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Soybean Glycinin Subunits: Characterization of Physicochemical and Adhesion Properties

XIAOQUN MO,† ZHIKAI ZHONG,† DONGHAI WANG,‡ AND XIUZHI SUN*,†

Bio-Material and Technology Lab, BIVAP, Department of Grain Science and Industry, and Department of Biological and Agricultural Engineering, Kansas State University, Manhattan, Kansas 66506

Soybean proteins have shown great potential for applications as renewable and environmentally friendly adhesives. The objective of this work was to study physicochemical and adhesion properties of soy glycinin subunits. Soybean glycinin was extracted from soybean flour and then fractionated into acidic and basic subunits with an estimated purity of 90 and 85%, respectively. Amino acid composition of glycinin subunits was determined. The high hydrophobic amino acid content is a major contributor to the solubility behavior and water resistance of the basic subunits. Acidic subunits and glycinin had similar solubility profiles, showing more than 80% solubility at pH 2.0−4.0 or 6.5−12.0, whereas basic subunits had considerably lower solubility with the minimum at pH 4.5−8.0. Thermal analysis using a differential scanning calorimeter suggested that basic subunits form new oligomeric structures with higher thermal stability than glycinin but no highly ordered structures present in isolated acidic subunits. The wet strength of basic subunits was 160% more than that of acidic subunits prepared at their respective isoelectric points (pI) and cured at 130 °C. Both pH and the curing temperature significantly affected adhesive performance. High-adhesion water resistance was usually observed for adhesives from protein prepared at their pI values and cured at elevated temperatures. Basic subunits are responsible for the water resistance of glycinin and are a good starting material for the development of water-resistant adhesives.

KEYWORDS: Soybean protein; adhesion strength; pH; thermal properties; hydrophobic amino acids

INTRODUCTION

The development of bio-based adhesives from renewable resources has attracted considerable attention from the public and scientific society in recent years. Because of limited petroleum resources and the pollution caused by commonly used formaldehyde-based adhesives, the demand for environmentally friendly adhesives from plant resources is increasing. Soybean protein is one of the most abundant and renewable polymers in the world. Enhancing and exploring utilization of soybean protein for adhesive applications could help to alleviate environmental problems and add value to agricultural products.

Soybean protein has shown great potential as a good alternative to formaldehyde-based adhesives for wood and nonwood applications (*1*−*3*). It has been used to partly replace phenol resorcinol formaldehyde in finger-joint applications for fresh wood (*4*) and in the fabrication of medium-density composite fiberboards, particleboards, and wood composites (*3*−*10*). Columbia Forest products, the largest manufacturer of hardwood plywood and hardwood veneer, has begun conversion of all its veneer-core hardwood plywood plants to formaldehyde-free manufacturing processing using soy-based adhesives (*11*).

However, water resistance of soy-based adhesives is still inferior to petroleum-based adhesives for exterior applications (*12*). Great effort has been made in trying to improve water resistance of soy adhesives. Research showed that water resistance could be improved by introducing functional groups, incorporating curing agents, or adjusting the pH at which the adhesive was prepared (*13*−*16*). Wood composites bonded with adhesives made from soy protein−polyamide−epichlorohydrin (weight ratio of 1.33:1) had a boiling-water wet shear strength of about 2 MPa (*16*). Adhesives from soy protein prepared at a pH close to its isoelectric point (pI = 4.5) had a good water resistance with a wet strength of 3.2 MPa. Surface-charge distributions and the net charge of a protein played important roles in adhesion strength and water resistance (*17*).

Glycinin and conglycinin are two major components in soybean proteins. Research has shown that glycinin is an important component for adhesion strength, especially for water resistance. Soy protein-based adhesives with high glycinin content gave higher adhesion strength and water resistance than those with low glycinin content. Wet strength from glycinin was about 50% higher than that of soy protein isolate and about 35% higher than that of conglycinin (*18*, *19*). Glycinin is

* To whom correspondence should be addressed. E-mail: xss@ksu.edu. Telephone: 785-532-4077.
† Department of Grain Science and Industry.
‡ Department of Biological and Agricultural Engineering.

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a heterogeneous oligomeric protein with a molecular mass ranging from 340 to 375 kDa and consists of acidic and basic subunits with pI values of 4.8−5.4 and 8−8.5, respectively (*20*, *21*). Acidic subunits were found to have higher viscosity and emulsification activity than those of basic subunits (*22*). However, no information is available on the adhesive behavior of glycinin acidic and basic subunits. The objectives of this study were to isolate glycinin subunits and characterize their physicochemical properties and to examine the effects of pH and processing temperatures on the adhesion strength and water resistance. The information obtained from this study should be valuable for future design and improvement of protein-based adhesives.

MATERIALS AND METHODS

Materials. Defatted soy flour (protein content of 52.4%, carbohydrate content of 34.3%, crude fiber content of 3.4%, ash content of 6.4%, and moisture content of 4.6%) with a protein dispersion index of 90 was obtained from Cargill (Cedar Rapids, IA). Cherry wood samples with dimensions of 50 mm (width) × 127 mm (length) × 3 mm (thickness) were a product from Veneer One (Oceanside, NY). The orientation of the wood grain was perpendicular to the length of the wood samples.

Isolation of Acidic and Basic Subunits. Glycinin was separated from the soy flour according to the method of Thanh and Shibasaki (*23*). The yield of glycinin was 17.1 g from 140 g of soy flour.

Acidic and basic subunits were separated from the glycinin according to methods described by Damodaran and Kinsella with some modifications (*24*). The glycinin was prepared at a 0.5% protein concentration using 30 mM Tris buffer (pH 8.0) containing 15 mM β-mercaptoethanol. The protein solution was heated to 90 °C for 30 min and then centrifuged at 10000*g* at 4 °C for 20 min. The precipitate was washed twice with 30 mM Tris buffer (pH 8.0), suspended in distilled water, and then freeze-dried and collected as basic subunits. The supernant was concentrated using a Centricon Plus-80 centrifugal filter (Millipore Corp., Bedford, MA), and the concentrate was freeze-dried and collected as acidic subunits.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). To estimate the purity of extracted acidic and basic subunits, SDS−PAGE was performed using a discontinous buffer system on a 12% separating gel and 4% stacking gel as described by Laemmli (*25*). 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 Company, Rochester, NY).

Amino Acid Composition Analysis. Proteins were hydrolyzed in 6 N hydrochloric acid at 110 °C for 24 h before analysis. Hydrolyzed proteins were run on Beckman System Gold high-performance liquid chromatography (HPLC) with a fluorescence detector (Beckman Coulter, Fullerton, CA) at $\lambda_{ex} = 340$ nm and $\lambda_{em} = 450$ nm. Separation was completed using a Lithium buffer on a 3 mm × 25 cm spherogel AA-Li^+ column. Elution was performed using a flow rate of 0.3 μL/min for 0−15 min and then 0.375 μL/min for 15−150 min. The column temperature was maintained at 39 °C at 0−30 min, 63 °C at 30−110 min, and then cooled down back to 39 °C from 110−150 min. The hydrolyzed samples were detected by postcolumn derivatization with *O*-phthaldialdehyde. Results were analyzed using Beckman System Gold Software. The reported values are the average of two replicates.

Solubility of Acidic and Basic Subunits. The pH solubility profiles were obtained by measuring the absorbance of the supernant of centrifuged protein solutions at 280 nm, as described by German et al. (*26*). The protein (0.1%) was dissolved in 10 mM Tris (pH 8.0). 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 supernant was measured at 280 nm using a Hitachi U-2010 spectrophotometer (Tokyo, Japan).

Differential Scanning Calorimetry Measurements. Thermal properties of the proteins were studied using a differential scanning calorimeter (DSC) (DSC 7, Perkin-Elmer, Norwalk, CT), which was calibrated with indium and zinc. Approximately 5 mg of protein was weighed (to an accuracy of micrograms) into the large-volume stainless-steel DSC pan. Distilled water weighing 10 times that of the protein was added, and then the pan was airtight-sealed. The samples were kept at 4 °C overnight before measurement at a temperature range from 10 to 160 °C and a heating rate of 10 °C/min. Reported values for enthalpies and peak temperatures of thermal transition were 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 for at least 7 days before use. Adhesives were prepared followed the method described by Mo et al. (*18*) with modification. Specifically, proteins at a 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. Next, 600 μL of the adhesive was brushed onto a marked area of 127 × 20 mm of the wood sample with dimensions of 50 × 127 mm. Two wood pieces were prepared and allowed to rest at room temperature for 15 min and then were assembled and pressed using a hot press (model 3890 Auto "M"; Carver, Inc., Wabash, IN), which had been preheated to the experimental temperatures (130, 150, 170, or 190 °C) before use. After the sample was pressed for 5 min at 1.4 MPa, it was removed promptly from the hot press, cooled down at room temperature, and then stored in the environmental chamber at 23 °C and 50% relative humidity.

Shear Strength Measurements. Wood specimens for shear strength testing were prepared and tested using an Instron (model 4465, Canton, MA) according to the standard test method for strength properties of adhesive in two-plywood construction in shear by tension loading (ASTM D2339-98, 2002) (*27*). Wood specimens were preconditioned at 23 °C and 50% relative humidity for 3 days, cut into pieces with a dimension of 20 × 50 mm, then further conditioned for 4 days before testing for dry strength. The crosshead speed for testing was 1.6 mm/min. Stress at a maximum load was recorded. Wood failure is one of the principal means for determining the quality of an adhesively bonded wood joint. Wood failure was estimated according to the standard practice for estimating the percentage of wood failure in adhesive bonded joints (ASTM D5266-99) (*28*). The results were the average of five samples.

Water Resistance Measurements. Water resistance was measured according to standard test methods for resistance of adhesives to cyclic laboratory aging conditions (*29*) and standard test methods for the effect of moisture and temperature on adhesive bonds (*30*). The preconditioned specimens were soaked in water at 23 °C for 48 h. The wet strength was measured immediately after 48 h of soaking, and the soaked strength was obtained by testing the specimens after they were dried and conditioned at 23 °C and 50% relative humidity for 7 days. The shear strength was tested as described previously.

RESULTS AND DISCUSSION

Isolation of Glycinin Subunits. The isolation of acidic subunits from basic subunits was based on the cleavage of the disulfide bonds between acidic and basic subunits at a higher temperature (90 °C) in the presence of β-mercaptoethanol, causing the selective aggregation and precipitation of basic subunits. The yield of acidic and basic subunits was 7.7 and 8.2 g, respectively, from 140 g of soy flour. Image analysis of the protein SDS−PAGE pattern revealed that purity was more than 90% for acidic subunits and more than 85% for basic subunits. Glycinin showed major bands corresponding to acidic and basic subunits (**Figure 1**). Isolated acidic subunits showed two major bands with a molecular mass around 42 kDa (A3), 36−38 kDa (A1a, A1b, A2, and A4), and a minor band at 12 kDa (A5). Isolated basic subunits showed major bands with a molecular mass around 20−22 kDa (**Figure 1**).

Amino acid composition of the isolated acidic and basic subunits is shown in **Table 1**. As is typical of most seed proteins, glutamine−glutamate (Glx) and asparagines−aspartate (Asx)

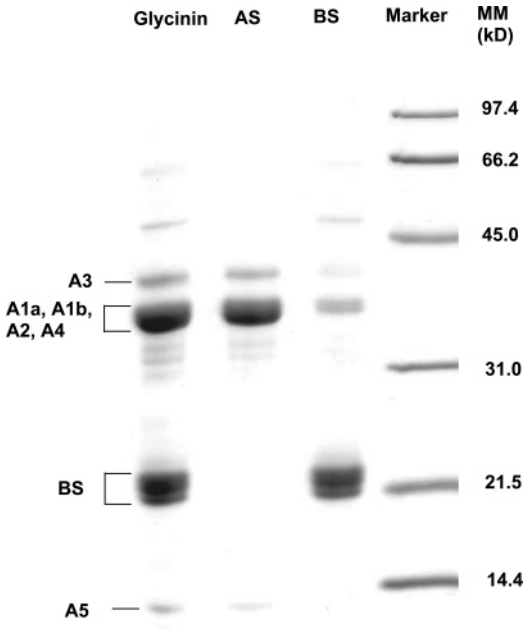

Figure 1. SDS–PAGE pattern of acidic subunits (AS), basic subunits (BS), and glycinin.

Table 1. Amino Acid Composition of Acidic Subunits and Basic Subunits

amino acids	acidic subunits (%)	basic subunits (%)
Asx	13.01 ± 0.34	13.25 ± 0.26
Thr	3.43 ± 0.03	3.98 ± 0.11
Ser	6.55 ± 0.07	7.26 ± 0.02
Glx	25.37 ± 0.36	15.15 ± 0.12
Gly	9.61 ± 0.31	8.11 ± 0.10
Ala	4.17 ± 0.06	7.44 ± 0.16
Val	4.44 ± 0.08	7.52 ± 0.09
Met	1.17 ± 0.02	1.12 ± 0.06
Ile	5.32 ± 0.06	5.87 ± 0.05
Leu	6.54 ± 0.07	9.55 ± 0.10
Tyr	2.68 ± 0.05	3.45 ± 0.11
Phe	3.79 ± 0.05	5.15 ± 0.09
Lys	5.63 ± 0.31	4.49 ± 0.12
His	2.27 ± 0.07	1.99 ± 0.01
Arg	6.03 ± 0.25	5.67 ± 0.20

were abundant residues. Acidic subunits contained more Glx but similar amounts of Asx to the basic subunits. However, the basic subunits had considerably more hydrophobic amino acids than the acidic subunits. Hydrophobic amino acids accounted for 47.1% of total amino acid content in the basic subunits but 36.6% in the acidic subunits, suggesting that basic subunits are more hydrophobic than acidic subunits.

Solubility. Glycinin and its subunits showed U-shaped, solubility−pH profiles (**Figure 2**). Acidic subunits and glycinin had a similar solubility profile. Acidic subunits had a sharp base with excellent solubility at pH above 6.0 or below 4.0 and with the minimum solubility at pH 5.0 in agreement with the reported pI value determined by using the isoelectric focusing technique (*21*). The basic subunits had the minimum solubility in a range of pH from 4.5 to 8.0, which was a much wider pH range than the reported pI values of 8.0−8.5.

It is well-known that the solubility of a protein is affected by the equilibrium between protein−solvent and protein−protein interactions. Chemical environments that favor protein−protein interactions decrease solubility, whereas conditions that favor protein−solvent interactions increase solubility. Two major forces, electrostatic and hydrophobic interactions, are usually

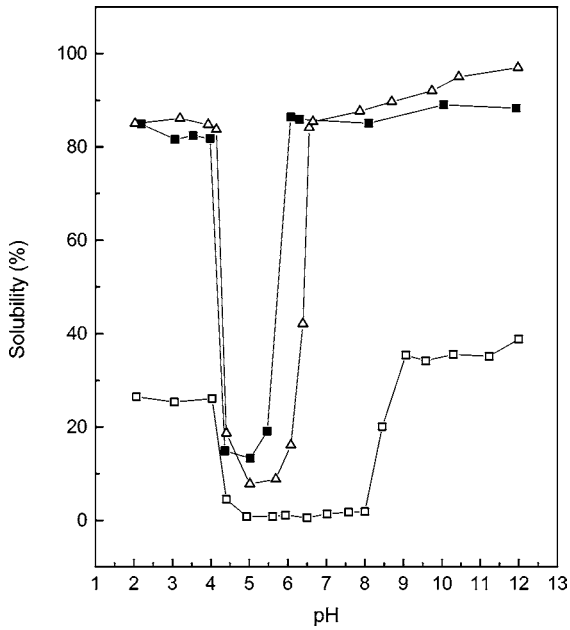

Figure 2. pH solubility profile of acidic subunits (■), basic subunits (□), and glycinin (△).

involved in and affect protein solubility. Basic subunits are highly hydrophobic and have 28.1% certain hydrophobic amino acids (valine, leucine, isoleucine, and phenylalanine) (**Table 1**), which is above the theoretical critical value of 28% for protein self-associations and the formation of insoluble protein aggregates (*31*). Experimentally, German and co-workers found that basic subunits were able to form aggregates via hydrophobic interactions (*26*). The strong hydrophobic interactions offset the electrostatic repulsions, giving a minimum solubility over a wide pH range. Conversely, acidic subunits have limited hydrophobic associations because their particular hydrophobic amino acid content is 20.1%, which is lower than the theoretical critical value for hydrophobic self-associations. The solubility of acidic subunits is mainly affected by electrostatic interactions. Their minimum solubility lies in the pI, where the protein electrostatic repulsions are the minimum. For basic subunits, both hydrophobic associations and electrostatic interactions influence solubility. At pH 4.5−7.5, hydrophobic associations in basic subunits appear to dominate electrostatic repulsions and give the observed minimum solubility. At pH values lower than 4.5 or greater than 8.5, increased electrostatic repulsions partially weaken the protein hydrophobic associations and result in increased solubility. However, basic subunits still had significantly lower solubility than acidic subunits did over the pH range studied, suggesting hydrophobic associations prevail over the electrostatic repulsions in basic subunits. Therefore, protein hydrophobic associations appear to cause decreased solubility of basic subunits. The aggregates resulting from such association were reported to have a molecular mass larger than 100 kDa as analyzed using nonreduced SDS−PAGE (*32*).

Thermal Properties. Disrupting and unfolding of highly ordered protein structures are usually accompanied by a significant uptake of heat. This thermal transition can be observed as an endothermic peak in the DSC thermogram. Acidic subunits showed no thermal transition, indicating that their native structures were destroyed during isolation. Thermal transition of basic subunits had a peak temperature of 128.8 °C, which was higher than the peak temperature of 89.7 °C for glycinin. Strong hydrophobic interactions in the basic subunits possibly exclude the water from the protein hydrophobic region

Table 2. Effects of the Adhesive pH on the Shear Strength (MPa) of Soybean Glycinin Subunits[a,b]

	adhesive pH			
	2.0	5.0	8.0	12.0
dry strength				
acidic subunits	2.78 c	4.57 a	4.14 b	2.52 c
basic subunits	3.18 b	4.98 a	4.72 a	4.88 a
glycinin	2.67 c	4.92 a	5.08 a	4.04 b
wet strength				
acidic subunits	0.37 b	0.92 a	0.93 a	0.41 b
basic subunits	0.60 d	2.04 b	2.40 a	1.68 c
glycinin	0.31 b	2.14 a	1.92 a	0.32 b
soaked strength				
acidic subunits	0.58 c	3.77 a	3.49 a	0.91 b
basic subunits	3.01 c	4.67 a	4.66 a	3.73 b
glycinin	2.62 c	4.56 b	5.04 a	2.57 c

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

Table 3. Effects of the Curing Temperature on the Shear Strength (MPa) of Soybean Glycinin Subunits[a,b]

	temperature (°C)			
	130	150	170	190
dry strength				
acidic subunits	4.57 b,c	4.38 c	5.12 a	4.65 b
basic subunits	4.72 a	4.69 a	4.66 a	4.90 a
glycinin	4.92 c	5.08 c	5.50 b	5.98 a
wet strength				
acidic subunits	0.92 c	1.12 b,c	1.34 b	2.30 a
basic subunits	2.40 b	2.29 b	2.45 b	2.91 a
glycinin	2.14 c	2.32 b,c	2.56 b	3.06 a
soaked strength				
acidic subunits	3.77 c	4.33 b	4.78 a	4.47 a,b
basic subunits	4.66 a	4.63 a	4.45 a	4.72 a
glycinin	4.56 c	4.97 b,c	5.21 b	5.91 a

[a] Adhesives from acidic and basic subunits were prepared at pH 5.0 and 8.0, respectively. The adhesive from glycinin was prepared at pH 6.4. Then, samples were pressed at 1.4 MPa for 5 min at different temperatures. [b] ANOVA and LSD tests were performed using SAS. Means with the same letters in the same row are not significantly different at $\alpha = 0.05$.

and favor hydrogen bonding between protein amide groups. The result is an increased stability of the oligomeric structure. The enthalpy of the thermal transition for basic subunits had a value of 3.07 J/g; however, this was lower than the value of 12.1 J/g for glycinin. Similar to the acidic subunits, the highly ordered native structures of basic subunits were disrupted, as evidenced by the precipitates of basic subunits when undergoing heating to 90 °C for 30 min during isolation. The thermal transition of basic subunits is possibly caused by the newly formed aggregates, which have greater thermal stability and less order than those of glycinin.

Effects of pH on Adhesion Strength. Both acidic and basic subunits consist of large amounts of amino acids with ionizable residues. They carry different types and amounts of charges as the pH value varies, inducing protein interactions. Two pH values, 5.0 and 8.0, close to the pI of acidic and basic subunits, respectively, and two extreme pH values, 2.0 and 12.0, were selected to investigate the effects of pH on adhesion strength. Adhesives from glycinin were also tested for a comparison.

The dry strength of the basic subunits was higher than that of the acidic subunits over the pH range studied (**Table 2**) and was affected by pH to a less degree than the acidic subunits. It increased as the pH increased from 2.0 to 5.0 and then leveled off. There was no statistically significant difference in strength observed as pH increased from 5.0 to 12.0. Acidic subunits showed a significantly higher dry strength at pH 5.0 and 8.0 than those at extreme pH values. Similar trends were observed for glycinin (**Table 2**).

Water resistance is an important property that determines the durability of adhesion. Wet strength of all protein adhesives had a dramatic drop off when compared to their respective dry strength (**Table 2**). During water soaking, water molecules penetrate into the glue area, interact with wood surfaces, and plasticize cured-protein adhesives through hydrogen bonding, dipoles, and induced dipoles, as well as dispersive forces, resulting in weakened adhesion bonds and reduced adhesion strength. Upon drying, the adhesion strength was recovered to a large degree as observed in soaked strength, indicating most of the adhesion bonds disrupted by water can be regained after the removal of water. Basic subunits and glycinin had considerably higher wet strength than that of acidic subunits, at pH 5.0 or 8.0. Their wood failure was around 30%, while it was 0 for acidic subunits at pH 5.0. The above results suggested that basic subunits are the main contributor to the water resistance of glycinin.

Glycinin and its subunits usually had their maximum adhesion strength at pH values close to their pI values, where proteins have strong interactions as indicated by the low solubility (**Figure 2**). Those protein associations are favorable for further protein−protein interactions during the curing process and give high adhesion strength. On the other hand, at extreme pH values such as pH 2.0 or 12.0, increased electrostatic repulsions among protein molecules weaken protein associations, which in turn reduce the adhesion strength. Basic subunits showed a higher adhesion strength than those of acidic subunits. As discussed in the solubility study, hydrophobic interactions and electrostatic repulsions are two opposite forces in protein associations. At extreme pH values, acidic subunits have weak protein associations because of dominant electrostatic repulsions. In contrast, strong hydrophobic interactions in basic subunits exert against electrostatic repulsions and favor protein associations, resulting in a relatively high adhesion strength.

Effects of Curing Temperature on Adhesion Strength. Thermal treatment is one of the commonly used processes causing protein association and aggregation. To minimize electrostatic repulsion effects on adhesion strength, adhesives were prepared from proteins at pH values close to their pI values. The dry strength of basic subunits showed no statistically significant difference as curing temperatures increased from 130 to 190 °C (**Table 3**). However, the dry strength of acidic subunits was affected by the curing temperature to a larger degree, and the maximum adhesion strength of 5.12 MPa was observed at 170 °C. The wet strength of basic subunits was comparable to those of glycinin, which are greater than those of acidic subunits for all curing temperatures. However, acidic subunits showed a greater improvement in the adhesion strength than basic subunits did when the temperature increased from 130 to 190 °C. The wet strength for adhesives from acidic subunits cured at 190 °C was about 250% of that from adhesives cured at 130 °C, whereas it was about 120% of that from basic subunits at the same conditions. It is possible that high curing temperatures greatly promote the association of acidic subunits and result in a large improvement in the adhesion strength. Basic subunits are highly associated and exist mainly in the form of large molecular aggregates. Further protein association appears

limited at elevated curing temperatures, giving less improvement in the adhesion strength. At a curing temperature of 130 °C, basic subunits had a higher wet strength than glycinin. However, glycinin showed a slightly higher wet strength than basic subunits when the temperature increased to 150 °C or higher (**Table 3**). A high curing temperature could possibly promote glycinin in the formation of large aggregates, which would improve water resistance.

In conclusion, protein physicochemical properties such as solubility, amino acid composition, and protein−protein interactions are important in adhesion properties. Increased hydrophobic interactions and minimized electrostatic repulsions promote protein associations and contribute to high adhesion strength. Basic subunits have similar wet and soaked strengths to those of glycinin, whereas acidic subunits show much lower water resistance, suggesting that the water resistance of glycinin is contributed by basic subunits. The maximum wet strength of basic subunits and soy protein isolate is 2.9 and 3.2 MPa (*17*), respectively. Therefore, basic subunits are also the major contributor to the water resistance of soy protein isolate. Commercial adhesive urea formaldehyde has a wet strength of 3.5 MPa and a dry strength of around 5.0 MPa, which are similar to the soy protein isolate adhesive prepared at its pI.

Chemical environments, such as pH, can affect protein interactions, which in turn affect adhesion strength. The best adhesion performance was usually observed at pH values close to the protein pI values, at which protein molecules had the least electrostatic repulsions. The curing temperature is another important factor affecting adhesion strength. The adhesion performance of protein adhesives with less molecular interactions before curing, such as acidic subunits, can be significantly improved by applying elevated curing temperatures. Factors that strengthen protein−protein associations improve adhesion performance. High adhesion strength can be achieved by selecting proteins with high hydrophobic associations, by preparing adhesives at the pI values of proteins, or by exposing adhesives to elevated curing temperatures.

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