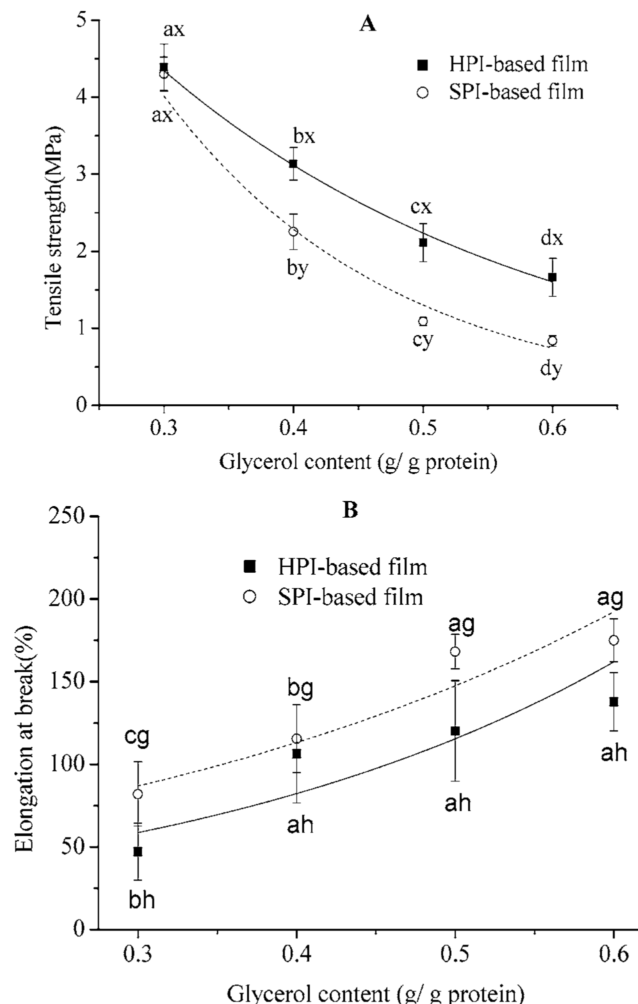

Figure 1. Tensile strength (**A**) and elongation at break (**B**) as a function of glycerol concentration. Each value is the mean and standard deviation of triplicate measurements. Dashes and lines were fitted with exponential equation. Different characters (**a**–**d**) above or below the curve indicate significant ($P < 0.05$) difference due to the plasticizer content. Different characters (**x**, **y**) indicate the significant ($P < 0.05$) difference of TS between two protein films. Different characters (**g**, **h**) indicate the significant ($P < 0.05$) difference of EAB between two protein films.

According to classic polymer science, the plasticizer would weaken the intermolecular force between the chains of adjacent macromolecules, increasing the free volume of the system (*44*). Thus, the increase in plasticizer concentration causes a reduction in tensile strength due to the decrease in intermolecular interactions between protein molecules and an increase in elongation at break due to the increase in the mobility of the molecules. The increase in moisture content of films due to the increase in

Table 2. Empirical Parameters and the Related Coefficient for TS and EAB of Both Films Calculated by Nonlinear Regression

mechanical properties	HPI film			SPI film		
	a	*b*	R^2	*a*	*b*	R^2
TS	11.77	−3.32	0.991	21.76	−5.63	0.969
EAB	21.25	3.34	0.673	39.21	2.65	0.922

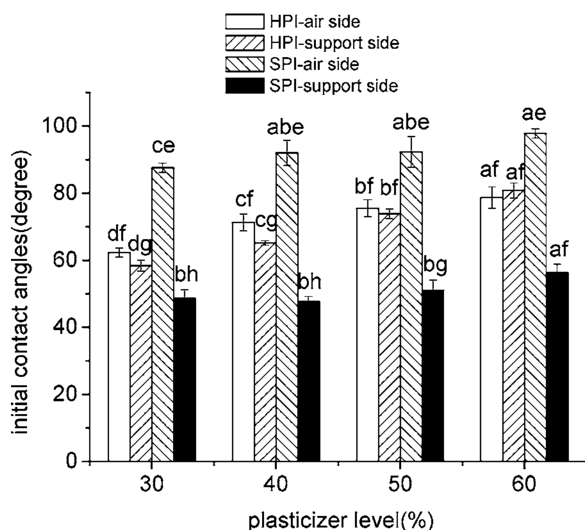

Figure 2. Initial contact angle values at time 0 s for HPI- and SPI-based films. Each value represents the mean and standard deviation of ten measurements. **a**–**d**: Contact angle values with different letters are significantly different within the same film type due to the plasticizer level ($P < 0.05$). **e**–**g**: Contact angle values with different letters are significantly different within the same plasticizer level ($P < 0.05$).

plasticizer concentration may also contribute to the reduction of the force between the adjacent protein macromolecules.

Surface Hydrophobicity. The surface hydrophobicity of film was evaluated using contact angle of water upon film surface by sessile drop method. The information given by contact angle measurements can be exploited in a static manner at time 0 s when the drop is just deposited onto the test surface. In theory, this contact angle may be from 0° up to 180°, that is to say, from complete spreading of liquid onto the solid surface up to the unrealistic limit of absolutely no wetting. Practically, a large contact angle (or small cos θ) represents a hydrophobic surface, whereas a small contact angle (or large cos θ) implies a hydrophilic surface. The quantitative definition of the relative terms "hydrophobic" and "hydrophilic" surfaces has been done respectively for surfaces exhibiting a water contact angle $\theta >$ 65° and $\theta <$ 65° (*45*).

The initial contact angle values with water for air and support sides of HPI-based films were similar, which were positively dependent upon the glycerol concentration (**Figure 2**). At a glycerol concentration higher than 0.4 g/g of protein, most of the contact angle values were above 65°. Thus, HPI films can be considered to have hydrophobic surfaces. A diversity of the contact angle results have been reported for various kinds of proteins, e.g., pea protein films in the range of 14−40°, sunflower protein films in the range of 12−30°, gliadin- and gelatin-based films above 80° (*12*, *46*, *47*). Interestingly, SPI films showed different surface hydrophobicity patterns. In this film, the contact angle values for air side, nearly independent of the glycerol concentration, were much higher than that of HPI films. However, the contact angle values for the support side were much lower (relative to HPI films). The data suggested that the surface hydrophobicity of SPI films is dependent upon the choice of tested surfaces (air or support sides).

An unexpected effect of glycerol, a plasticizer with hydrophilic nature, on the initial contact angle of water was observed, that is to say, plasticization resulted in increase in surface hydrophobicity of the HPI-based films (**Figure 2**). The underlying mechanism for this phenomenon is unknown yet. However, it may be associated with the reorientation of hydrophobic moieties of the side chain in polymer molecules. The increase in the hydrophilic plasticizer concentration may strengthen the interactions between protein polymer and plasticizer molecules by hydrogen bonds. As a result, the hydrophobic moieties of the proteins may relatively preferably orient at the air−film or support matrix−film interfaces. A preferred orientation of hydrophobic moieties at the film− or hydrogel−air interface was observed in the case of gelatin-based films or hydrogel (*47*).

The effect of the side choice of film surface in the SPI case was very outstanding. Unexpectedly, the contact angle values for the air side were over 85°. A similar phenomenon has been observed in gelatin films (*47*, *48*), where this is attributed to the orientation of functional groups and special hydration of the film. In our previous paper, we reported that the contact angle values of native SPI film (air side), cast with 0.4 g of glycerol/g of protein, were 59.3 ± 2.5° (*49*). The differences in contact angle values may be attributed to the differences of nature of protein present in film-forming solutions, and the influence of heat pretreatment (70 °C, 20 min for Tang et al. (*49*), and 90 °C, 30 min in the present study).

Protein Solubility of Film. To reveal the interactive forces involved in the formation and maintenance of three-dimensional film network, the protein solubility of HPI and SPI films in various kinds of solvents was analyzed. In this case, the films cast with 0.5 g of glycerol/g of protein, and four kinds of solvents (DW, deionized water; B, Tris-buffer; BSU, B additionally containing 8 M urea and 0.5% SDS; BSUM, BSU plus 2-ME) were used. Usually, solvent B disrupts the electrostatic interactions, solvent BSU disrupts the hydrogen bonds and hydrophobic interactions, and BSUM can disrupt hydrogen bonds, hydrophobic interactions, and disulfide bonds (*31*, *32*, *50*).

There was no significant difference of protein solubility for HPI films between solvents DW and B (**Figure 3**), suggesting that the electrostatic interactions are nonsignificant for the film network formation. The protein solubility in B was significantly increased by the addition of 8.0 M urea and 0.5% SDS, or plus 1% (v/v) 2-ME. Especially in the BSUM, the solubility of HPI films was above 70%, much higher than that in BSU (about 18%). These results suggest that the disulfide bonds (especially those newly formed during film formation) are the predominant interactive forces involved in the film network formation and maintenance, while hydrogen bonds and hydrophobic interactions should be to a lesser extent involved. Additionally, there was still about 30% protein of films which could not be disrupted by 8.0 M urea plus 0.5% SDS and 1% 2-ME, suggesting the presence of insoluble macromolecules (or protein aggregates), or possible formation of covalent bonds other than disulfide bonds in the film formation.

In the case of SPI films, the solubility in B was on the contrary significantly ($P < 0.05$) lower than that in DW (**Figure 3**). This may be due to the effect of salting-out. Similar phenomena have been reported in SPI gels or films (*51*). The solubility in BSU (about 70%) was much significantly higher than that in B (∼15%), and the proteins of films were almost completely solubilized in the BSUM. The results suggest that

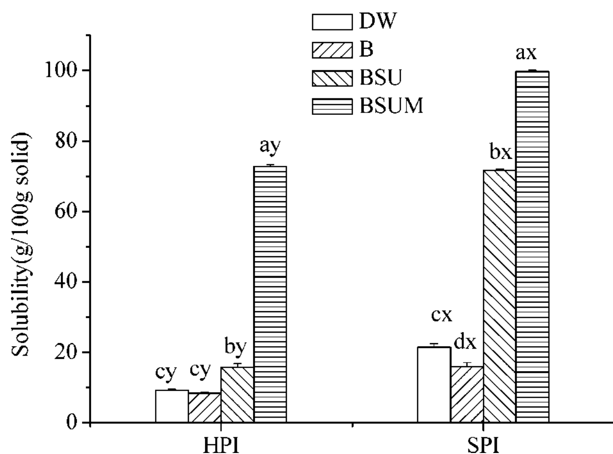

Figure 3. Protein solubility patterns of HPI and SPI films in various solvents. The protein film was plasticized by 0.5 g of glycerol/g of protein. DW: deionized water (pH 8.0). B: Tris-Glycine buffer (0.086 M Tris, 0.09 M glycine, and 4 mM Na_2EDTA, pH 8.0). BSU: solvent B with 0.5% SDS and 8 M urea. BSUM: solvent BSU plus 1% (v/v) 2-ME. Each value represents the mean and standard deviation. Different characters (**a**–**d**) on the top of the column indicate significant difference ($P < 0.05$) due to the denaturing buffer used. Different characters (**x**, **y**) on the top of the column indicate significant difference ($P < 0.05$) due to the protein type.

Table 3. Free Sulfydryl Group Content in Isolates, as Well as Films, pH-Adjusted, or Heated Film-Forming Solutions of HPI and SPI[a]

protein samples	total free sulfydryl (μmol/g of protein)
HPI	
isolates	7.45 ± 0.31 bx
pH-adjusted solutions	8.95 ± 0.15 ax
heated solutions	2.41 ± 0.19 cx
films	1.95 ± 0.02 dx
SPI	
isolates	4.14 ± 0.19 by
pH-adjusted solutions	5.41 ± 0.13 ay
heated solutions	2.10 ± 0.09 cy
films	1.52 ± 0.12 dy

[a] The protein film was plasticized by 0.5 g of glycerol/g of protein. All data are expressed as the means and standard deviations of triplicate measurements. Different superscript characters (a–d) indicate significant ($P < 0.05$) difference within the same protein type. Different superscript characters (x, y) indicate significant ($P < 0.05$) difference due to protein type.

the interactive force patterns are different among SPI and HPI films. In the SPI films, hydrogen and hydrophobic bondings as well disulfide bonds are to a similar extent involved in the film network formation. However, Rhim and others (*52*) observed that soy protein films were mainly stabilized by disulfide bonds, and to a minor extent, by hydrophobic interactions and hydrogen bonds. The differences may be attributed to the differences of pH of the film-forming solutions. In this work, pH 10 was used, while in the present case, pH 9.0 was used. Different from the case of HPI films, in this case, the formation of insoluble aggregates nearly did not occur.

Free Sulfydryl Content. To further confirm the significance of newly formed disulfide bonds for the film network maintenance of HPI, we analyzed the changes of free sulfydryl groups (SH_f) of protein isolates before and after film formation. HPI and SPI had total SH_f contents of 7.45 and 4.14 μmol/g of protein, respectively (**Table 3**). This data is consistent with amino acid composition of these proteins reported by Tang et al. (*20*) and Callaway (*19*). In the film-forming solutions (at pH 9.0), significantly higher SH_f contents were observed for both HPI snd SPI (**Table 3**). Beveridge and Arntfield (*53*) attributed this increment in SH_f to the extensively alkaline hydrolysis of disulfide bonds. Factually, the unfolding of protein structure at alkaline conditions may also in part account for the increase in SH_f content, since the SH_f initially buried in the interior of protein structure would be exposed.

The heat pretreatment (90 °C, 30 min) resulted in significant decrease in SH_f from 8.95 to 2.41 μmol/g of protein for HPI film-forming solutions (**Table 3**). A similar decrease was observed in the SPI case, but the extent of the decrease was relatively lower (from 5.41 to 2.1 μmol/g of protein). This pretreatment may cause the protein molecules to unfold, and as a result, many SH groups previously buried in the interior of protein molecules would be exposed (*53*−*56*). These exposed sulfhydryl groups are rather reactive at alkaline pH and therefore can be expected to be oxidized to form new intermolecular disulfide bonds. Similar results have been obtained in the egg white proteins (*53*, *56*). In this case, the decrease in SH_f content was attributed to the oxidation by oxygen contained in the egg white solutions. Furthermore, the film-forming process (air-drying) resulted in further decrease in the SH_f (**Table 3**).

In conclusion, HPI-based cast films had some superior characteristics, e.g., much lower total soluble mass, relatively higher surface hydrophobicity (support matrix side), as compared to SPI-based films. The moisture content, mechanical, and surface hydrophobic properties of the HPI films were dependent upon the used plasticizer level. Disulfide bonds played a predominant role in the formation and maintenance of the HPI film network, while in the SPI case, besides the disulfide bonds, both hydrophobic interactions and hydrogen bonds were also involved.

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