Effect of Drying Methods on Molecular Properties and Functionalities of Disulfide Bond-Cleaved Soy Proteins

U. Kalapathy[a], N.S. Hettiarachchy[a,*], and K.C. Rhee[b]

[a]Department of Food Science, University of Arkansas, Fayetteville, Arkansas 72704, and [b]Food Protein Research and Development Center, Texas A&M University, College Station, Texas 77843

ABSTRACT: Effects of drying methods on hydrophobicity, solubility, water hydration capacity, viscosity, and adhesive strength of soy protein isolates treated with Na_2SO_3 (disulfide bond-cleaving agent) were investigated. Treatment with 0.1 M Na_2SO_3 resulted in 28% decrease in disulfide linkages in soy proteins. While hydrophobicity and solubility increased, water-holding capacity of soy proteins decreased due to the treatment. Spray-dried product had higher hydrophobicity, solubility, water hydration capacity, and viscosity compared to freeze-dried product. Adhesive strength on wood increased due to modification; however, the drying process had no significant effect on this property. Viscosities of spray-dried product, freeze-dried product, and unmodified soy proteins were 2,200, 100, and 240 cP, respectively. Fluorescence spectra of spray-dried and freeze-dried products indicated a partial folding of molecules around tryptophan. High-performance liquid chromatographic elution profiles showed no significant differences in molecular sizes of unfolded molecules of spray-dried and freeze-dried proteins.
JAOCS 74, 195–199 (1997).

KEY WORDS: Disulfide cleavage, freeze drying, soy proteins, spray-drying.

Soy proteins have long been used as ingredients in wood adhesives (1,2). However, petrochemical products have replaced the use of soy proteins in wood adhesives because these are comparatively inexpensive and have superior qualities. High adhesive strength, water resistance, and low viscosity are the basic requirements for a wood glue. Improved adhesive strength and water resistance have been observed for adhesives prepared from alkali-modified soy proteins (3,4). However, alkali-modified soy proteins had higher viscosity (>30,000 cP at 14% solids concentration). Cleavage of disulfide (SS) bonds with sulfites, followed by alkaline treatment, has been used to produce adhesives with low viscosity from soy proteins (4). However, when freeze-drying was replaced with spray-drying, the viscosity of the product increased from 110 to an undesirably high level of over 5000 cP. Hence, elimination of alkali treatment may minimize excess unfolding and lead to lower viscosity. A lower viscosity is desired to allow easy handling and sufficient penetration of glue through wood surfaces.

Sulfites (5,6) and thiols (7,8) have been widely used to cleave inter- and intradisulfide bonds in proteins. The presence of SS bonds in native protein molecules affects their flexibility and unfolding properties. Hence, drying methods that involve severe heat treatments may have more pronounced effects on the physicochemical properties of SS-cleaved soy proteins compared to native proteins.

The objective of this work was to investigate the effect of drying methods on adhesion and viscosity of SS-cleaved soy proteins.

EXPERIMENTAL PROCEDURES

Materials. Soy protein isolate (SPI) (ARDEX D), a general-purpose food-grade protein) was obtained from Archer Daniels Midland Co. (Decatur, IL). All chemicals were from Sigma Chemical Company (St. Louis, MO). Soft maple wood blocks (5 × 2 × 0.3 cm) were purchased from White River Hardwoods (Woodworks, Inc., Fayetteville, AR).

SS-cleaved soy proteins. SS-cleaved soy proteins were prepared by the method of Kalapathy *et al*. (4). Ten-gram amounts of SPI were dispersed in 140 mL of deionized water and stirred for 10 min to obtain uniform dispersions. Dispersions were then adjusted to 0.1 M Na_2SO_3 and a pH of 8.0 with 1 N NaOH, followed by incubation and shaking at 180 rpm for 1 h at 50°C. The product was divided into two portions, one portion was frozen at −5°C and freeze-dried, and the other portion was spray-dried with inlet and outlet temperatures of 210 and 82°C, respectively. The spray-dried and freeze-dried products were stored at ambient temperature (23°C) until further analysis.

Adhesive strength. The procedures used for gluing wood pieces and determining adhesive strengths were described by Kalapathy *et al.* (9). One-hundred milligrams of 8.0% (w/w, pH adjusted to 6.8 with 1 N HCl) protein solution were placed on opposite ends of a wood piece (5 × 2 × .3 cm) and spread on an area of 2 × 2 cm to give a protein concentration of 2.0 mg/cm^2. Two other wood pieces of similar size were superimposed on these glued areas and pressed with a load of 5 kg for 2 h. A total of five blocks per treatment were prepared.

*To whom correspondence should be addressed at University of Arkansas, Department of Food Science, 272 Young Ave., Fayetteville, AR 72704.

The glued wood pieces were allowed to dry overnight at ambient conditions. The force (in Newton, N) required to shear the glued wood pieces was measured with an Instron (Model 1011; Instron Corporation, Canton, MA) by pulling apart from two edges at a loading rate of 20 mm/min, and expressed as adhesive strength of protein glue. All values for reported adhesive strength are means of five measurements.

Hydrophobicity determination. Surface hydrophobicity of modified proteins was determined by a hydrophobic fluorescence probe, 1-anilino-8-naphthalene sulfonate (ANS) method (10). A stock solution with a protein concentration of 0.015% in 0.01 M phosphate buffer (pH 7.0) was prepared by diluting soluble proteins prepared as described below (for solubility determination). Protein solutions with concentrations ranging from 0.0015 to 0.015% were prepared by serially diluting the stock solution. Ten microliters of ANS (8 mM in 0.01 M buffer) were added to 2.0 mL of the protein solutions. Fluorescence intensity of ANS–protein conjugates was measured with a Kontron Model SF23/B spectrofluorometer (Kontron Ltd., Zurich, Switzerland), at excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the fluorescence intensity vs. percentage protein concentration was calculated by linear regression and was used as an index of protein hydrophobicity.

Protein solubility. Protein solubility was determined by the method of Franzen and Kinsella (11) with a slight modification. The pH of a 1% protein sample was adjusted to 6.8 (the pH of the unmodified SPI) with 1 N HCl, and the solution was stirred for 30 min. The suspension was centrifuged at 10,000 rpm for 10 min. The protein content of the supernatant was determined by the Biuret method (12). SPI solubilized at a pH of 12.0 served as a standard.

Water hydration capacity (WHC). WHC of proteins was determined by the method of Quinn and Paton (13). Protein sample (about 1 g) was accurately weighed into a polycarbonate centrifuge tube, and water was added in small amounts and stirred with a spatula till the samples were thoroughly wet (3.3, 4.0, and 4.9 mL/ g of protein for freeze-dried, spray-dried, and unmodified proteins, respectively). The tubes were centrifuged at 5, 000 rpm for 10 min, the supernatant was discarded, and the tubes were reweighed. Because a minimum amount of water is used to wet the proteins, little supernatant (about 100–200 μL) was discarded after centrifugation. Hence, the protein solubilized in this small amount of supernatant was not taken into account for the determination of WHC. The amount of water absorbed per gram of protein was determined from the difference in sample weight and reported as water-holding capacity.

Viscosity. Viscosity of modified soy protein adhesives at 14% solid dispersions (pH adjusted to 6.8 with 1 N HCl) was determined with a Brookfield viscometer (Stoughton, MA). Industrial formulation of adhesives requires a viscosity of <5000 cP at 20% solids. However, for soy protein adhesives, 20% solids gave a paste rather than a dispersion. Hence, the highest possible concentration of 14% that gave uniform dispersions with all protein samples was selected for viscosity measurement. An 8% dispersion was used to glue wood pieces because it gave a uniform spread on wood. The spindle speed was 20 rpm. All measurements were made in duplicate at ambient temperature (23°C).

Sulfhydryl (SH) and SS content of proteins. The sulfhydryl and total SH/SS contents were determined with 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB) according to the method of Beveridge *et al*. (14). Modified soy protein samples (100 mg) were suspended in 10.0 mL of 0.08 M *Tris*-glycine buffer (pH 8.0) that contained 8 M urea and 3 mM EDTA (*Tris*-gly/urea/EDTA) and were stirred for 1 h. To this solution, 10 mL of 12% TCA was added, vortexed, and incubated for 1 h at 23°C. The solution was then centrifuged at 12,000 rpm for 10 min. The precipitate was washed twice with 5 mL 12% TCA to remove Na_2SO_3 and resuspended in 10 mL *Tris*-gly/urea/EDTA buffer.

For SH determination, to 0.5 mL of the above solution, 4.5 mL of *Tris*-glycine buffer with urea and EDTA, and 0.05 mL DTNB reagent (4 mg/mL) were added. Absorbances were measured at 412 nm, and the SH contents were calculated by using an extinction coefficient of 13.6 mM^{-1}.

For total SH/SS, to SPI suspended in *Tris*-gly/urea/EDTA buffer, 50 μL mercaptoethanol was added and stirred for 1 h at 23°C. Total SH/SS was determined from this solution as described above for modified soy proteins.

SS contents of the protein samples were obtained by subtracting free SH contents from total SH/SS contents.

Fluorescence spectral measurements. Fluorescence spectra of unmodified and modified proteins were recorded with a Shimadzu PC-1501 spectrofluorometer (Shimadzu Corp., Columbia, MD). Protein samples (1 mg/mL in 0.05 M phosphate buffer of pH 7.0) were excited at 280 nm, and the emissions were measured in the range of 290–400 nm.

High-perfomance liquid chromatography (HPLC). HPLC protein separations were performed with a Synchropak GPC300 (300 Å, 5 μm, 4.6 × 250 mm) size-exclusion column (Synchrom, Inc., Lafayette, IN) on a Hewlett-Packard HP 1090L System (Hewlett-Packard, Palo Alto, CA), equipped with a diode array ultraviolet detector and an HP analytical DOS Chemstation controller.

Statistical analysis. The general linear models procedure (15) was used for data analysis. The differences between means were tested with Tukey's studentized range test at 5% level.

RESULTS AND DISCUSSION

Hydrophobicity and water hydration properties of modified soy proteins. Hydrophobicity is an important property that influences the interaction of protein molecules with other components in addition to protein-protein interaction (16). Hence, it governs the functionality of proteins. Hydrophobicities of soluble portions of protein samples are listed in Table 1. The hydrophobicity of modified soy proteins increases due to unfolding of protein molecules by cleavage of disulfide bonds. Disulfite treatment results in a cleavage of 28% of the disul-

TABLE 1
Hydrophobicity, Solubility, and Water Hydration Capacity (WHC) of Modified and Unmodified Soy Proteins

Soy protein samples	ANS hydrophobicity[a,b]	Solubility[a,b]	WHC[b,c]
Unmodified SPI	7[c]	51[c]	4.76[a]
MSPSD[d]	17[a]	70[a]	3.75[b]
MSPFD[e]	12[b]	64[b]	3.09[c]

[a]Means of two measurements.
[b]Values in the same column with different superscripts are significantly different from each other at $P < 0.05$; ANS, 1-anilino-8-naphthalene sulfonate.
[c]Means of three measurements.
[d]Spray-dried product of modified soy proteins.
[e]Freeze-dried product of modified soy proteins.

fide bonds in soy proteins (4). The drying method had no significant effect on the SS content of modified soy proteins. However, spray-dried products had significantly ($P < 0.05$) higher hydrophobicity compared to freeze-dried products, indicating the excess unfolding due to heat treatment during spray-drying. Previous studies have indicated that modified proteins with enhanced hydrophobicity had improved water resistivity when these proteins were used as a wood adhesive (3,4).

Water solubility of proteins is mainly governed by the net result of electrostatic repulsion and hydrophobic interaction (6). Although, in general, an increase in hydrophobicity results in a decrease in solubility, exceptions to this trend have also been reported (17). Solubilities of modified soy proteins and unmodified soy protein are shown in Table 1. Protein solubility significantly ($P < 0.05$) increased due to SS bond cleavage. For SS-cleaved soy proteins, higher solubility was observed for more hydrophobic proteins. The high solubility of hydrophobic proteins may be attributed to the increased interaction with water due to greater unfolding of proteins.

WHC is another important property that determines the performance of protein gels (18). As shown in Table 1, modified soy proteins had significantly lower WHC compared to unmodified soy proteins. An inverse relationship between WHC and solubility has been reported in the literature (19). This could be due to an increase in molecular size of hydrated protein molecules. However, freeze-dried samples had significantly ($P < 0.05$) lower water-holding capacity and lower solubility than spray-dried samples. This differences could be due to the differences in protein conformation that results from the varying degree of unfolding.

While hydrophobic measurement provides more general information on protein unfolding, fluorescence emission by tryptophan residues may indicate specific structural changes due to its intrinsic nature. Fluorescence spectra of modified and unmodified soy proteins due to tryptophan emission are shown in Figure 1. Fluorescence spectra of soy proteins treated with mercaptoethanol (10 mM) and urea (6 M) are also included for comparison. The emission maximum was shifted toward lower wavelength (red shift) for all SS-cleaved soy proteins, indicating exposure of tryptophan to a less polar environment after SS cleavage. Further, wavelengths at which maximum emission occurred were identical for spray-dried,

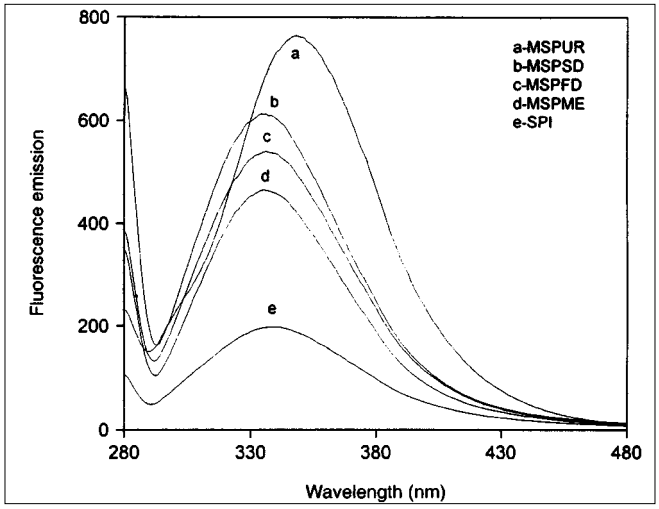

FIG. 1. Fluorescence spectra of modified and unmodified soy proteins. SPI, unmodified soy proteins; MSPSD, spray-dried product of modified soy proteins; MSPFD, freeze-dried product of modified soy proteins; MSPME, soy proteins treated with mercaptoethanol; MSPUR, soy proteins treated with urea.

freeze-dried, and mercaptoethanol-treated products. However, treatment of all protein samples (unmodified and modified) with urea resulted in a shift in the emission maximum to a higher wavelength (blue shift; only the spectrum of urea-treated unmodified soy proteins is shown in Fig. 1), indicating increased exposure of tryptophan to polar solvent compared to unmodified and SS-cleaved soy proteins. Exposure of tryptophan to a less polar environment in SS-cleaved soy proteins indicates a partial folding of molecules around tryptophan. This may be due to exclusion of water molecules due to increased intermolecular interaction between protein molecules promoted by hydrogen bonding, electrostatic, and hydrophobic interactions. This observation also supports the trends observed in WHC values of protein samples.

Adhesive strength. Table 2 shows the effect of drying method on adhesive strength of modified soy proteins. Both freeze-dried and spray-dried products had enhanced adhesive strengths as compared to the control. The drying method had no significant ($P < 0.05$) effect on adhesion of modified soy

TABLE 2
Adhesive Strength and Viscosity of Modified and Unmodified Soy Proteins

Soy protein samples	Adhesive strength[a,b] (Pascal)	Viscosity[b,c]
Unmodified SPI	95[b]	240[b]
MSPSD[d]	325[a]	2,200[a]
MSPFD[e]	336[a]	100[c]

[a]Means of five measurements; SPI, soy protein isolate.
[b]Values in the same column with different superscripts are significantly different from each other at $P < 0.05$.
[c]Means of three measurements.
[d]Spray-dried product of modified soy proteins.
[e]Freeze-dried product of modified soy proteins.

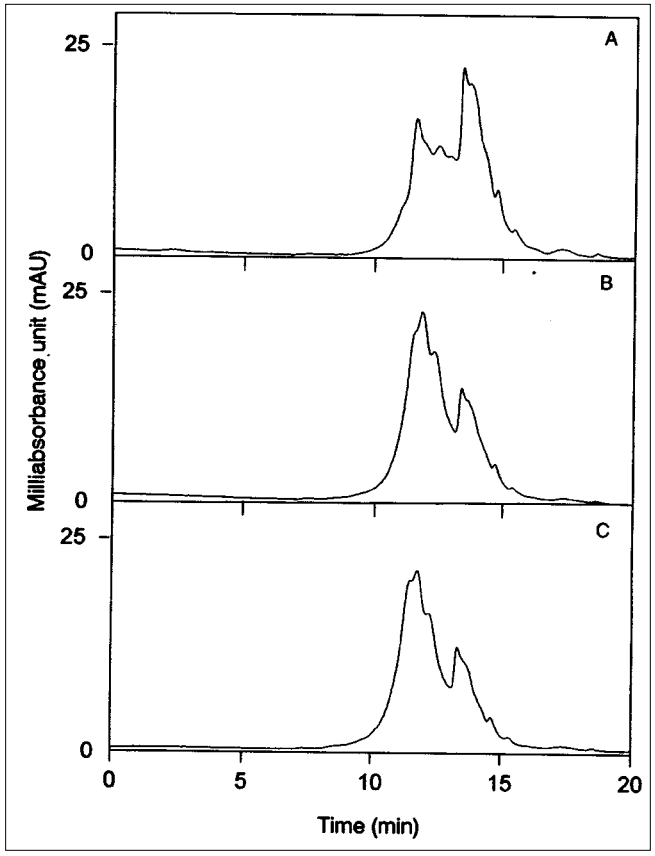

FIG. 2. Size-exclusion chromatography of A) unmodified soy proteins, B) freeze-dried, and C) spray-dried product of disulfide-cleaved soy proteins.

proteins, indicating that additional unfolding of protein molecule did not have any significant effect.

Viscosity. The viscosity of the protein dispersion significantly ($P < 0.05$) decreased from 240 to 100 cP when the SS-cleaved soy proteins were freeze-dried (Table 2). This decrease may be due to decreased intermolecular interaction that results from cleavage of disulfide bonds. However, when the disulfide-cleaved soy proteins were spray-dried, viscosity of the product increased to 2,200 cP. This may be attributed to increased electrostatic and hydrophobic intermolecular interactions due to exposure of amino acid side-chains that result from the unfolding of proteins by heat treatment during spray-drying.

HPLC separation. Separation of unmodified and modified soy proteins on a size-exclusion column with Tris-HCl buffer (pH 6.8) containing 0.2M NaCl and 0.1% sodium docecyl sulfate (SDS) as a mobile phase is shown in Figure 2. SDS was used to disperse and unfold all proteins. All protein samples were completely solubilized in this buffer. The increase in larger molecular size components and the decrease in smaller molecular size components in the chromatograms of unfolded soy proteins indicate the increase in molecular size due to SS cleavage. However, there were no significant differences in molecular size of unfolded molecules of spray-dried and freeze-dried proteins, as shown by their chromatograms. This observation, in addition to confirming the SS content of spray-dried and freeze-dried products, also eliminated the notion of possible changes in molecular size due to SS/SH interchange during spray-drying.

In conclusion, the data show that drying methods have a significant effect on the molecular and functional properties of SS-cleaved soy proteins, although the molecular size of the modified soy proteins were similar. Cleavage of 28% of the SS bonds in soy proteins resulted in a product with moderate viscosity and enhanced adhesive and hydrophobic properties.

ACKNOWLEDGMENT

This research was supported by Grant USB 4007 from the United Soybean Board.

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[Received July 3, 1996; accepted November 14, 1996]