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Physicochemical Properties of β and α′α Subunits Isolated from Soybean β-Conglycinin

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ABSTRACT: Soy protein has shown great potential for use in biobased adhesives. β-Conglycinin is a major component of soy protein; it accounts for 30% of the total storage protein in soybean seeds. β-Conglycinin was isolated and purified, and its subunits' (β, α′α) physicochemical and adhesive properties were characterized. Crude β-conglycinin was isolated from soy flour and then purified by the ammonium sulfate precipitation method. The α′α and β subunits were isolated from the purified β-conglycinin by anion exchange chromatography. Yields of α′α subunits and β subunits from 140 g of soy flour were 1.86 g (1.3%) and 0.95 g (0.67%), respectively. The minimum solubility for α′α subunits, β subunits, and β-conglycinin occurred in pH ranges of 4.1−5.4, 3.5−7.0, and 4.8−5.3, respectively. Transmission electron microscopy showed that the β subunits existed as spherical hydrophobic clusters, whereas α′α subunits existed as uniformly discrete particles at pH 5.0. Differential scanning calorimetry showed that β subunits had higher thermal stability than α′α subunits. The pH had a lesser effect on adhesion strength of the β subunits than on that of the α′α subunits. The adhesives made from β subunits also showed greater water resistance than those from α′α subunits and β-conglycinin. Soy protein rich in β subunits is likely a good candidate for developing water-resistant adhesives.

KEYWORDS: Soy protein, β-conglycinin subunits, anion exchange chromatography, solubility, thermal stability, adhesion strength, water resistance

INTRODUCTION

Soy proteins have shown great potential for use in biobased wood adhesives,[1] but the water resistance of soy protein-based adhesives is still inferior to that of petroleum-based adhesives for exterior applications. Developing a better understanding of the relationship among structural, functional, and compositional properties of adhesion may lead to development of soy protein adhesives with improved water resistance.

Soy protein contains two major globulin components, β-conglycinin (7S) and glycinin (11S), which account for about 30 and 40%, respectively, of the total protein in soybean seeds. Glycinin is a heterogeneous oligomeric protein with a molecular mass of approximately 360 kDa and consists of acidic and basic subunits. β-Conglycinin is a trimeric glycoprotein with a molecular weight of 150−200 kDa and consists of three types of subunits with distinct physicochemical properties: α′ (72 kDa), α (68 kDa), and β (52 kDa).[2] Together, these subunits form seven heterotrimers with random combinations, as well as three homotrimers. Each of these three subunits is rich in aspartate/asparagine, glutamate/glutamine, arginine, and leucine. The α′ and α subunits have very similar amino acid compositions and are composed of extension regions and a core region; the extension regions are highly hydrophilic and rich in acidic amino acids. The β subunits consist of only the core region. Core regions in all subunits exhibit high absolute homologies.[3]

Adhesives made from glycinin had higher adhesion strength and water resistance than those made from β-conglycinin protein at pH 7.6,[3] and the basic subunits from soy glycinin had higher adhesion water resistance than acidic subunits from soy glycinin.[4] Sun et al.[5] reported that hydrophobic polypeptides formed spherical clusters at pH 7.0 and exhibited continuous network morphology at pH close to their isoelectric pH value. This hydrophobic network promoted wet adhesion strength. The basic subunit from glycinin is more hydrophobic than the acidic subunit, which results in higher wet adhesion strength. Of the three subunits in β-conglycinin, the β subunit is considered to be more hydrophobic than the α′ and α subunits because of its hydrophobic amino acid composition.[5] The β subunit should have higher wet adhesion strength than the α′ and α subunits, but this hypothesis is difficult to verify. Obtaining sufficient subunits for analysis is technically difficult, so there is limited information available on subunits isolated from β-conglycinin. Therefore, the objectives of this study were to quantitatively prepare β and α′α subunits from β-conglycinin and characterize their physicochemical and adhesion properties.

MATERIALS AND METHODS

Materials. Defatted soy flour with a protein dispersion index of 90 was obtained from Cargill (Cedar Rapids, IA). Soy flour contains about 50% protein and 10% moisture, and 98% of the particle size of this soy flour was <150 μm. Cherry wood samples with dimensions of 50 mm (width) × 127 mm (length) × 3 mm (thickness) were obtained from

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Veneer One (Oceanside, NY). The wood grain was perpendicular to the length of the wood samples.

Preparation and Purification of β-Conglycinin. Crude β-conglycinin was separated from soy flour according to the method of Thanh and Shibasaki.[6] The crude β-conglycinin was then purified by ammonium sulfate fraction as described by Iwabuchi and Yamauchi.[7] The crude β-conglycinin fraction was dissolved at 3% concentration in phosphate buffer (2.6 mM KH_2PO_4, 32.5 mM K_2HPO_4, 0.4 M NaCl, 10 mM mercaptoethanol, 1 mM EDTA). Ammonium sulfate was added to the protein solution to 75% saturation. The precipitate was centrifuged off, and additional ammonium sulfate was added to the supernatant to 90% saturation. After centrifugation, the precipitate was collected and desalted with a Centricon Plus-80 centrifugal filter (Millipore Corp., Bedford, MA); the resultant material was the purified β-conglycinin fraction.

Isolation of β and α′α Subunits. The β-conglycinin subunits were isolated by subjecting the purified β-conglycinin fraction to anion exchange chromatography. Purified β-conglycinin (1 g) was applied to a DEAE-Sepharose Fast Flow column (2.6 × 40 cm) equilibrated with 20 mM Tris buffer (pH 7.5) containing 6 M urea. Gradient elution was carried out with a linear increase of NaCl concentration from 0 to 0.4 M (800 mL each). Column effluents were collected in 15.6 mL fractions at a flow rate of 1.3 mL/min. The collected fractions were analyzed with sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). Fractions of subunits were pooled and dialyzed against deionized water and freeze-dried.

SDS-PAGE. SDS-PAGE was performed using a discontinuous buffer system on a 12% separating gel and 4% stacking gel as described by Laemmli.[8] Protein samples were mixed with SDS-PAGE sample buffer solution containing 5% β-mercaptoethanol, 2% SDS, 25% glycerol, and 0.01% bromphenol blue. Approximately 5 μg of protein sample was loaded per well. Gel electrophoresis was carried out at a constant voltage of 100 V. The gel was stained with 0.25% Coomassie Brilliant Blue-R250 and destained with a solution containing 10% acetic acid and 40% methanol. Molecular weight marker proteins were run along with the samples. The purity of isolated subunits was estimated by analyzing the gel image with Kodak 1D Image analysis software, version 4.6 (Eastman Kodak Co., Rochester, NY).

Solubility. The pH solubility profiles were obtained by measuring the absorbance of supernatants of centrifuged protein solutions at 280 nm.[9] The protein (0.1%) was dissolved in 10 mM Tris (pH 8.0), and then the protein solutions were adjusted to various pH values, stirred for 30 min, and centrifuged at 20000*g* for 15 min. The absorbance of the supernatants was measured at 280 nm with a U-2010 spectrophotometer (Tokyo, Japan).

Transmission Electron Microscopy (TEM). A model CM 100 (FEI Co., Hillsboro, OR) was operated at 100 kV. β and α′α subunits were diluted to 1% with distilled water, adjusted to pH 5.0, and sonicated for 3 min in an L&R 320 ultrasonic stirrer (L&R Manufacturing Co., Keary, NJ). Samples were absorbed for approximately 30 s at room temperature onto Formvar/carbon-coated 200 mesh copper grids (Electron Microscopy Sciences, Fort Washington, PA) and stained with 2% (w/v) uranyl acetate (Ladd Research Industries, Inc., Burlington, VT) for 60 s at room temperature before being viewed by TEM.

Confocal Laser-Scanning Microscopy (CFLSM). A model Axioplan 2 MOT research microscope (Carl Zeiss, Inc., Thornwood, NY) was used to obtain CFLSM images. This model was equipped with a Zeiss Axiocam HR digital camera, a fully motorized stage with mark-and-find software, plan neofluor objectives, plan apochromat objectives, an achroplan objective, differential contrast interference, phase contrast, dark field, bright field, and Axiovision 3.1 software with interactive measurements and D deconvolution modules. A drop of sample with 10% solid content was placed onto a 3 in. × 1 in. glass slide (Fisher Scientific) without spreading force. The sample was allowed to set at room conditions for 12 h to ensure it was completely dry, then soaked in water at room temperature for 24 h, and finally dried for 2 h. Differential contrast interference images were taken at various magnifications and locations in the sample. Images were obtained for β and α′α subunits.

Thermogavimetric Analysis (TGA). The thermal degradation patterns of β-conglycinin and its subunits were characterized with a thermal gravimetric analyzer (Perkin-Elmer TGA pyis 7, Norwalk, CT). Approximately 10 mg of sample was scanned from 20 to 900 °C at a heating rate of 10 °C/min under a nitrogen atmosphere. Onset (T_o) and peak temperatures (T_p) were calculated using TGA software. The maximum degradation rate was calculated by dividing the mass (%) at peak temperature by the peak temperature. Reported results are the average of two replicates.

Adhesive and Specimen Preparation. Cherry wood samples were preconditioned in a controlled environment chamber (Electro-Tech Systems, Inc., Glenside, PA) at 23 °C and 50% relative humidity (RH) for at least 7 days before use. Proteins at 4% concentration were prepared in distilled water and stirred for 2 h, and then the pH of the adhesives was adjusted to various values by adding 1 N sodium hydroxide or 1 N hydrochloric acid. About 600 μL of the adhesive was brushed onto a marked area of 127 mm × 20 mm of the wood sample (sample dimensions were 127 mm × 50 mm). Two wood pieces were prepared and allowed to rest at room temperature for 15 min and then assembled and pressed with a hot press (model 3890 Auto "M"; Carver Inc., Wabash, IN) that had been preheated to the experimental temperatures (130 °C). After being pressed for 5 min at 1.4 MPa, the sample was removed promptly from the hot press, cooled at room temperature, and then stored in the controlled environment chamber at 23 °C and 50% RH.

Shear Strength Measurements. Wood specimens for shear strength testing were prepared and tested with an Instron (model 4465, Canton, MA) according to the standard test method for strength properties of adhesive in two-ply wood construction in shear by tension loading.[10] Wood specimens were preconditioned at 23 °C and 50% RH for 3 days, cut into pieces with dimensions of 20 mm × 50 mm, and then further conditioned at 23 °C and 50% RH for 4 days before dry strength testing. The crosshead speed of the Instron for shear strength testing was 1.6 mm/min. Stress at the maximum load was recorded as adhesion strength. Wood failure was estimated according to the standard method for estimating the percentage of wood failure in adhesive-bonded joints.[11] Reported results are the average of five samples.

Water Resistance Measurements. Water resistance was measured according to standard test methods for determining the resistance of adhesives to cyclic laboratory aging conditions[12] and the effect of moisture and temperature on adhesive bonds.[13] The preconditioned specimens were soaked in water at 23 °C for 48 h. Wet strength was measured immediately after the 48 h soaking, and soaked strength was determined after specimens were dried and conditioned at 23 °C and 50% RH for 7 days. Shear strength was tested as described previously.

RESULTS AND DISCUSSION

Isolation and Purification of β-Conglycinin Subunits. A method for large-scale isolation of β-conglycinin subunits was developed. The β subunits and the mixture of α and α′ subunits (α′α) were successfully isolated by ammonium sulfate precipitation and then passed through anion exchange chromatography. This procedure gave two peaks (Figure 1), which were identified as the β subunits and a mixture of α′ and α subunits (α′α) by SDS-PAGE (Figure 2). About 1 g of β-conglycinin was fractionated on a 2.6 × 60 cm column of the DEAE-Sepharose Fast Flow with phosphate buffer. The size of the column permitted the fractionation of up to 3 g of the protein without loss of resolution. Yields of the β and α′α subunits were 0.95 and

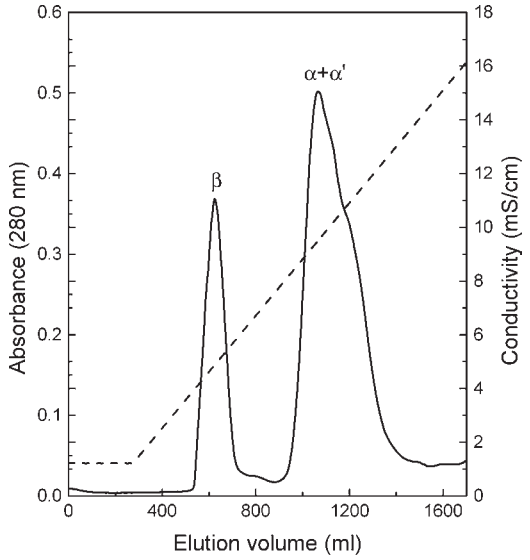

Figure 1. Anion exchange chromatography of purified β-conglycinin fraction on a DEAE-Sepharose Fast Flow column, 2.6 × 60 cm.

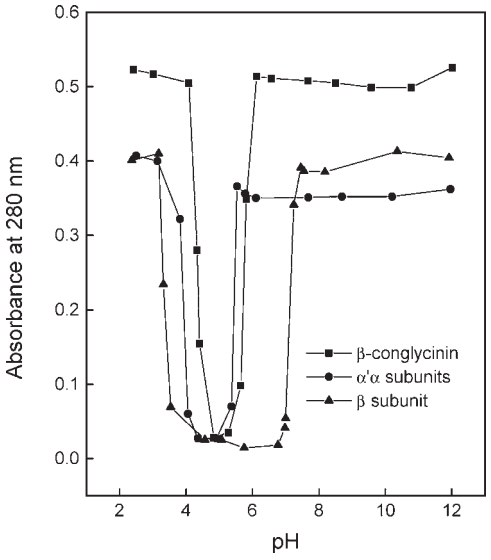

Figure 3. pH dependence of solubility of β-conglycinin and the constituent subunits.

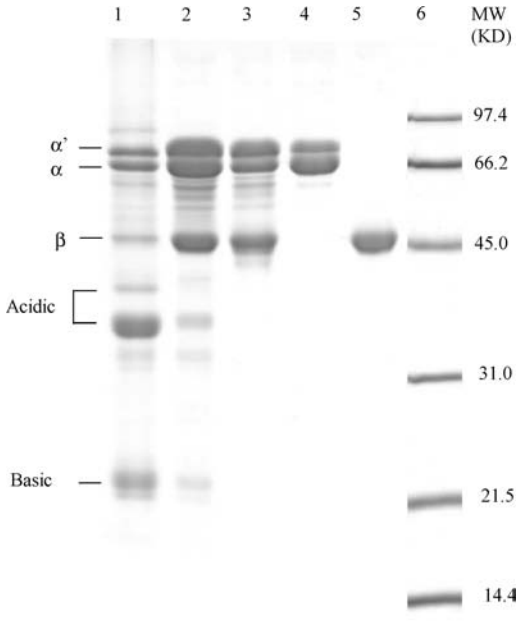

Figure 2. SDS-PAGE pattern of soy protein and the subunits on a 12% separating gel and 4% stacking gel. Lanes: 1, soy flour; 2, crude β-conglycinin; 3, β-conglycinin after ammonium sulfate precipitation; 4, α′α subunits; 5, β subunit.

1.86 g, respectively, from 140 g of soy flour. The β subunits had a molecular weight of 46 kDa, and the α′ and α subunits had molecular weights of 77 and 68 kDa (Figure 2), respectively, which are in agreement with previously reported values.[2]

The SDS-PAGE gel pattern also indicated that β-conglycinin subunits were enriched after each purification step. The protein in soy flour has approximately 16% α′α subunits and 6% β subunits. Content of the α′α and β subunits in the crude β-conglycinin significantly increased to 42 and 20%, respectively. After ammonium sulfate precipitation, the β subunits were enriched to 36%, whereas the α′α subunits remained at a level similar to that in the crude β-conglycinin. Both α′ and α are composed of a core region and extension regions and have high homology and similar amino acid compositions, whereas the β subunits consist of only the core region.[3] Therefore, further characterization was conducted on α′α subunits as one component and on the β subunits as another component.

Solubility. β-Conglycinin showed the minimum solubility at pH 4.8−5.3 (Figure 3), which is in agreement with findings reported by Thanh and Shibasaki.[6] The α′α subunits exhibited the minimum solubility at pH 4.1−5.4. Similar results were obtained for subunits expressed in an *Escherichia coli* system.[14] The β subunits exhibited the minimum solubility across a broad pH range (3.5−7.0). Solubility of a protein is affected by the equilibrium status between protein−solvent and protein−protein interactions. Protein hydrophobic interactions are the major force that favors protein−protein interactions in an aqueous system. Of the proteins studied, the β subunits had the largest amount of hydrophobic amino acids. Certain hydrophobic amino acids that favor strong hydrophobic interaction (i.e., valine, proline, leucine, and phenylanine) are present at 27.7, 30.2, and 34.2% in α′, α, and β subunits, respectively.[15] The strong hydrophobic associations probably contributed to the wide pH range for the β subunit's minimum solubility.

Morphology of β and α′α Subunits. The α′α subunits were uniformly dispersed in water (Figure 4A), whereas the β subunits formed spherical clusters with diameters ranging from 5 to 10 μm at pH 5.0. Sun et al.[5] observed clusters formed from native heterogeneous hydrophobic soy protein polypeptides, but clusters observed in the present study had a clear solid line with their surroundings, which suggests that pure homogeneous hydrophobic polypeptides will form more cohesive clusters. The CFLSM analysis further confirmed that β subunits were more water resistant than α′α subunits (Figure 5). After 24 h of water soaking, most α′α subunits dissolved in water (Figure 5A), whereas most β subunits were still stuck to the glass panel surface (Figure 5B).

Thermal Gravimetric Analysis. Thermogravimetric analysis is a continuous process that involves the measurement of sample

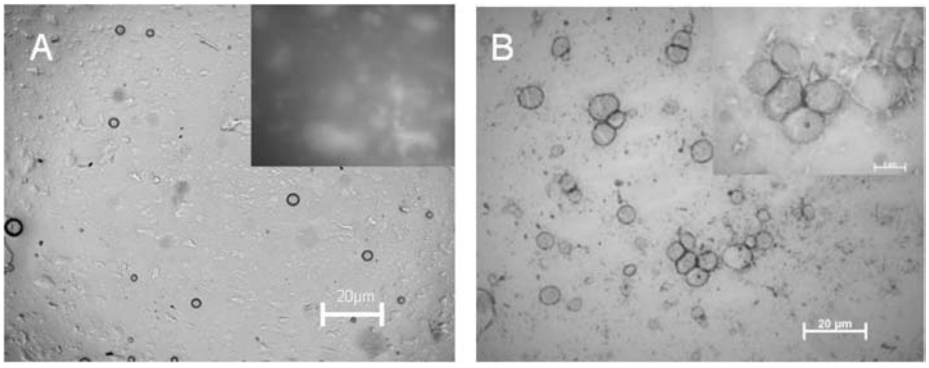

Figure 4. TEM images of (A) α′α subunits and (B) β subunit.

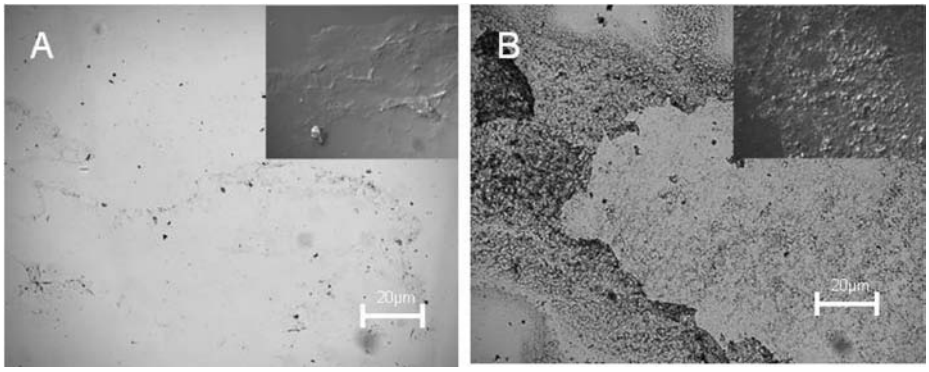

Figure 5. CFLSM images of (A) α′α subunits after 24 h of water soaking and (B) β subunits after 24 h of water soaking.

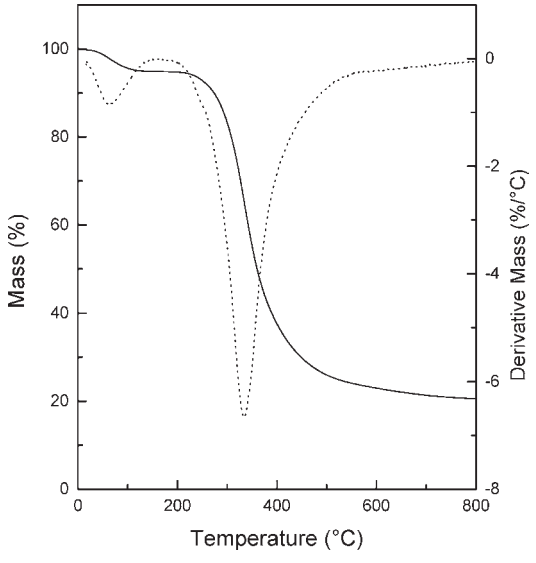

Figure 6. Thermal gravimetric (—) and derivative thermal gravimetric (- - -) curves for β subunit.

mass change as a function of increasing temperature. The thermal behaviors of all samples exhibited three similar regimes (as shown for β subunits in Figure 6). The first regime was observed primarily between 50 and 110 °C and is related to the loss of free and physically absorbed water. All samples lost about 2% of their weight at their respective peak temperatures (T_p). The α′α subunits and β-conglycinin had similar T_p values that were higher than that of the β subunits (Table 1). Hydrophilic amino acid content was approximately 63% in α′α subunits and 58% in β subunits.[15] The increased interactions between hydrophilic amino acids and water in α′α subunits require more energy to remove water from protein, which leads to a high T_p value.

The second regime was observed between 290 and 410 °C. Thermal degradation in this regime involves broken intermolecular and intramolecular hydrogen bonds and electrostatic bonds, rupture of weak bonds (C−N, C(O)-NH, C(O)−NH_2, and NH_2), and decomposition of protein side chains.[16,17] The β subunits had a higher T_o than the α′α subunits (Table 1). The high hydrophobic amino acid content in β subunits favors strong hydrophobic associations and gives rise to formation of a compacted oligomeric structure, which protects protein side chains by increasing the activation energy required to break the covalent bonds. However, β subunits and α′α subunits had similar T_p values that were lower than that of β-conglycinin. Also, the maximum degradation rates for these subunits were higher than that of β-conglycinin. Molecular conformation affects maximum degradation rates and the corresponding temperatures. The β-sheet conformation and intermolecular secondary bonds (parallel hydrogen bonding and van der Waals forces) help maintain protein stability. β-Conglycinin contains about 34% β-sheet structures,[18] but those ordered structures in α′α and β subunits were destroyed by urea denaturation during isolation. The lack of β-sheet structures in subunits led to low T_p and high degradation rates. Similar results were reported

Table 1. Thermal Gravimetric Analysis of Soybean β-Conglycinin Subunits

	peak 1		peak 2			residue (%) at different temperatures	
protein subunit	T_p[a] (°C)	residue (%)	T_o[b] (°C)	T_p[a] (°C)	MR[c] (%/°C)	340 °C	600 °C
α′α	68.0	97.5	288	336	0.191	61.8	25.4
β	61.9	97.9	294	337	0.192	62.8	25.6
β-conglycinin	67.2	97.4	289	346	0.181	64.9	23.7

[a] T_p, peak temperature. [b] T_o, onset temperature. [c] MR, maximum rate of degradation.

Table 2. Effects of Adhesive pH on the Shear Strength (MPa) of Soybean β-Conglycinin Subunits[a]

	adhesive pH			
	3.0	5.0	8.0	12.0
dry strength				
α′α subunits	3.46c	4.71a	4.48b	4.66ab
β subunits	4.39a	4.68a	4.68a	4.51a
β-conglycinin	2.19c	5.07a	5.10a	4.62b
wet strength				
α′α subunits	1.00c	2.02a	1.30b	1.80a
β subunits	1.28c	1.93b	2.41a	1.90b
β-conglycinin	0.79c	2.17a	1.67b	1.78b
soaked strength				
α′α subunits	3.02c	4.64a	3.98b	4.56a
β subunits	4.16b	3.92c	4.57a	3.41d
β-conglycinin	1.37d	4.66b	5.12a	4.07c

[a] Samples were pressed at 130 °C and 1.4 MPa for 5 min. ANOVA and LSD tests were performed using SAS. Means with the same letters in the same row are not significantly different at α = 0.05.

for silk fibroin,[16] which had improved thermal stability when it adopted β-sheet structures. The mass retention at 340 °C was reported because it is close to the T_p for all of the samples studied. Again, β-conglycinin possessed the highest residues (Table 1), which suggests that secondary interactions associated with β-sheet conformation improve thermal stability.

The third regime corresponded to the decomposition of protein backbone peptide bonds at a temperature above 420 °C. The mass retention values for all samples were similar at 600 °C. The residual mass probably represents inorganic compounds derived from thermal degradation.[19]

Adhesion Strength. The dry adhesion strength of the β-conglycinin and α′α subunits was low at pH 3.0, increased sharply at pH 5.0, and leveled off as pH increased from 5.0 to 8.0 (Table 2). The β subunits were affected by pH to a lesser degree than the α′α subunits and maintained adhesion strength values around 4.5 MPa over the pH range studied. Adhesives made from α′α subunits and β subunits showed similar dry adhesion strength from pH 5.0 to 12.0 (Table 2). However, the cohesive wood failures for β subunits and α′α subunits at those pH levels were about 95 and 70%, respectively. Therefore, adhesives made from β subunits had higher adhesive cohesiveness than those made from α′α subunits, even though both subunits showed similar adhesion strengths.

One of the commonly used methods to evaluate adhesion performance is to measure water resistance. Wet adhesion strength of all protein adhesives decreased to a large degree compared with their respective dry adhesion strengths. During water soaking, water molecules interact with protein adhesives, weaken interactions among protein adhesives and the wood surface, and ultimately result in reduced adhesion strength. The wet adhesion strength of β-conglycinin and its subunits was also affected by pH to a much larger degree than dry adhesion strength (Table 2). Both β-conglycinin and α′α subunits exhibited the maximum wet adhesion strength of 2.0 MPa at pH 5.0, whereas β subunits exhibited the maximum wet adhesion strength of 2.4 MPa at pH 8.0; these values are similar to those of basic polypeptides in glycinin. Maximum wet adhesion strength generally was observed at pH levels close to those at which proteins have minimum solubility and are strongly associated with each other. Cohesive wood failure for β subunits and α′α subunits at their respective maximum wet adhesion strengths was 64 and 20%, respectively. Again, as observed in the dry adhesion strength tests, the β subunits had relatively high adhesive cohesiveness. The high hydrophobic amino acid content in β subunits seems to contribute to adhesion water resistance. Soybean glycinin basic subunits, which are rich in hydrophobic amino acids, were also found to have better water resistance than glycinin acidic polypeptides, which are rich in hydrophilic amino acids.[4] Soaked adhesion strength, which was measured after wet samples were dried and conditioned, recovered to a large degree (Table 2), indicating that most adhesion bonds disrupted by water can be re-formed after removal of water.

In summary, β and α′α subunits were successfully isolated from soy flour and their physicochemical properties were characterized. The β subunits had a wider minimum solubility pH range than α′α subunits. pH affects protein adhesion strength through varying electrostatic interactions among proteins. Proteins rich in hydrophobic subunits had higher wet adhesion strength across a broad pH range than those rich in hydrophilic subunits, and protein with more hydrophilic subunits had higher wet adhesion strength at pH close to their minimum solubility. The β subunits in β-conglycinin had high water resistance similar to basic subunits in glycinin, but both subunits are rich in hydrophobic amino acids. Proteins with higher hydrophobic amino acid content promote protein−protein associations and could be favorable for producing adhesives with higher water resistance. These results indicate that designing protein polymers with a more hydrophobic composition would increase water resistance of adhesives made with these proteins.

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