Soy Protein Adhesion Enhanced by Glutaraldehyde Crosslink

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ABSTRACT: Soy protein has been considered as an alternative to partly replace petroleum-based polymers for adhesive applications. The weakness of protein-based adhesive is poor water resistance, which limits its outdoor applications. The objective of this research was to improve the water resistance of soy protein adhesive by introducing crosslinkage between amino groups of amino acid residue. Laboratory prepared soy protein isolate (SPI) was used in this study. Glutaraldehyde at concentrations of 4, 20, 40, and 80 μ*M* was used as the crosslinking reagent for SPI modification. Adhesive properties of soy protein modified by glutaraldehyde, as well as thermal and morphological properties, were investigated. Crosslinking-induced protein conformation and structure changes through decrease of amino groups and adding of hydrophobic groups, subsequently affect adhesive performance of SPI. At optimum glutaraldehyde concentration (20 μ*M*), dry, wet, and soak strengths of modified SPI increased to 31.5, 115, and 29.7%, respectively, compared with unmodified SPI. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 130–136, 2007

Key words: adhesive property; crosslink; glutaraldehyde; soy protein; protein modification

INTRODUCTION

Soybean contains 30–45% protein.[1] Most of soy protein is used in food and feed applications. Soy protein can also be used for industrial applications, including composites, adhesives, plastics, and other applications.[2] Because of the natural limitation of petroleum resources, environment issues, and health concerns, protein-based adhesives is a desirable alternative to replace petroleum-based adhesives. One of the major drawbacks of protein-based adhesive is its relatively poor water resistance.[3]

Designed for biological roles, soy protein doesn't necessarily possess desirable functional properties.[4] Proteins are made of combinations of 20 different amino acids. Functionality of proteins arises from primary structure, conformation determined by amino acid sequence, and their environment.[5] Protein functions can be altered through amino acid modification. Chemical modification has been used to alter soy protein structures to increase its adhesive performance. Sodium hydroxide, urea, guanidine, and HCl have been used frequently for protein modification.[6,7] Kalapathy et al. used an alkali-modification method and obtained improved adhesive strength and water resistance.[6] Huang and Sun used sodium dodecyl sulfate and sodium dodecylbenzene sulfonate to modify soy proteins.[7] It was suggested that proteins were partly unfolded after denaturation by the binding of detergent, and that the extent of unfolding was important to the adhesive strength; excess unfolding could be detrimental to adhesiveness. Huang and Sun used urea and guanidine hydrochloride (GH) as a modifier to study the soy protein adhesive. Results showed that urea and GH modification improved adhesive performance.[8]

Crosslinking is a specific chemical modification by which two molecular components can be joined together by a covalent bond.[9,10] Glutaraldehyde is an extensively used reagent for inactivating, stabilizing, or immobilizing proteins.[11] Glutaraldehyde polymerizes through a Schiff base. The glutaraldehyde polymer can couple two amino groups from two amino residues; methylene bridges are formed in the process (Fig. 1).[12] It mainly reacts with the ε-amino group of lysine and N-terminal of polypeptides, but also reacts with other nucleophilic groups in proteins, such as the sulfhydryl group of cysteine, the imidazole ring of histidine, and the phenolic hydroxyl group of tyrosine.[13] Crosslinking has been used for food and industrial applications to increase the mechanical properties of protein. Park et al. reported that soy protein crosslinked by glutaraldehyde had a higher tensile strength and greater elongation than the native soy protein.[14] Gerrard et al. reported that glutaraldehyde could strengthen the gluten network, providing better functional properties.[15] Bae et al. developed a gelatin-based glue through glutaraldehyde crosslinking.[16] Zhang

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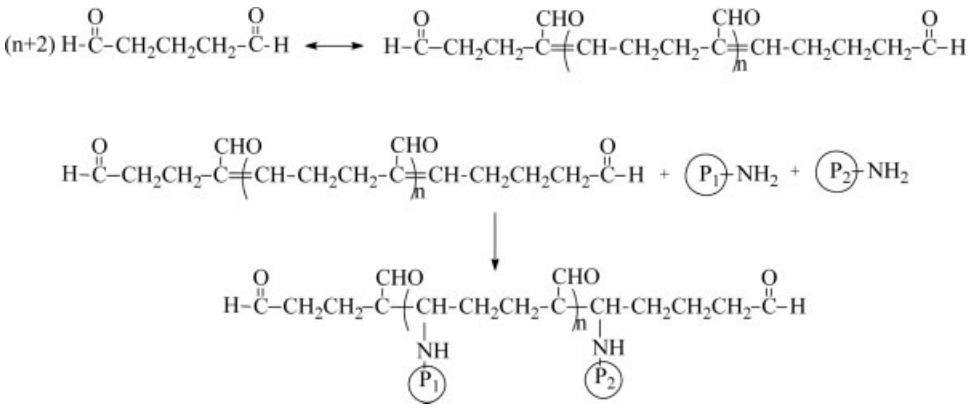

Figure 1 The reactions between protein and glutaraldehyde.

et al. increased mechanical properties of soy plastics by using glutaraldehyde.[17] The previous researches on glutaraldehyde crosslinking showed a potential for protein adhesive application. The objective of this research was to improve the water resistance of soy protein adhesive by introducing crosslinkage between amino groups of amino acid residue and to study the effect of crosslinking on thermal and morphological properties of soy protein adhesive.

EXPERIMENTAL

Materials

Soybean protein isolate (SPI), containing 85% (dry basis) protein and 3% moisture, was extracted from defatted soybean flour (Cargill, Cedar Rapids, IA) by isoelectric point precipitation at pH 4.2, then adjustment to neutral pH. The precipitate was freeze-dried (Model 62,111-0495 Freeze-Dryer, Virtis, Gardiner, NY) and then milled (Cyclone Sample Mill, UDY, Fort Collins) into powder. Cherry woods with dimensions of 127 mm (length) × 50 mm (width) × 5 mm (thickness) were provided by Veneer One (Oceanside, NY). Glutaraldehyde was purchased from Sigma Aldrich Company (St. Louis, MO).

SPI modification and preparation of adhesive testing specimens

SPI was suspended in distilled water to make a suspension at room temperature and was stirred (magnetic stirrer) for 1 h. Then stock glutaraldehyde solutions (25%, w/v) were added to make 4, 20, 40, or 80 μ*M* concentrations needed and the mixture were stirred for another 3 h. The final 10% (w/v) SPI suspensions were used as adhesive. Cherry woods were preconditioned in a controlled environment chamber (Model 518, Electro-tech Systems, Glenside, PA) for 7 days at 25°C and at a relative humidity (RH) of 50%. The SPI suspension was brushed onto one end of a piece of cherry wood until the entire area was completely wetted. Amount of adhesive applied on each piece was about 0.06 g, controlled by using a pipette and a consistent brushing procedure. Area of application on each end was 127 × 20 mm^2. The brushing and setting procedure described by Mo et al. was used.[18] The two pieces of slurry-brushed cherry wood were allowed to rest open at room temperature for 15 min and then were assembled and pressed at a pressure of 3.57 MPa at 130°C for 10 min, with a Hot Press (Model 3890 Auto 'M', Carver, Wabash, IN).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

To verify the effect of the crosslink on protein molecular weight, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by using a discontinuous buffer system on a 12% separating gel and 4% stacking gel as described by Laemmli.[19] The SPI suspension was mixed with SDS–PAGE sample buffer solution containing 5% β-mercaptoethanol, 2% SDS, 25% glycerol, and 0.01% bromphenol blue. The mixture containing about 13 μg SPI was loaded per well. The gel electrophoresis was carried out at 100 V constant voltage for 1.5 h. The gel was stained with 0.25% Coomassie Brilliant Blue-R250 and destained with solution containing 10% acetic acid and 40% methanol. Molecular weights of marker proteins were run along with the samples.

Thermal properties

The transition of protein from a native to denatured conformation is accompanied by rupture of inter- and intramolecular bonds. The energy involved in this denaturation process can be detected by differential scanning calorimetry (DSC). Thermal properties of modified protein adhesives were studied by using DSC (DSC 7, PerkinElmer, Norwalk, CT), which was calibrated with indium and zinc. All measurements were

conducted under a nitrogen atmosphere. A large DSC pan was used to hold about 50 mg of modified SPI. All samples were held at 25°C for 1 min and then scanned from 25 to 150°C at a heating rate of 10°C/min. The denaturation enthalpies were calculated as the area of denaturation peaks. All experiments were done in duplicate, and the average values were reported.

Rheological properties

Rheological measurements of the glutaraldehyde-modified SPI suspensions were performed by using a Bohlin CVOR 150 rheometer (Malvern Instruments, Southborough, MA) with a CP 4/40 cone and plate fixture (4° cone angle, 40-mm cone diameter). The distance between cone and plate was set to 150 μm for all measurements. Experiments were conducted under steady shear flow at 23°C. Shear rates ranged from 10 to 240 s^{-1}, at 10 s^{-1} increment. All experiments were done in duplicate, and average values were reported.

Morphological properties

A Hitachi *S*-3500 N (Hitachi Science System, Ibaraki, Japan) scanning electron microscope (SEM) was used to investigate the microstructure of modified and non-modified SPI. The adhesive solution was freeze-dried (Model 62,111-0495 Freeze-Dryer, Virtis, Gardiner, NY) and ground (Cyclone Sample Mill, UDY, Fort Collins, CO). The ground powder was used as a specimen. Specimens were affixed to an aluminum stub with two-sided adhesive tape, and were coated with an alloy of 60% gold and 40% palladium with a sputter coater (Desk II Sputter/Etch Unit, Moorestown, NJ). The microstructure of the SPI was observed with operation conditions at an accelerating voltage of 5 kV.

Shear strength measurement

The glued wood specimens were conditioned at 23°C and 50% RH for 2 days after hot pressing, and then were cut into five 20-mm-wide specimens. The cut specimens were continually conditioned for another 5 days before dry strength test. The dry strength was measured according to ASTM Standard Method D2339-98.[20] Three adhesion shear strengths, including dry strength, soak strength, and wet strength, were measured by using an Instron (Model 4465, Canton, MA). Water resistance was measured according to ASTM Standard Methods D1183-96 and D1151-00.[21,22] The preconditioned specimens were soaked in tap water at 23°C for 48 h, and then the specimens were tested immediately for wet strength. For the soak strength test, the specimens after 48 h soaking were conditioned for another 7 days before shear strength testing.

The crosshead speed for shear strength testing was 1.6 m/min. Stresses at maximum load were recorded as shear strength. The reported results are the average of five measurements.

RESULTS AND DISCUSSION

SDS–PAGE

Protein composition was analyzed by SDS–PAGE (Fig. 2). The main subunits of SPI were developed successively into α′, α, and β subunits of β-conglycinin and acidic (A) and basic (B) subunits of glycinin. SDS–PAGE patterns for crosslinked SPI obtained with glutaraldehyde were different from the control SPI. The characteristic bands were still visible for glutaraldehyde concentration of 4 μ*M*. The residues can be seen in the loading well of all glutaraldehyde concentrations; the amount was less in cells of 40 and 80 μ*M* glutaraldehyde than in cells of 4 and 20 μ*M* glutaraldehyde. The crosslinked protein molecules were greater than 97.4 kDa, resulting in an seemingly new band, which showed at the top of the resolving gel in lanes A, B, and C. Some of them could not migrate through the stacking gel, stacking at the bottom of the loading well.

The larger molecules of crosslinked protein posed a problem in maintaining the loading amount of the sample. The small opening of pipette tip allowed to do the

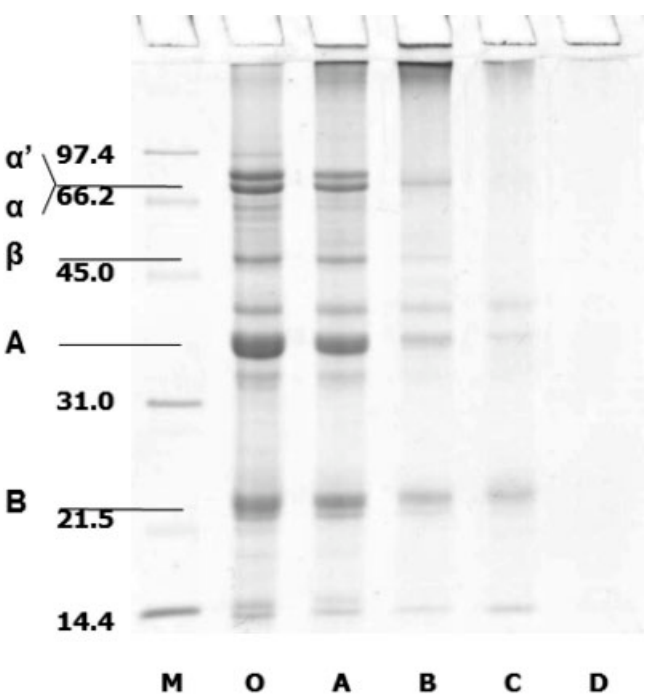

Figure 2 SDS–PAGE analysis for crosslinked soy protein samples. Numbers on left are the molecular masses of marker proteins in kDa. Letters on bottom are crosslinking conditions. M - marker proteins, O - no glutaraldehyde, A - 4 μ*M* glutaraldehyde, B - 20 μ*M* glutaraldehyde, C - 40 μ*M* glutaraldehyde, and D - 80 μ*M* glutaraldehyde. Glycinin acidic and basic subunits are A and B, respectively. The β-conglycinin subunits are labeled α′, α, and β.

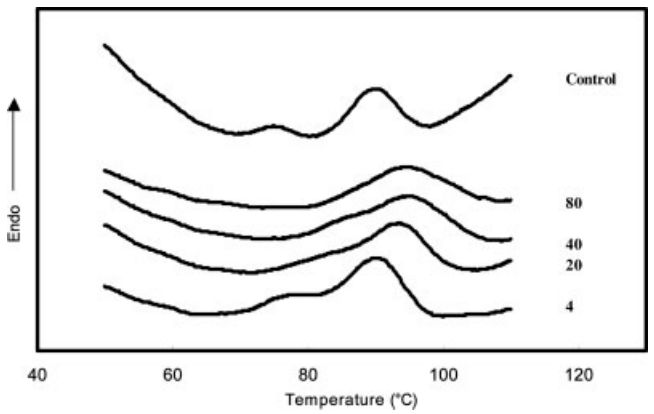

Figure 3 DSC thermogram of unmodified soy protein isolate (SPI, designated by control) and SPI crosslinked by glutaraldehyde at different concentrations (designated by the concentrations in μ*M*).

loading in sample well could not transfer enough crosslinked proteins because the crosslinked proteins presented as a cluster. Crosslinked proteins formed new covalent bonds (Fig. 1). These covalent linkages cannot be reduced by the reducing agent in the denaturing step because they are not disulfide bonds. The formation of covalent bonds could restrain proteins from adopting the random-coil configuration necessary for separation in PAGE. Breaking covalent bonds presenting in protein quaternary structure, i.e., reducing disulfide bonds in protein is usually necessary before they can adopt the random-coil configuration.[23] The bands in lane A are lighter than in lane O, followed by B and C, and eventually disappearing in lane D, indicating that more and more proteins have been crosslinked to form larger molecules through lanes A and B, as indicated by heavier color in the B loading well than in the lane A loading well. For lanes C and D, glutaraldehyde concentration was so high (40 and 80 μ*M*, respectively) that crosslinking was intensified in these two samples; not enough sample had been loaded in the loading well.

Thermal properties

DSC can determine the thermal transitions of materials by measuring the amount of energy absorbed or released by a sample. Protein denaturation can be detected in this way by measuring an endothermic peak in the DSC thermogram. Proteins with an ordered native structure undergo a transition with modification, lose a certain degree of native structure, and will be denatured by DSC further and present a random coil conformation.[24] The temperatures and heat flow associated with transitions were recorded by DSC (Fig. 3). The denaturation temperatures (T_d) and denaturation enthalpies (ΔH_d) were determined from the maximal peak temperature and the area of the peak, respectively. There are two peaks dedicated to denaturation of 7S and 11S in the thermogram of control SPI. The T_d of 73.8–88.5°C and ΔH_d of 0.55–3.52 J/g were observed for 7S and 11S, respectively. At the low glutaraldehyde concentration (4 μ*M*), two peaks with T_d (°C)/ΔH_d (J/g) values 76.7/0.29 and 90.0/4.29 presented, indicating higher molecular weight polymers formed due to crosslinking between 7S globulins and 11S globulins. When the concentration of glutaraldehyde increased to 20 and 40 μ*M*, only one peak was detected, with values of T_d (°C)/ΔH_d (J/g) of 93.1/7.53 for 20 μ*M* and 94.4/7.26 for 40 μ*M*. A very tiny shoulder merging to the peak showed that higher molecular weight polymers were produced, which could correspond to the disappearing bands on the upper part of lanes B and C in Figure 2. As glutaraldehyde concentration continued to increase (80 μ*M*), the values of T_d (°C) and ΔH_d (J/g) were 94.3 and 5.43, respectively. At this concentration, as more amino groups and N-terminal were being crosslinked and intertwined, some native structure of the protein held by noncovalent bonds may have been destroyed because of the stretch from the covalent bonding groups (crosslinked).

Rheological properties

The rheological behaviors of crosslinked protein differed with the different concentrations of glutaraldehyde. Viscosity increased as glutaraldehyde concentration increased. The 20 μ*M* glutaraldehyde modified SPI seemed to have a very good flowability. At higher concentrations (40 and 80 μ*M*), the protein became a gel and the viscosity could not be tested with the cone and plate fixture. Figures 4 and 5 showed the flow behavior and viscosity curves of control SPI and SPI modified by a low concentration of glutaraldehyde. Apparent viscosity decreased as shear rate increased, exhibiting a shear thinning behavior, which can be expressed by the Herscher-Bulkley model: $\tau = \tau_0 + K\dot{\gamma}^n$ where τ is the shear stress (N/m^2), τ_0 is the yield stress (N/m^2), $\dot{\gamma}$ is the shear rate (s^{-1}), and n and K are the flow behavior index and the consistency index, respec-

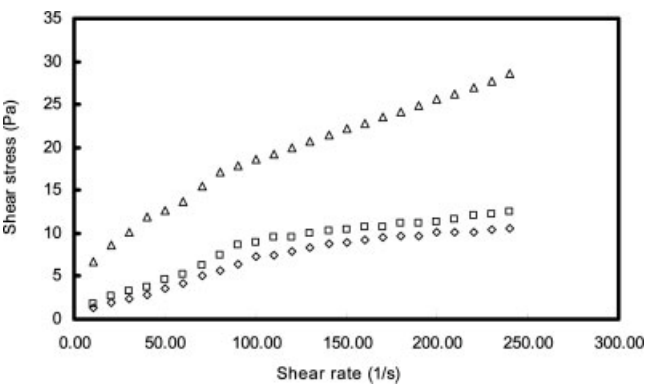

Figure 4 Shear behavior of crosslinked SPI. Glutaraldehyde concentration: 0 (◇); 4 (□); and 20 μ*M* (△).

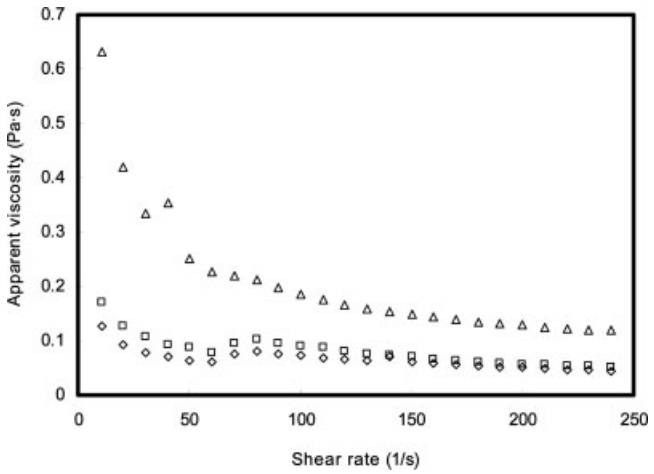

Figure 5 Shear rate-dependence of apparent viscosity of crosslinked SPI. Glutaraldehyde concentration: 0 (◇); 4 (□); and 20 μ*M* (△).

tively. The method of least squares was used to find the best-fitting equation: Estimate a τ_0 ($=\tau_{01}$) by extrapolating the plot of τ and $\dot{\gamma}$, plotting $\ln\tau$ and $\ln\dot{\gamma}$, and getting $k1$ and $n1$ from linear regression by using Microsoft® Excel. Then put $k1$ and $n1$ back into the equation, plot $\ln\tau$ and $\ln\dot{\gamma}^n$, and get τ_{02} and $k2$ from linear regression. Compare τ_{01} and τ_{02} until $\tau_{01} = \tau_{02}$, then τ_0 ($= \tau_{01}$), k ($= k1 = k2$), and n ($= n1$) are obtained. The values of τ_0, n, and K are summarized in Table I. Apparent viscosity of the glutaraldehyde-modified SPI adhesives increased as glutaraldehyde concentration increased at the same shear rate.

Morphological properties

Microstructure of SPI is displayed in Figure 6. The SPI particles were irregular compact disks for control and lower glutaraldehyde concentrations (A–C). Añón et al. reported similar observations of the particles for soy protein.[25] The particle size decreased until (C) (40-μ*M* glutaraldehyde modified SPI), then changed into bigger chunks when glutaraldehyde concentration increased (D and E). Figure 6(a–e) shows the surface morphology of the protein particles. Surface of control SPI and SPI modified with very low glutaraldehyde concentration (4 μ*M*) was coarse and fluctuant, displaying a rough appearance. The surface became very smooth and homogeneous as glutaraldehyde concentration increased to 20 μ*M* (c), then again became coarse and fluctuant with higher glutaraldehyde concentrations (d and e). Protein globulins were not so compact, and had some flexibility before the crosslink reaction occurred. At the low glutaraldehyde concentrations (4 and 20 μ*M*), amino groups accessible on the surface of protein were crosslinked, resulting in decreased movement freedom of protein molecules and reduced flexibility. Therefore, the protein structure of modified SPI was more compact, more rigid, and more brittle than that of the control SPI. It is easier for the brittle protein to break up and form smaller fragments as shown in (B) and (C), compared with control SPI fragments shown in (A). Compact protein structure of SPI modified at the low glutaraldehyde concentration presented a smoother surface on the fragment (b and c) than that from control SPI (a). At higher glutaraldehyde concentrations, the stretch of the crosslinked groups unfolded some protein structures held by noncovalent bonds. More protein surface area might be exposed, bigger fragments (D and E) and a rough surface (d and e) on it could be developed. On the surface of control SPI particles, scattered holes can be observed (a). Fewer holes presented on the surface of SPI modified at lower concentration, and no holes were observed on the surface of SPI modified at higher concentrations. These holes could be the result from vaporized ice during lyophilization. With the crosslinking proceeding, the number of amino groups and N-terminals in polypeptide chains decreased. Polar groups that can interact with water molecules lessened, and decreased water could be attached as the result. The decreased ice would leave fewer holes while lyophilizing.

Mechanical properties

Dry, wet, and soak strength of modified SPI showed the same trends (Fig. 7). Crosslinked SPI had significantly higher adhesive strengths than the unmodified SPI. At optimum concentration of glutaraldehyde (20 μ*M*), dry, wet, and soak strengths were 6.81, 3.04, and 6.27 MPa, and increased to 31.5, 115, and 29.7%, respectively, compared with those of unmodified SPI. At optimum concentration, the amino groups and N-terminal were crosslinked, hydrophobic groups were attached between the amino ends, and hydrophilicity of the amino ends was reduced. The structure of protein might be more compact (Fig. 6) at lower glutaraldehyde concentrations. This compact structure with more hydrophobic groups attached could induce more entanglements and crosslinking during thermal setting, and these structures would maintain themselves better than the unmodified SPI after water soaking. Shear strength decreased at higher glutaraldehyde concentrations, indicating that a high degree of crosslinking was not beneficial for adhesive performance. The protein mechani-

TABLE I
Rheological Parameters of Glutaraldehyde (G) Modified SPI

G concentration (μ*M*)	Yield stress, τ_0 (N/m^2)	Flow behavior index, n	Consistency index, K
0	0.30	0.7876	0.1578
4	0.30	0.7005	0.2928
20	2.00	0.5750	1.2410

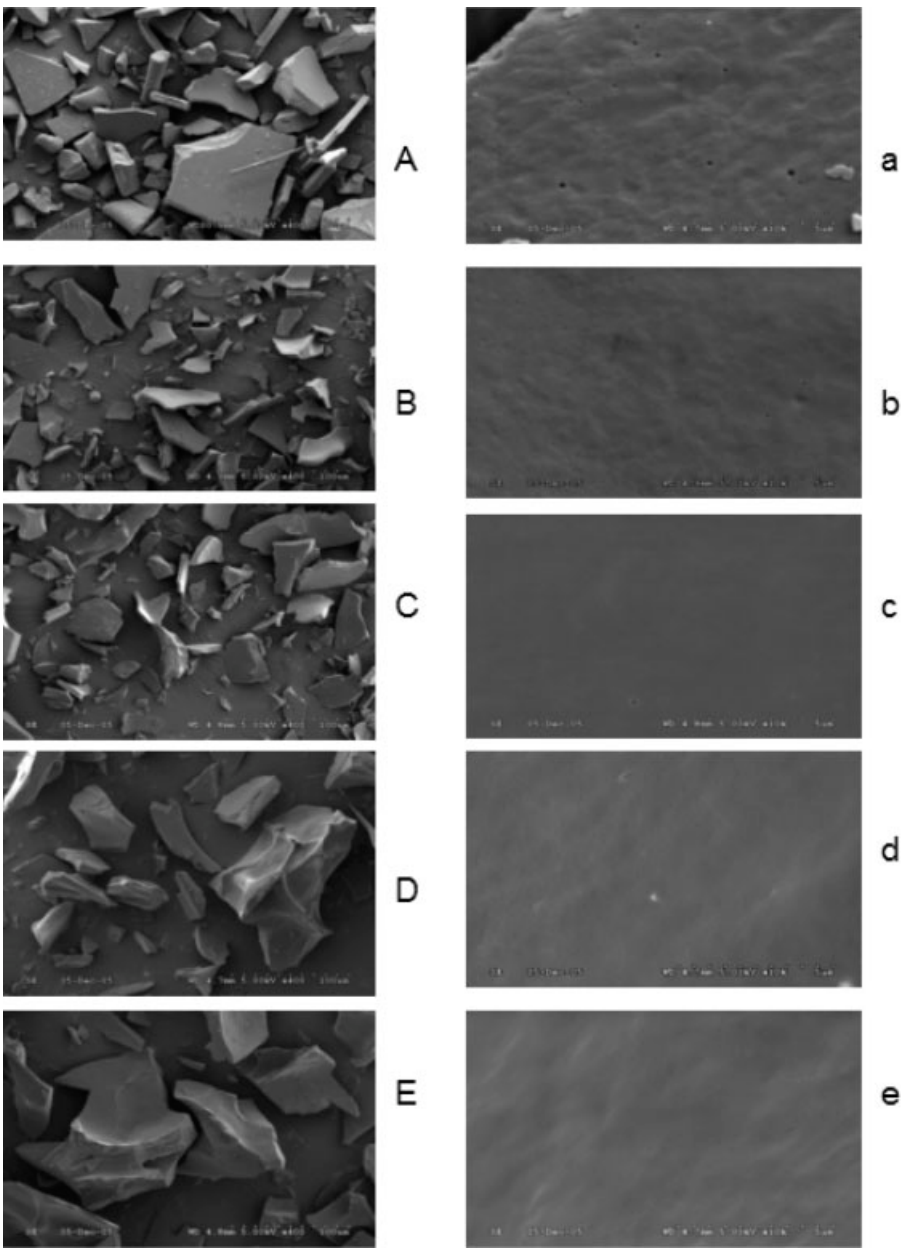

Figure 6 SEM micrographs of crosslinked soy protein. (A) a - control; (B) b - 4 μ*M*; (C) c - 20 μ*M*; (D) d - 40 μ*M*; and (E) e - 80 μ*M* glutaraldehyde modified SPI. Magnification: (A)–(E), ×400; (a)–(e), ×10k. Horizontal bars represent 100 and 5 μm for ×400 and ×10k magnifications, respectively. Figures are reproduced at 16% of the original size.

cally interlocks with wood (spreads and penetrates into the porous structure of the wood interface) before physical attraction and chemical bonding begin.[26] The mechanical interlock would happen while sitting in ambience. The physical attraction and chemical bonding would happen mainly when the thermal setting procedure begins. With higher glutaraldehyde concentration (40 and 80 μ*M*), more covalent bonds were formed and some local structure might be distorted. The SPI became a gel with a poor flowability, which affected its spread and penetration. Protein was entangled and crosslinked when it was subjected to heat and pressure in the hot press. The more hydrophobic groups attached on soy protein with higher glutaraldehyde concentration would affect the physical attraction and chemical bonding between the protein and the wood surface because the wood surface is polar in nature.[27] Excess hydrophobicity and distorted structure could be detrimental to the adhesive strength.

CONCLUSIONS

Glutaraldehyde crosslinks protein by reacting with amino groups. Crosslink increased the molecular weight and changed the conformation of protein. Cross-

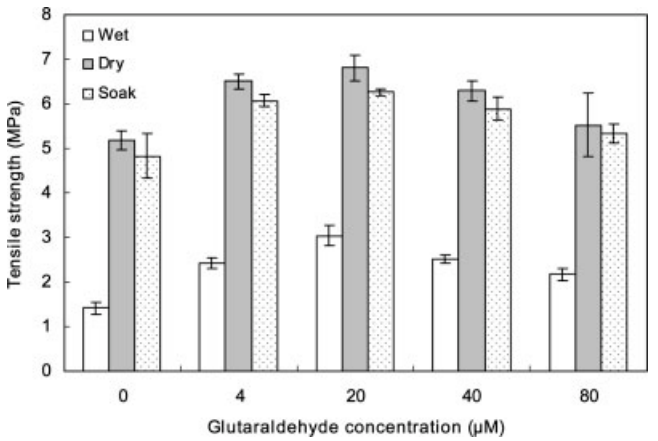

Figure 7 Effect of glutaraldehyde concentration on protein adhesive performance.

linkage resulted in a decreased number of amino groups and an increased number of hydrophobic groups from glutaraldehyde in protein. Concentration of glutaraldehyde had a significant effect on adhesion of modified SPI. The mild conformation change resulting from cross-link could benefit adhesive performance. Higher glutaraldehyde concentration could induce more conformation and structure changes that might not be favorable for adhesive performance. At optimum glutaraldehyde concentration (20 μ*M*), wet strength of modified SPI was 115% greater that that of control SPI.

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