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Silk Fibroin/Chitosan Conjugate Crosslinked by Tyrosinase

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Abstract: Two biopolymers, silk fibroin (SF) and chitosan, were conjugated by tyrosinase (EC 1.14.18.1), a polyphenolic oxidase, to improve their physicochemical properties, such as their thermal properties and morphological stabilities in organic solvents. The crosslinking between SF and chitosan took place mainly through Michael addition reactions. A main reaction between the amino groups in chitosan and *o*-quinone, the oxidation product of the tyrosyl residue in SF, was confirmed by UV spectroscopy. Measurements of viscosity and light scattering indicated that the crosslinked SF/chitosan conjugate was compact: it had a smaller particle size because of tight bonding forces between the SF and chitosan molecular chains. Thermal decomposition of SF/chitosan conjugates crosslinked by tyrosinase occurred at higher temperatures. The adhesiveness of the SF/chitosan conjugates decreased steadily as the crosslinking reaction progressed. We propose that this new crosslinking method be used for the preparation of silk fibroin/chitosan conjugates using tyrosinase. We expect that SF/chitosan conjugates crosslinked by tyrosinase can be used preferentially in biomedical applications because of its unique properties and non-toxicity.

Keywords: silk fibroin, chitosan, conjugate, tyrosinase, crosslinking.

Introduction

Silk fibroin (SF) and chitosan are well known as biocompatible polymers capable of being easily and largely accessible from nature. SF, a natural protein from *Bombyx mori*, is a semicrystalline polymer mainly composed of glycine, alanine, serine and tyrosine.[1] Recently, SF has been studied in fields of bio-industry, such as fibers,[2] hydrogels for controlled drug release,[3] scaffolds for cell culture,[4] films for enzyme-immobilized biosensors[5] due to its biocompatible properties and durability in a biological environment. Additionally, SF was examined vigorously as supplemental dietary foods since amino acids and peptides composing SF had specific functions.[6] And chitosan rich in nature is a polysaccharide prepared by *N*-deacetylation of chitin obtained from shells of crabs or shrimps. Chitosan is also applied as hydrogels, films, scaffolds, nutrients and so on.[7,8]

There have also been many researches on blends[9] and crosslinked conjugates[10] of these two polymers. SF/chitosan blend can be prepared by strong hydrogen bonding between carbonyl groups in SF and amino groups in chitosan while crosslinked conjugate of SF and chitosan, which has more stable structure than blend, is formed by the addition of crosslinking agents,[10] commonly glutaraldehyde or isocyanate. However, a toxicity of chemical crosslinking agents can limit its application to biomedical fields. Therefore, more biocompatible crosslinking agents or other crosslinking methods without any chemical reagents may be necessary for the application of crosslinked SF/chitosan conjugate to biomaterials.

Tyrosinase (EC 1.14.18.1), a polyphenol oxidase, oxidizes the tyrosine or 3,4-dihydroxyphenylalanine (DOPA) to *o*-quinone.[11] *o*-Quinone can be reacted with hydroxyl group or amino group through Michael addition reaction[12] or Maillard reaction[13] since it is a highly reactive substance. Mussel adhesive protein can adhere strongly to a variety of substrates in wet environments[14] and insect cuticle is also hardened through above reaction pathway.[15] Tyrosine is abundant in SF (about 12 mol%) and chitosan has many amino groups.

Therefore, in this study, we tried to prepare the crosslinked conjugate of SF and chitosan without any chemical crosslinking agent for nontoxic and biocompatible materials. The reaction pathways are proposed as follows. The tyrosyl residues in SF are oxidized by tyrosinase to form the *o*-quinone residues. These oxidized residues can be nonenzymatically reacted with amino groups in chitosan through Michael addition reaction or Maillard reaction, resulting in the formation of a 3-dimentional structure. The crosslinked SF/chitosan conjugate was characterized by UV/visible spectroscopy, viscosity measurement, light scattering, DSC, and adhesiveness.

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Experimental

Materials. To obtain a pure silk fibroin (SF), raw cocoon of *Bombyx mori* was degummed using an aqueous sodium carbonate solution (0.3% o.w.f.) with *Marseillus* soap (0.5% o.w.f.) for 30 min at the boiling condition to remove sericin and other impurities enveloping SF. Chitosan (Brookfield viscosity 800.000 cps, 14624DB) and tyrosinase (EC 1.14.18.1) (2,870 units/mg, T-7755) were purchased from Sigma-Aldrich, Korea.

For the preparation of SF solution, degummed SF was treated with a solution of calcium chloride, water and ethanol ($CaCl_2$: water : ethanol = 1 : 8 : 2, molar ratio) for 30 min at 70 °C under reflux. Subsequently, the solution was dialyzed in distilled water for 3 days to remove the neutral salts using semipermeable cellulose tubing (MWCO 12,000-14,000, Sigma). After filtration of the dialyzed solution, it was diluted to reach 1.0 wt% of final concentration.

In case of chitosan solution, 1.2 g of chitosan granule was dispersed in water and then pH value was lowered to 2.0 by the addition of hydrochloric acid. After the mixture was stirred for 24 hrs, insoluble parts were removed from the chitosan solution by vacuum filtration apparatus. The pH value of the solution was checked and adjusted since the value was changed during dissolving process. Finally, the chitosan solution was controlled to reach 1.0 wt% of the concentration.

Reaction of SF/Chitosan by Tyrosinase. Before conducting the enzymatic reaction, the pH of the chitosan was adjusted to 5.0-5.5 using 1.0 M sodium hydroxide due to an inactivation of tyrosinase in low pH values. SF (1.0 wt%) and chitosan (1.0 wt%) solutions were mixed at various proportions. Tyrosinase solution was added in the mixed solution and then, the solution was incubated at 28 °C for certain times. The amount of tyrosinase was controlled to be 28.7 U/1 mL of the final solution. As a control, the solution without tyrosinase was incubated under the same condition.

Analysis.

Viscosity Measurement: SF (1.0 wt%) and chitosan (1.0 wt%) solution were mixed at a volume-ratio of 2:8. After initiating the reaction by the addition of tyrosinase, the change of shear viscosity during the reaction was monitored using a Brookfield DV-E viscometer with S01 spindle at a rotation speed of 100 rpm.

Light Scattering: To determine the variation of particle size during the crosslinking reaction of SF/chitosan by tyrosinase, light scattering measurement was performed using Electrophoretic Light Scattering Spectrophotometer (ELS-8000) at 50 times of beam-canning and repeated 3 times.

UV/Visible Spectroscopy: An 1.98 mL of SF/chitosan (2:8) solution was prepared in 2.5 mL cuvette and then, 0.02 mL of tyrosinase solution was added to the mixed solution to initiate the reaction. From UV/VIS spectrophotometer (UVIKON 923, KONTRON, Italy), the change in absorbance was monitored at the range of 200-600 nm.

DSC: The SF/chitosan solution (2:8) was incubated sufficiently at 28 °C after initiation by tyrosinase and then, lyophilized. The thermal behavior of lyophilized sample was determined using DSC 2910 (TA instruments, USA). The measurement was carried out in the temperature range of 50 to 400 °C under nitrogen at a scanning rate of 10 °C/min.

Adhesiveness: Glass slides (Marienfeld, Germany), which were selected as an adherend, were cleaned by soaking for 24 hrs in a mixture of water, H_2SO_4, and K_2CrO_7 (10:5:1) and then, washed with deionized water. After drying fully, the reaction mixture was spread onto each face of two glass slides, and the faces were placed in contact with 25 × 25 mm overlapping surface area. After the overlapping surfaces were pressed, the samples were clipped together with a binder clip and dried in air. Shear strength was measured using mechanical tester (MMT-2000, Rheometric Scientific, Inc., USA).

Results and Discussion

Viscosity Measurement. It is known that the crosslinking reaction between solutes in solution commonly results in an increase of viscosity.[16,17] However, if the solutes exist as a colloidal state by crosslinking reaction, the viscosity tends to be decreased. Figure 1 shows the change of the viscosity

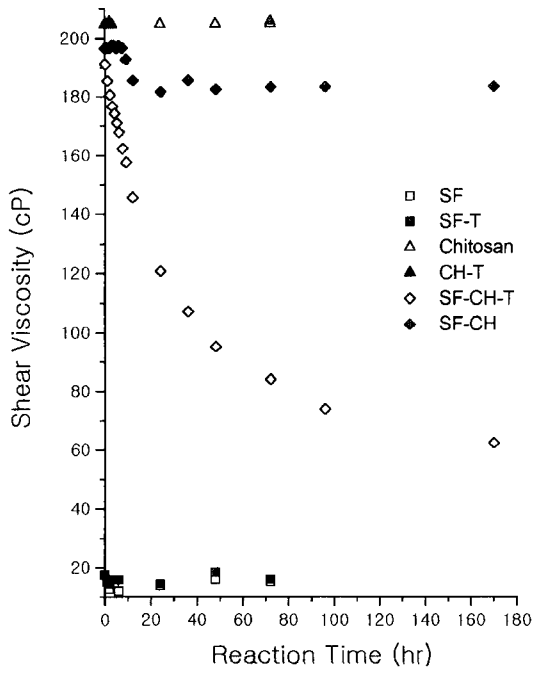


Figure 1. Changes in the viscosity of SF, chitosan, SF/chitosan blend (SF-CH), and SF/chitosan conjugate reacted by tyrosinase (SF-CH-T) with reaction times. Here, SF-T and CH-T represent the SF and chitosan solution incubated with tyrosinase, respectively.

of various solutions with a reaction time. In case of the solutions of SF and chitosan (0.8 wt%), the values of viscosity were maintained constantly around 18 and 205 cPs, respectively, regardless of the reaction time and the existence of tyrosinase. Chitosan was not affected at all by tyrosinase due to no existence of specific substrate for the enzyme in chitosan while SF, which may be capable of the generation of the enzymatic reaction, already existed as a colloidal state before the reaction and no viscosity changed during the reaction.

When the incubation of SF/chitosan (volume ratio 2:8) was carried out without tyrosinase, there was little change of viscosity during entire reaction times (170 hrs). However, the viscosity was slightly decreased after about 10 hrs, indicating that dissolved chitosan might be aggregated with colloidal SF particles by means of entanglement of linear chains or hydrogen bonds.

On the other hand, the viscosity of the mixed SF/chitosan solution with tyrosinase decreased sharply and continuously with a reaction time. This result can be explained as follows. Tyrosine residues in SF are mostly transformed to *o*-quinone, a reactive group, by tyrosinase. Subsequently, the dissolved chitosan is crosslinked nonenzymatically with the reactive sites in SF. Considering that tyrosine is originally contained as much as 10-12 mol% among the amino acids composing SF, the reactive groups of *o*-quinone in SF are produced by tyrosinase as large amounts. As a result of nonenzymatic crosslinking reaction between colloidal SF and dissolved chitosan, the amount of the chitosan remaining in a solution state is decreased, resulting in a decrease of the viscosity of the mixed solution.

If the crosslinked chitosan exists in dissolved state, the viscosity will increase with a reaction time.[16,17] However, SF is insoluble in water and exists as aggregated colloidal state. Moreover, crosslinked chitosan with SF might also exist as a colloidal state. Colloidal particles of SF and SF/chitosan conjugate cannot affect the viscosity of SF/chitosan solution. Therefore, when the enzymatic and subsequently nonenzymatic reaction occurred in the mixed SF/chitosan/tyrosinase solution, the amount of chitosan dissolved in water was reduced through the crosslinking reaction, resulting in a continuous decrease of the viscosity.

Particle Size Analysis. Light scattering can inform about the particle size and its distribution in a solution. Figure 2 shows the particle size distributions of SF, chitosan, SF/chitosan blend, and SF/chitosan conjugate crosslinked by

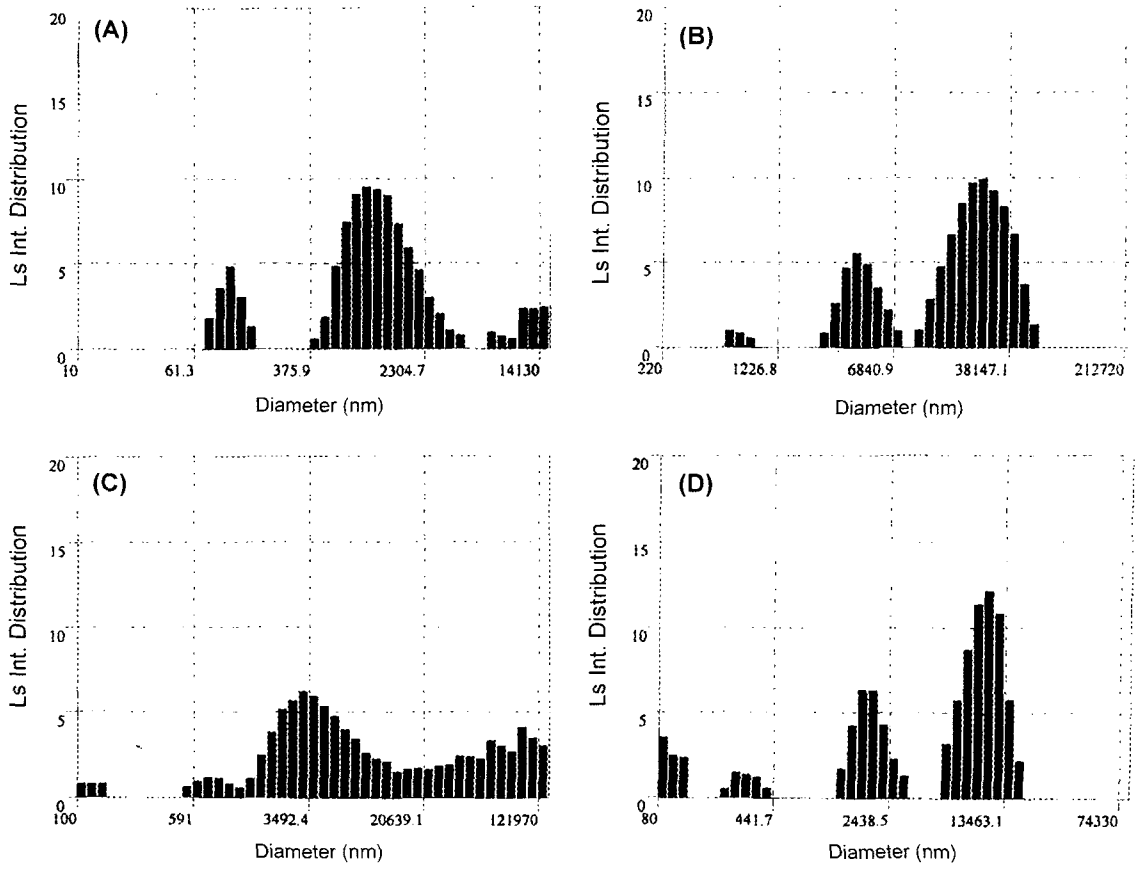


Figure 2. Particle size distribution of (A) SF, (B) chitosan, (C) SF/chitosan blend, and (D) crosslinked SF/chitosan conjugate. The mixing ratio of SF and chitosan is 5:5 and the reaction was carried out at 28 °C for 48 hrs.

tyrosinase. Here, the particle size means a hydrodynamic radius because SF and chitosan are not globular proteins but linear ones. As shown in Figure 2(A), the size of SF one molecule was about 80~140 nm. When tens or hundreds of molecules were aggregated each other by the entanglement of linear polymer or hydrogen bonds, the lump can be considered as one particle. The most frequent size value of SF particle was about 1,000 and 11,000 nm.

In case of chitosan (Figure 2(B)), the hydrodynamic radius of one molecule was around 3,600 nm and those of lumps around 23,000 nm. The size of chitosan was turned out to be larger than that of SF since chitosan was a series of polysaccharide composed of cyclic sugar structure with larger volume as well as its higher molecular weight.

When SF and chitosan solution was mechanically blended without tyrosinase, the sizes of particles appeared in a broad range (Figure 2(C)) due to various types of aggregations of SF-SF, chitosan-chitosan, and SF-chitosan. On the other hand, the size distribution of the particles from the SF/chitosan solution with tyrosinase (Figure 2(D)) was much different from that without tyrosinase. The particle sizes in SF/chitosan solution with tyrosinase were 100, 250, 1,500, and 9,800 nm while the maximum size of particle in SF/chitosan blend solution was 120,000 nm, meaning that the conjugate was contracted strongly.

UV/Visible Analysis. Figure 3 shows the characteristic UV/visible spectra of the SF/chitosan conjugates crosslinked by enzymatic initiation followed by nonenzymatic reaction. It has been reported that the absorption peaks at 300 and around 360 nm exhibit *o*-quinone residues and *p*-crosslinked quinones by Michael-addition reaction, respectively.[18] The absorption intensity at 300 nm increased sharply when tyrosinase was added to the SF and SF/chitosan solution. This means that tyrosine residues in SF were wholly transformed to *o*-quinone residues by tyrosinase.

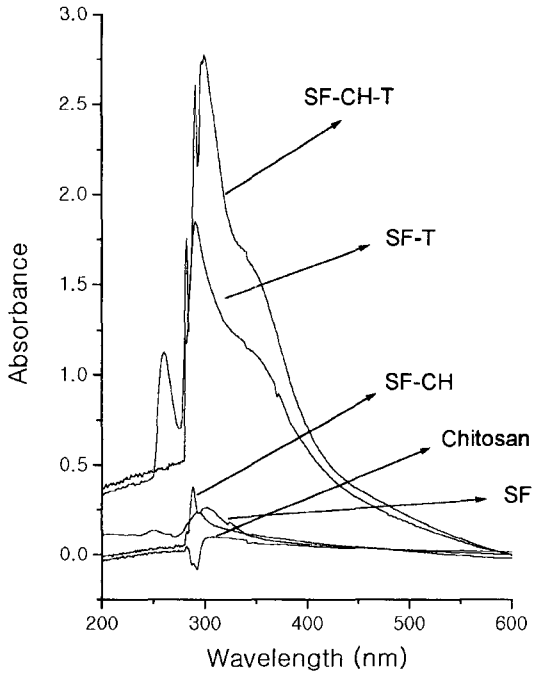
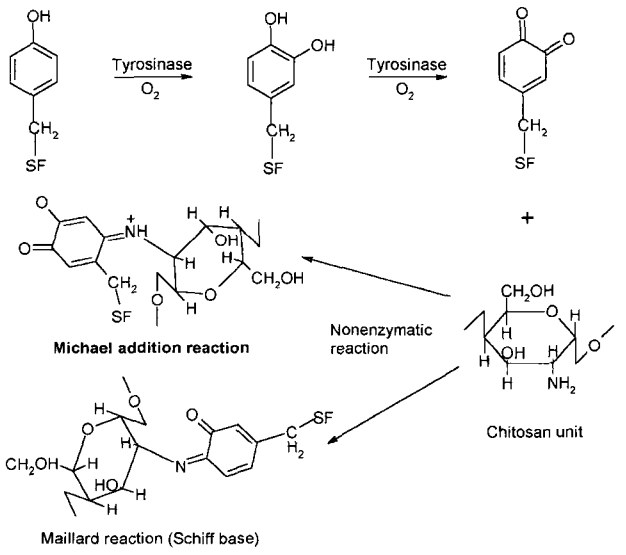


Figure 3. Spectroscopic observation of the transformation of tyrosyl residues in SF to quinone residues and subsequent crosslinking reaction with chitosan from UV/visible measurement. Here, SF-CH-T and SF-T represent the SF/chitosan (SF-CH) and SF reacted by tyrosinase, respectively.

Moreover, strong shoulder peak at around 360 nm also appeared, indicating that the crosslinking reaction occurred via the formation of *p*-crosslinked quinones. It can be explained that amino groups of chitosan and *N*-terminal amino groups in SF reacted with enzymatically generated *o*-quinone residues in SF.[18] The crosslinking formation of SF and chitosan would be superior to that of SF itself since the amino groups in chitosan are more reactive than *N*-terminal amino groups in SF. The possible pathways of the enzymatic and nonenzymatic reactions between chitosan and SF are given in Scheme I. Here, Maillard reaction,[13] a reaction of carbonyl group of quinone and amino group to form Schiff base, is also proposed for the possible reaction, although there is no direct evidence in this study.

Thermal Analysis. Differential scanning calorimetry was carried out for obtaining the thermal properties of the crosslinked SF/chitosan conjugates. As shown in Figure 4, SF shows typical thermal transitions. Glass transition temperature (T_g), recrystallization temperature (T_c), and thermal decomposition temperature (T_d) were 170, 220, and 280 °C, respectively.[19] However, T_g and T_c disappeared and T_d shifted to 320 °C for the SF crosslinked by tyrosinase (sample SF-T). In case of SF/chitosan blend (sample SF-



Scheme I. Reaction pathways for enzymatic oxidation of tyrosyl residues in SF and nonenzymatic crosslinking reaction with chitosan.

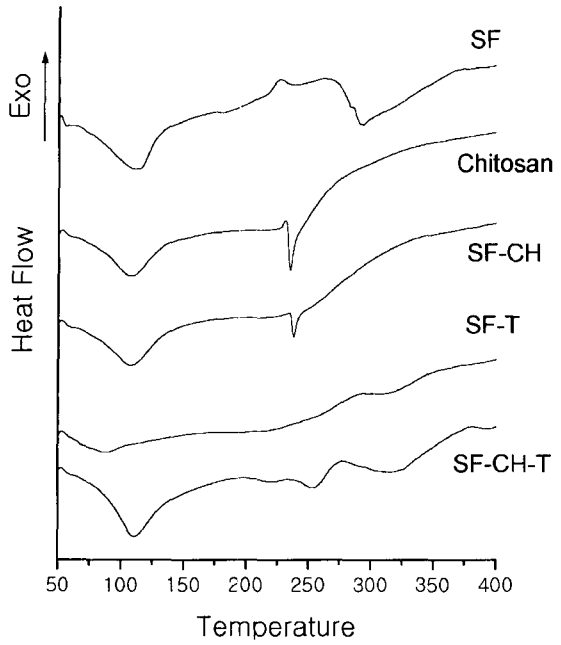


Figure 4. DSC thermal data of SF, chitosan, SF/chitosan blend (SF-CH), crosslinked SF (SF-T), and crosslinked SF/chitosan conjugate (SF-CH-T).

CH), T_d was clearly observed at 220 °C, which was same as T_d of chitosan.[20]

On the other hand, the thermal behavior of SF/chitosan conjugate crosslinked by tyrosinase (sample SF-CH-T) was markedly different from that of SF/chitosan blend. The endothermic peaks were observed at 250 and 320 °C attributed to the thermal decomposition of chitosan and SF, respectively. T_ds of chitosan and SF were slightly shifted to higher temperatures due to the compact size of conjugate formed by crosslinking with strong chemical interactions. The different thermal behavior also indicates that the reactions occurred between SF and chitosan through the formation of *p*-crosslinked quinone by tyrosinase.

Adhesive Properties. Figure 5 shows the adhesive shear strength of the samples dried in air with or without tyrosinase. Chitosan had no changes of adhesiveness regardless of the existence of tyrosinase because no reaction occurred by tyrosinase. However, the adhesiveness of SF and SF/chitosan treated with tyrosinase decreased as compared with those containing no enzyme. Moreover, the adhesiveness of SF/chitosan crosslinked with tyrosinase for longer reaction times (60 hrs) was much lower. This is another indirect evidence of the crosslinking reaction between SF and chitosan by tyrosinase.

Different adhesive properties can be explained by the difference in particle sizes of polymers. As mentioned before, the particle size, a hydrodynamic radius of particle, becomes small due to tight compression by the crosslinking reaction.

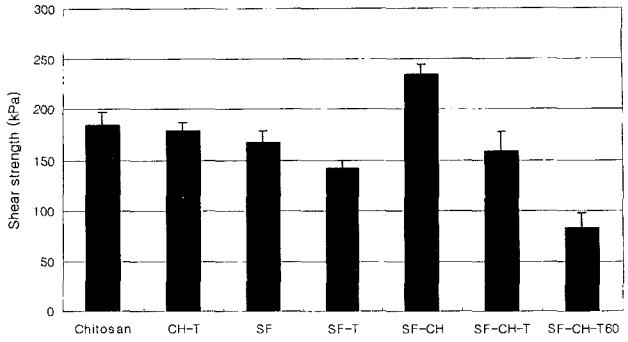


Figure 5. Adhesive shear strength of chitosan, chitosan with tyrosinase (CH-T), SF, crosslinked SF by tyrosinase (SF-T), SF/chitosan blend (SF-CH), and SF/chitosan conjugate incubated with tyrosinase for 24 hrs (SF-CH-T) and for 60 hrs (SF-CH-T60).

The crosslinked SF/chitosan conjugates are also dispersed as a colloidal state. The larger the particle size is, the more broadly the particle can be spread and the more strongly it is bonded onto adherend. Therefore, it was observed that the adhesiveness of SF/chitosan blend, which had the largest size as shown in Figure 2, appeared most highly among the samples. If crosslinked polymers can exist as a solution state, the adhesiveness as well as viscosity may increase,[16] which is different from our results.

Conclusions

On the base of theoretical background for the reaction mechanism induced by enzyme, we carried out the crosslinking reaction of SF and chitosan using tyrosinase. From the measurements of viscosity, UV, light scattering, DSC, and adhesiveness, it could be confirmed directly and indirectly that the chemical reaction occurred between SF and chitosan by tyrosinase. Tyrosyl residues in SF are transformed to quinone residues, very reactive groups, by tyrosinase and then, amino groups in chitosan are crosslinked onto the reactive site in SF through nonenzymatic pathways. Moreover, the SF/chitosan conjugate was contracted strongly and tightly due to the joint effect of adjacent molecular chains by the crosslink formation. Conclusively, the crosslinked SF/chitosan conjugate can be prepared through enzymatic process, which is biocompatible process without any use of chemical crosslinking agent. The crosslinked conjugate will be applied to biomaterial fields, especially, where the hardness would be requested, such as scaffolds.

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