Solubility and Molecular Properties of Heat-Cured Soy Protein Films†

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Changes in solubility and molecular weight distribution of protein in heat-cured soy protein film were investigated to understand the mechanism of protein−protein interactions that occur during heat treatment. Soy protein films were heated at 65, 80, or 95 °C for 6, 18, or 24 h. The solubility of the proteins decreased with increasing temperature of heat treatment in various buffers. Buffers containing urea, a hydrogen bond-disrupting agent, and 2-mercaptoethanol, a disulfide bond-disrupting agent, dissolved more than 95% of protein in all the heat-treated samples. SDS−PAGE patterns indicated aggregation of proteins during film formation and in heat-treated films. The combined effects of urea and 2-mercaptoethanol suggested that proteins were aggregated primarily through hydrogen bonds and intermolecular disulfide bonds. This aggregation increased molecular weight and decreased film solubility.

Keywords: *Protein film; heat curing; solubility; disulfide bonds*

INTRODUCTION

Biopolymer films and coatings hold promise for innovative uses in food protection and preservation. They can prevent quality changes in food by acting as selective barriers to control moisture transfer, oxygen uptake, loss of flavors, and lipid oxidation. Such films have found applications in confectionery (Andres, 1984), fresh produce (Kaplan, 1986), meat (Hood, 1987), and pharmaceutical industries (Viro, 1980). Several biopolymers, including polysaccharides, proteins, or a combination of these materials have been used to prepare edible films and coatings (Kester and Fennema, 1986; Guilbert, 1986; Krochta, 1992; Gennadios et al., 1994). Edible packaging also could lead to further utilization of agricultural commodities employed as sources of film-forming materials.

Films and coatings based on proteins such as wheat gluten, soy protein, corn zein, milk proteins, peanut proteins, and collagen have been reviewed by Gennadios et al. (1994). A major disadvantage of protein films is their high water vapor permeability, which generally is undesirable in edible coatings and packaging materials. A number of recent studies have concentrated on the development and property evaluation of protein films, soy films in particular. Various methods employed to improve properties of soy protein films include treatment with alkali (Bradenburg et al., 1993), alkylation with sodium alginate (Shih, 1994), enzymatic treatment with horseradish peroxidase (Stuchell and Krochta, 1994), acylation with acetic and succinic anhydride (Ghorpade et al., 1995) and treatment with formaldehyde (Ghorpade et al., 1995). Gennadios et al. (1993) studied the effect of pH of soy protein isolate (SPI) film-forming solutions on the physical properties of soy films and reported that soy protein films prepared at pH 6−11 had higher tensile strength, higher percentage elongation at break, and lower water vapor permeability than films at pH 1−3. Heating film-forming protein solutions or prepared films also had a noticeable effect on film properties (Ali et al., 1996). Films prepared from heated SPI film-forming solutions had lower water vapor permeability than those prepared from unheated film-forming solutions (Stuchell and Krochta, 1994). Heating soy protein films at 80 or 95 °C for various period of time resulted in films with increased tensile strength and *b* Hunter color values and reduced percentage elongation at break, moisture content, and water vapor permeability values (Gennadios et al., 1996).

There are various reports on improvements in physical properties of soy protein films as a result of heat curing. However, information on protein−protein interactions in such heat-cured films is limited. Therefore, this investigation was undertaken to study the solubility, disulfide bond content, and molecular weight distribution of soy protein isolate, soy protein films, and heat-cured soy protein films.

MATERIALS AND METHODS

Film Preparation. Film-forming solutions were prepared by mixing 100 mL of distilled water, 5 g of soy protein isolate (Arpro 1100, 93.5% db protein content, obtained gratis from Archer Daniels Midland Co., Decatur, IL), and 3 g of glycerin (Fischer Scientific, Pittsburgh, PA). Sodium hydroxide (1 N) was used to adjust the pH of film-forming solutions to 11.0 ± 0.1. The solutions were held for 30 min in a 70 °C constant-temperature water bath, strained through grade 40 cheese cloth (Fischer Scientific, Pittsburgh, PA), and cast on level Teflon-coated glass plates. Films were peeled from plates after drying at ambient temperature for ∼30 h.

Heat Curing of Films. Dried films were mounted on glass plates by applying masking tape around the film edges and heated at 65, 80, or 95 °C for 6 , 18, or 24 h in an air-circulating oven. Masking tape was used to hold the film flat and to prevent curling and breaking of the film during heating. Following heat treatment, the films were ground, sifted (30 mesh), and stored at 4 °C.

Determination of Protein Solubility. Protein solubility in water at different pH's was determined according to the procedure of Coffmann and Garcia (1977). A 250 mg sample

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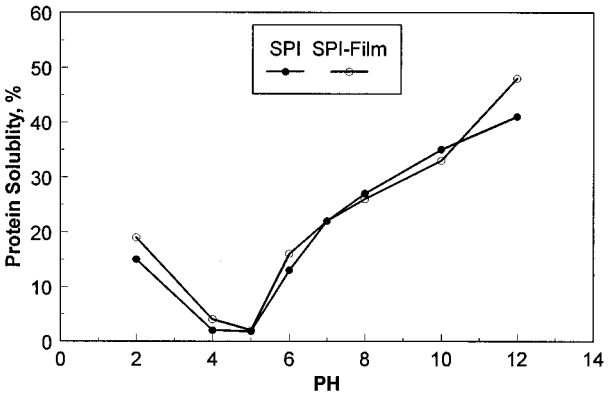

Figure 1. Solubility profile of SPI and SPI film in different pH buffers. Values are average of duplicate measurements.

was suspended in 3.5 mL of water. The pH of each sample was adjusted ranging from pH 2 to 12 using either HCl (1 N) or NaOH (1 N) and the volume made up to 5 mL with distilled water. Samples were incubated in a water bath at room temperature (25 °C) for 24 h with continuous agitation. The pH of each sample was checked at 9 and 24 h to ensure that there was no appreciable change during the course of study. The suspension was centrifuged at 9000*g* for 20 min and the protein content in the supernatant determined using a bicinchoninic acid (BCA) protein assay (Smith et al., 1985). Soluble protein content values were obtained in duplicate and expressed as percentage of initial protein concentration.

The solubility of samples in different buffer systems was based on the method described by Hager (1984). Again, 250 mg of sample was dispersed in 5 mL of buffer and shaken in a water bath at room temperature for 30 min. The suspensions were centrifuged at 9000*g* for 20 min, and the protein content in the supernatant was determined. The buffer systems used were as follows: (1) buffer BI, 2.6 mM KH_2PO_4 and 32.5 mM K_2HPO_4 (pH 7.6), (2) buffer BII 18.2 mM $NaHCO_3$ and 31.8 mM Na_2CO_3 (pH 10.6), (3) buffer BI containing 8 M urea, (4) buffer BII containing 8 M urea, (5) buffer BI containing 0.1 M 2-mercaptoethanol (2-ME), (6) buffer BII containing 0.1 M 2-ME, (7) buffer BI containing 8 M urea and 0.1 M 2-ME, and (8) buffer BII containing 8 M urea and 0.1 M 2-ME.

Determination of Sulfhydryl Groups and Disulfide Bonds. Assays of sulfhydryl groups and disulfide bonds were carried out according to the method of Thannhauser et al. (1987). The procedure was modified as described by Chan and Wasserman (1993). The principle of the method is to suspend the sample in the buffer containing urea and then react it with a color reagent that simultaneously reacts with both soluble and insoluble protein with the release of a soluble chromophore. Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$) were used for the determination of sulfydryal groups and disulfide bonds, respectively. Reaction with either color reagent results in release of the soluble 2-nitro-5-thiobenzoate anion (NTB^{2-}), which has an extension coefficient of 13 600 M^{-1} cm^{-1} at 412 nm.

For assaying sulfhydryl content, a 30 mg sample was suspended in 1 mL of reaction buffer consisting of 8 M urea, 10 mM DTNB, 3 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), and 0.2 M Tris-HCl, pH 8.0. The reaction mixture was allowed to stand for 15 min and was then centrifuged at 13800*g* to remove particulate matter; its absorbance was measured at 412 nm. The concentration of sulfhydryl groups was calculated by using of the extinction coefficient of NTB (13 600 M^{-1} cm^{-1}) at 412 nm.

For assaying total sulfhydryl group content, a 30 mg of sample was suspended in 1 mL of reaction buffer consisting of 8 M urea, 0.1 M sodium sulfite, 3 mM EDTA, 1% SDS, 0.2 M Tris-HCl, pH 9.5, and 10 mM $NTSB^{2-}$ synthesized from DTNB in the presence of sodium sulfite and O_2. The reaction mixture was incubated in the dark for 25 min at room temperature and centrifuged to remove particulate matter; the absorbance was read at 412 nm. Disulfide bond content was calculated as half the difference between thiol group content before and after reduction of disulfide bonds with sulfite.

Table 1. Protein Solubility (Percent of Total Protein Content) of Soy Protein Isolate (SPI) and SPI Film in Different Buffers[a]

buffer[b]	SPI	SPI film
1. BI	15.5 ± 6.3a	20.5 ± 2.1a
2. BII	22.0 ± 1.4a	21.5 ± 3.5a
3. BI + 8 M urea	45.5 ± 6.3b	47.5 ± 3.5b
4. BII + 8 M urea	50.0 ± 2.8b	44.5 ± 2.1b
5. BII + 0.1 M ME	87.0 ± 2.8c	79.0 ± 1.4c
6. BII + 0.1 M ME	79.0 ± 1.4c	82.5 ± 2.1c
7. BII + 8 M urea + 0.1 M ME	96.5 ± 4.9d	93.0 ± 4.2d
8. BII + 8 M urea + 0.1 M ME	97.0 ± 1.4d	98.0 ± 2.8d

[a] Solubility values are means of two replicates ± standard deviation. Any two solubility means followed by the same lower case letter are not significantly ($p > 0.05$) different according to Duncan's multiple range test. [b] BI buffer, 2.6 mM KH_2PO_4 and 32.5 mM K_2HPO_4, (pH 7.6); BII buffer, 18.2 mM $NaHCO_3$ and 31.8 mM Na_2CO_3, (pH 10.6); ME, 2-mercaptoethanol.

SDS−PAGE. Soy protein isolate and soy protein film extracts were prepared by suspending 50 mg of sample in 1 mL of different solvent buffer systems, BI, BI + 8 M urea, BI + 0.1 M 2-ME, or BI + 8 M urea + 0.1 M 2-ME. The suspensions were incubated for 30 min at 25 °C and centrifuged at 13800*g* for 10 min. To 0.5 mL of supernatant, 1 mL of ice-cold acetone was added, the resultant mixture allowed to stand for 15 min and centrifuged at 13800*g* for 10 min. To the precipitate was added 0.5 mL of sample buffer with pH 6.8 containing 0.063 M Tris-HCl, 2% (w/v) SDS, 5% (v/v) 2-ME, 10% (v/v) glycerol, and 0.025% (w/v) bromophenol blue. The suspension was heated for 5 min in a boiling water bath and allowed to cool; an aliquot of the top clear layer was diluted 1 in 5 times with sample buffer. The amount loaded into the gel was 6 μL for samples containing urea and 18 μL for samples without urea.

Extracts of heat-cured soy protein films were prepared by suspending 50 mg of sample in 1 mL of BI. The suspensions were incubated at room temperature (25 °C) for ∼9 h with occasional shaking and centrifuged at 13800*g* for 10 min. To 0.5 mL of the supernatant, 1 mL of ice-cold acetone was added; the mixture was allowed to stand for 15 min and then centrifuged at 13800*g* for 10 min. To the precipitate, 0.3 mL of sample buffer was added. The suspension was heated in a boiling water bath for 5 min and allowed to cool. An aliquot of the top clear layer was diluted 1 in 10 times with sample buffer and 10 μL of this was loaded into the gel.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of samples was performed on a 12% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) using a Mini-Protean II cell (Bio-Rad Laboratories). The gels were run at 150 V constant voltage for ∼60 min and then stained with 0.1% Coomassie Brilliant Blue G-250 for 2 h. Destaining was done with a solution of 40% methanol and 10% acetic acid.

RESULTS AND DISCUSSION

Soy Protein Solubility. The protein solubility profiles of soy protein isolate and soy protein films at various pH's are shown in Figure 1. A decrease in protein solubility with decreasing pH was observed, with the minimum solubility being between pH 4 and 5 and subsequent resolubilization of proteins at pH's lower than pH 4. A higher protein solubility (greater than 30%) was observed at pH values greater than 8 as compared to the acidic pH values at which the protein solubilities were 15%. The solubilities of proteins are known to vary considerably with pH. This is because above and below the isoelectric point proteins have either a positive or a negative charge, which enhances solubility. At the isoelectric point, pH 4.5 in the case

Table 2. Effect of Heat-Curing Temperature and Time on Solubility (Percent of Total Protein Content) of Soy Protein Films in Different Buffer Systems[a]

	65 °C			80 °C			95 °C		
buffer[b]	6 h	18 h	24 h	6 h	18 h	24 h	6 h	18 h	24 h
1. BI	40.0 ± 2.8a	41.0 ± 0.0a	41.5 ± 0.7a	43.5 ± 0.7a	28.5 ± 6.3a	21.0 ± 2.8a	21.0 ± 0.0a	20.5 ± 0.7a	16.5 ± 0.7a
2. BII	49.0 ± 4.2a	46.0 ± 1.4a	43.5 ± 0.7a	56.0 ± 4.2b	26.5 ± 2.1a	25.0 ± 2.8b	29.0 ± 2.8b	27.5 ± 2.1b	21.0 ± 1.4b
3. BI + 8 M urea	46.0 ± 1.4a	55.5 ± 2.1a	53.5 ± 6.3b	54.0 ± 8.4b	58.5 ± 4.9b	49.5 ± 0.7c	48.5 ± 0.7c	46.5 ± 0.7c	43.0 ± 1.4c
4. BII + 8 M urea	60.5 ± 3.5b	50.0 ± 4.2a	54.0 ± 4.2b	56.5 ± 0.7b	51.0 ± 4.2b	51.0 ± 2.8c	50.5 ± 2.1c	49.0 ± 1.4c	48.5 ± 3.5c
5. BII + 0.1 M ME	90.0 ± 5.6c	94.5 ± 7.7b	100.0 ± 0.0c	100.0 ± 0.0c	81.5 ± 0.7c	83.0 ± 2.8d	67.5 ± 4.9d	80.0 ± 1.4d	73.0 ± 11.3d
6. BII + 0.1 M ME	96.5 ± 3.5c	100.0 ± 0.0b	100.0 ± 0.0c	100.0 ± 0.0c	88.5 ± 9.1c	80.5 ± 3.5d	73.5 ± 2.1d	71.0 ± 5.6d	63.0 ± 1.4d
7. BII + 8 M urea + 0.1 M ME	100.0 ± 0.0d	100.0 ± 0.0b	100.0 ± 0.0c	100.0 ± 0.0c	100.0 ± 0.0d	100.0 ± 0.0e	100.0 ± 0.0e	87.0 ± 1.4d	94.5 ± 7.7e
8. BII + 8 M urea + 0.1 M ME	100.0 ± 0.0d	100.0 ± 0.0b	100.0 ± 0.0c	100.0 ± 0.0c	100.0 ± 0.0d	100.0 ± 0.0e	100.0 ± 0.0e	97.0 ± 4.2e	93.9 ± 8.9e

[a] Solubility values are means of two replicates ± standard deviation. Any two solubility means followed by the same lowercase letter are not significantly ($p > 0.05$) different according to Duncan's multiple range test. [b] BI buffer, 2.6 mM KH_2PO_4 and 32.5 mM K_2HPO_4, (pH 7.6); BII buffer, 18.2 mM $NaHCO_3$ and 31.8 mM Na_2CO_3, (pH 10.6); Me 2-mercaptoethanol.

Table 3. Effect of Heat-Curing Temperature and Time on Sulfhydryl Group and Disulfide Bond Contents of Soy Protein Films[a]

	SH (μmol/g of sample)			S−S (μmol/g of sample)		
temp (°C)	6 h	18 h	24 h	6 h	18 h	24 h
65	1.51 ± 0.12a	1.56 ± 0.03a	1.53 ± 0.03a	5.53 ± 0.80a	4.83 ± 0.75a	4.63 ± 0.98a
80	1.18 ± 0.06b	1.13 ± 0.08b	1.16 ± 0.03b	5.83 ± 0.70b	6.83 ± 1.09b	8.53 ± 0.73b
95	1.03 ± 0.05c	0.99 ± 0.03c	1.02 ± 0.03c	5.13 ± 0.25c	6.90 ± 0.47b	5.56 ± 0.49c

[a] SH and S−S values are means of two replicates ± standard deviation. Any two solubility means followed by the same lowercase letter are not significantly ($p > 0.05$) different according to Duncan's multiple range test.

of SPI (Gennadios et al., 1993), the net charge is zero, resulting in the association of molecules, thus reducing solubility.

Buffer systems with specific chemical action on proteins (simple buffers, buffers containing 8 M urea, buffer containing 0.1 M 2-ME, and buffer containing both urea and 2-ME) were used to investigate the types of aggregation forces in soy films, soy protein isolate, and heat-cured soy films. Simple buffers can dissolve proteins in their native states; urea can disrupt hydrogen bonds and dissolve small aggregates held together by such bonds. 2-Mercaptoethanol can cleave disulfide bonds to sulfhydryl groups and facilitate the solubilization of large protein aggregates held together by disulfide bridges.

As shown in Table 1, ∼20% of the proteins in soy isolate and film were soluble in simple buffers, phosphate buffer (pH 7.6) and carbonate buffer (pH 10.6). With the addition of urea, the solubility increased to 46−50%. The presence of 2-ME in the buffer dissolved about 80−85% of the protein, and the buffer containing both urea and 2-ME dissolved more than 95% of the total protein in the soy isolate and soy films. An increase in protein solubility of ∼20% with the addition of urea and ∼60% with the addition of 2-ME indicated that the forces leading to insolubilization of protein in simple buffers were probably a combination of hydrogen bonds and disulfide bonds.

The soy protein films were heated at 65, 80, or 95 °C for 6, 18, or 24 h in an air-circulating oven. The solubility of heat-cured film in different systems was determined (Table 2). Protein solubilities of soy films in simple buffer decreased with increasing temperature of heat treatment. The solubilities of films heated at 65 °C for 6, 18, and 24 h and 80 °C for 6 h were in the range of 42−50% of the total protein. However, prolonged heat treatment at 80 °C decreased solubility, less than 25% being soluble when films were heated for 24 h. Films heated at 95 °C also showed decreasing solubility with heating time (Table 2).

It was observed that unheated soy protein isolate and soy film had lower solubilities in buffers than soy film heated at 65 °C (Tables 1 and 2). Heating soy films at 65 °C probably caused dissociation of the quaternary structure, releasing smaller peptides and facilitating their solubilization. Heat treatment has been reported to disrupt the quaternary structure of 7S (conglycinin) and 11S (glycinin) (Kinsella, 1979), which are the major components of soy protein. Lower solubility of unheated soy films as compared to heat-treated films incubated with potassium sorbate was reported by Stuchell and Krochta (1994). Prolonged heating at 80 °C, or heating at high temperature (95 °C), however, resulted in a decrease in the solubility, probably due to the formation of large aggregates. Above 90 °C the 11S component of soy protein is known to form insoluble aggregates (Wolf, 1970; German et al., 1982).

In buffer with urea, the solubilities of films heated at 65 and 80 °C were between 50 and 60% and at 95 °C were between 45 and 50%. The addition of 2-ME dissolved more than 90% of total protein in films heated at 65 °C and about 80 and 65−75% in films treated at 80 and 95 °C, respectively. The presence of both urea and 2-ME in buffer solubilized more than 95% of the total protein in almost all heat-treated films. The decrease in solubility of heat-treated films could have been due to formation of disulfide bonds and hydrogen bonds as an increase in solubility was observed with the addition of 2- ME and urea. Wolf (1970) reported that when soy protein solutions were heated, a sulfhydryl−disulfide interchange occurred which may have resulted in intermolecular cross-linkage and gelation. The formation of new disulfide bonds in heat-induced gels of soy protein isolate also was reported by Shimada and Cheftel (1988). Disulfide bonds and hydrophobic inter-

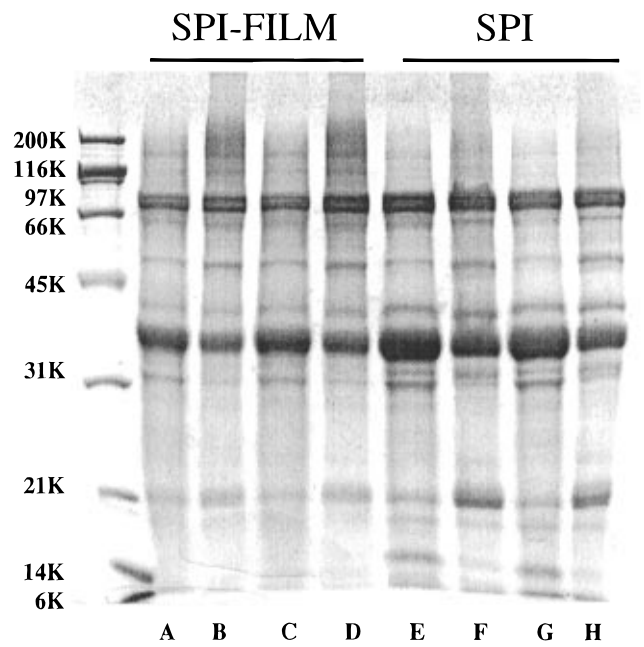

Figure 2. SDS−PAGE analyses for soy protein film samples (lanes, A−D) and native soy protein isolate (lanes, E−H): Lanes A and E contain proteins extracted in buffer BI (2.6 mM KH_2PO_4 and 32.5 mM K_2HPO_4 (pH 7.6)); lanes B and F contain protein extracted in BI + 8 M urea; lanes C and G contain protein extracted in BI + 0.1 M 2-mercaptoethanol (2-ME); and lanes D and H contain protein extracted in BI + 8 M in urea and 0.1 M in 2-ME.

actions have been shown to be responsible for decreased protein solubility during extrusion of soy (Prudencio-Ferreira and Areas, 1993), cornmeal (Camire, 1991), and wheat flour (Li and Lee, 1996).

Determination of Sulfhydryl Group and Disulfide Bonds. The disulfide bond contents and sulfhydral groups in soy protein isolate and soy protein film were determined. The SH and S−S bond contents for soy protein isolate were 2.56 ± 0.04 and 29.6 ± 0.95 μmol/g, respectively. These values were comparable to those reported in the literature (Petrucelli and Anon, 1995). The SH and S−S bond contents for SPI films were 1.79 ± 0.02 and 14.2 ± 1.25 μmol/g, respectively. The SH and S−S contents of heat cured soy films also were determined and the results are shown in Table 3. The data obtained in our experiments did not show a conclusive picture of the changes in SH and S−S bond as result of heat treatment. However, decreases in S−S bond contents was reported for extruded samples of soy (Hager, 1984) and wheat flour (Li and Lee, 1996).

SDS−PAGE. SDS−PAGE patterns of soy isolate and soy film proteins were obtained to examine the molecular weight distrubution of the proteins in different buffer systems: namely, phosphate buffer (pH 7.6), buffer + 8 M urea, buffer + 0.1 M 2-ME, and buffer + 8 M urea + 0.1 M 2-ME (Figure 2). SDS−PAGE of soy protein isolate extract revealed bands in the lower molecular weight region (<20 000) that were absent in the soy film extract, suggesting the possibility of protein aggregation during film formation. Two distinct bands corresponding to ~31K were observed in extracts from soy protein isolate whereas in the film extract, only one clear band was observed. Soy protein isolate, and film samples treated with 2-ME showed a distinct band corresponding to ~64K; whereas the corresponding region in soy film extracts without 2-ME, showed only a faint band, suggesting S−S bond formation during film formation.

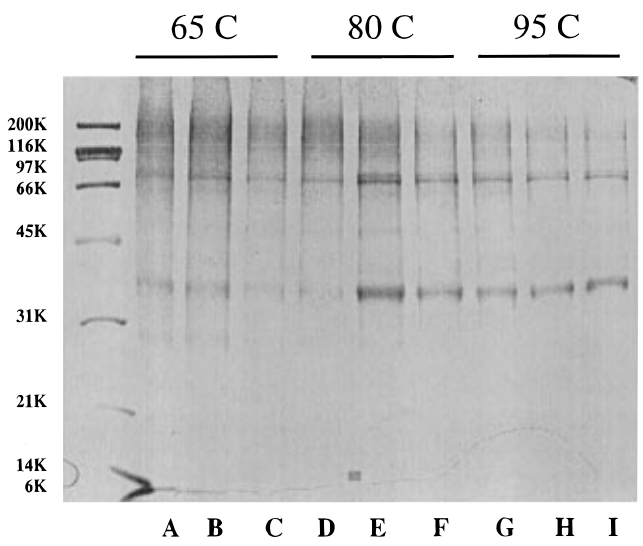

Figure 3. SDS−PAGE analyses of heat treated soy proteins films. Lanes: (A) 65 °C for 6 h, (B) 65 °C for 18 h, (C) 65 °C for 24 h, (D) 80 °C for 6 h, (E) 80 °C for 18 h, (F) 80 °C for 24 h, (G) 95 °C for 6 h, (H) 95 °C for 18 h, and (I) 95 °C for 24 h.

Molecular weight distrubution patterns in heat-cured soy samples are shown in Figure 3. Extracts of films heat cured at 65 and 80 °C for 6 h revealed a band in the low molecular weight region (<25K), which was either absent or appeared as a low-intensity band in samples heated at 95 °C. Samples heat cured at higher temperatures (80 and 95 °C for 24 h), however, revealed a band at ~45K, which was absent in the samples treated at 65 °C. Similarly, the intensity of the band corresponding to ~35K was more pronounced in the sample treated at 80 and 95 °C than that treated at 65 °C. These results indicated that, in samples heat cured at 80 and 95 °C, there were aggregations of protein molecules leading to decreased solubility.

In conclusion, both hydrogen bond and disulfide bond formations played an important role in aggregation of proteins during heat treatment. This aggregations increased molecular weight and decreased solubility.

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