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Crosslinking Reaction of Phenolic Side Chains in Silk Fibroin by Tyrosinase

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Abstract: Tyrosinase oxidizes the tyrosyl residues in silk fibroin (SF) with oxygen, resulting in the production of *o*-quinone residues. Subsequently, the inter- or intramolecular crosslinks are formed by reaction with amino groups in through nonenzymatic process. The measurement of oxygen consumption proved that the tyrosyl residues in SF were mostly oxidized to quinone residues by tyrosinase. The reaction mechanisms were proposed in this study and the crosslinking reaction of *o*-quinone residues and the enzymatic oxidation of tyrosyl residues could be confirmed by the measurements of UV, ^{1}H-NMR and GFC.

Keywords: Silk fibroin, Tyrosinase, Crosslinking reaction, UV-vis spectroscopy, ^{1}H-NMR

Introduction

Mussel adhesive protein (MAP) can adhere strongly to a variety of substrates in wet environments [1]. Although there has been no evidence how the MAP plays a key role exactly in forming strong cohesive bonds to the wetting surfaces, some presumable hypotheses were proposed. It was suggested that the oxidation of 3,4-dihydroxyphenylalanine (DOPA) was mainly responsible for the characteristic bonds [2]. In these reactions, tyrosinase (EC 1.14.18.1) oxidizes DOPA in the MAP (ca. 5-20 mol %) to form *o*-quinone, a very reactive group. The *o*-quinones may couple intra- or intermolecular quinones (quinone-coupling reaction) [2] or react with polar groups such as amino groups (Michael-addition reaction) [3] and hydroxyl groups [4], resulting in a new crosslinking formation. Many efforts for the synthesis of water-resistant adhesives using these reactions have been carried out [5,6]. Additionally, the reactions were applied for the post-translation processing in which biological molecules such as enzymes were coupled with chitosan rich in amino groups [7].

Silk fibroin (SF), which is a natural protein mostly composed of glycine, alanine, serine and tyrosine, has been studied in the fields of biomaterials [8] due to its specific functionality. The molecular weight of SF is about 300-350 kDa [9]. Repeating unit in the crystalline region of SF is known as Ala-Gly-Ser-Gly-Ala-Gly [10]. SF also contains tyrosine in semicrystalline or amorphous region [11]. Tyrosine is a precursor of DOPA against tyrosinase [12] and the content of this aromatic amino acid is about 10-12 mol % in SF [9]. Therefore, it is expected that the SF may be used as a water-resistant adhesive protein by itself or with other materials, which are abundant in functional groups, due to many tyrosyl residues contained in SF polymers.

Although crosslinking reactions of protein polymer, including SF, have been reported enormously, there is no study concerned with enzymatic crosslinking reaction of SF. Therefore, in this study, we investigated the reactions possibly occurred in phenolic side chain of SF by tyrosinase. The reaction pathways can be suggested as follows. The tyrosyl residues in SF were oxidized by tyrosinase to form the *o*-quinone residues in repeated positions. And then, the crosslinks were induced by the self-addition reaction between intra- or intermolecular quinone residues or between quinone residues and *N*-terminal amino groups of SF molecules. In order to confirm the reactions, the oxygen concentration in SF solution was monitored to for the oxidation of tyrosyl residues in SF molecules. UV/VIS spectroscopy, ^{1}H-NMR and gel filtration chromatography (GFC) measurements were used for the elucidation of the reaction mechanism. Using this reaction, it is expected that possibility for application of biomaterial would be elevated by improving its property.

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Experimental

Preparation of SF Solution

Raw cocoon of *Bombyx mori* was degummed using 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. Subsequently, the degummed SF was treated with a solution of calcium chloride, water and ethanol ($CaCl_2$:water:ethanol = 1:8:2, molar ratio) at 70 °C for 30 min under reflux.

Reaction of SF by Tyrosinase

After 10 m*l* SF solution containing 2 mg of tyrosinase (T-7755, Sigma) was incubated at 20 °C for a certain time, 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). Oxygen consumption and UV measurement were carried out in the salt-including solution, while a dialyzed solution was used for ^{1}H-NMR and GFC.

Measurements

The oxygen consumption: The sensor of dissolved oxygen

meter (inoLab Multi Level 1, Germany) was inserted in 9 m*l* of SF solution containing neutral salts. And then, 1 m*l* of tyrosinase solution (2 mg/m*l*) was added to the SF solution and stirred. The dissolved oxygen concentration was detected during the reaction at 20 °C for 40 min.

UV

1.8 m*l* of SF solution was prepared in 2.5 m*l* cuvette and 0.2 m*l* of tyrosinase solution was added to the solution to initiate the reaction. In standard cuvette, albumin was added as much as tyrosinase instead of the enzyme. From UV/VIS spectrophotometer (UVIKON 923, KONTRON, Italy), the change in absorbance was monitored at the range of 200-600 nm.

^{1}H-NMR

After SF solution was oxidized and coupled by tyrosinase, neutral salt was removed using dialysis. From H-NMR (Avance-600, Bruker, Germany), the transformations of aromatic side chains in SF molecules were observed.

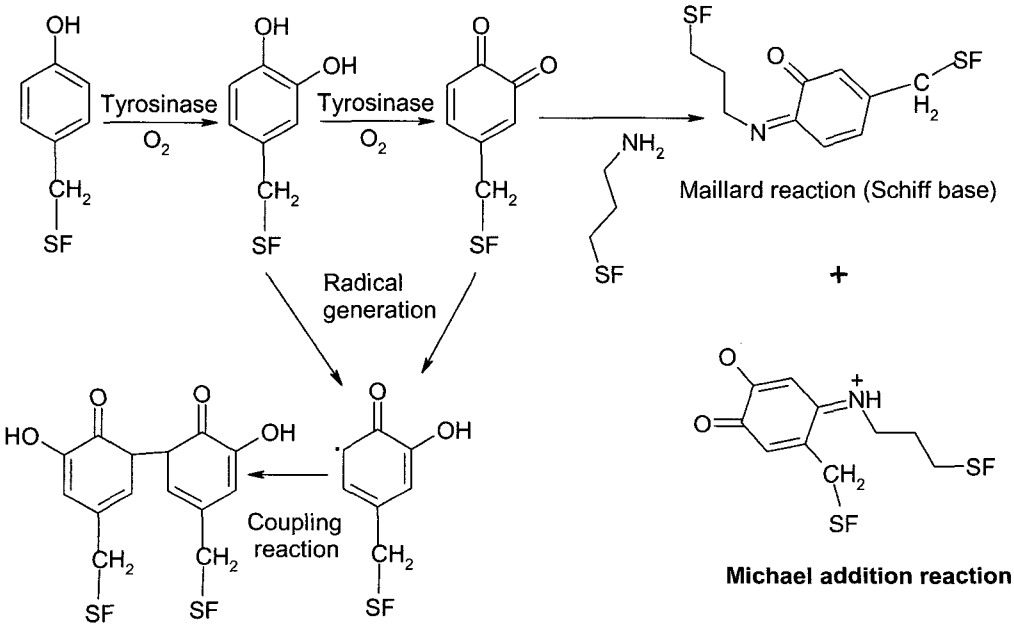
GFC

For a preparation of matrix, Sephadex-100 was fully swelled in buffer solution (pH 8.0) composed of guanidine-HCl and Trizma-base. Column was filled with the matrix and stabilized by flowing buffer solution. SF and reacted SF were dissolved in buffer solution and injected to the column. Using standard molecular kit (Sigma) contained with aprotinin (MW 6,500), cytochrome C (MW 12,400), carbonic anhydrase (MW 29,000) and bovine serum albumin (MW 66,000), retention time was monitored by UV detector (M720, Youngin, Korea) at 280 nm. Here, the flowing rate was controlled constantly to 0.37 m*l*/min.

Results and Discussion

It has been known that the tyrosine could form melanin by the action of tyrosinase [13]. However, when the aromatic amino acid was located in polypeptide or protein, the tyrosyl residue was oxidized to dihydroxyphenyl and, consequently, *o*-quinone residue by tyrosinase [1]. Here, *o*-quinone residue plays an important role in the formation of crosslinking bond [2] by means of nonenzymatic process. Enzymatic catalysis of the substrates requires the oxygen while oxygen is unnecessary for nonenzymatic reaction of *o*-quinone due to its self-reaction. Since *Bombyx mori* SF contains the aromatic amino acid (10-12 mol-%, tyrosine) [9], the crosslinking reaction can be occurred in SF proteins via several pathways (Scheme 1).

Figure 1 shows the amounts of the oxygen consumption during the reaction. The oxygen consumption reached the maximum (in 20-30 min) and leveled off after the reaction was completed. This result indicates that tyrosyl residues in SF are normally oxidized to *o*-quinone by tyrosinase as same as tyrosine is a free state [14]. The oxygen-consuming rate, the maximum amount of consumed oxygen and the time to reach a maximum concentration of oxygen consumed are different depending on the concentration of SF used. The oxygen concentration in the solution reached an equilibrium state at the maximum point. As well, the equilibrium state was maintained for a long time with an increase of the concentration of SF. The reason is that the oxygen-consuming rate in the solution during the oxidation of a few unreacted tyrosyl or



Scheme 1. Proposed reaction pathways for tyrosyl residues in silk fibroin (SF).

dihydroxyphenyl residues by tyrosinase is same as the oxygen-dissolving rate in air. The higher the concentration of SF is, the more the unreacted tyrosyl or dihydroxyphenyl residues remain at the maximum point. Therefore, the equilibrium state in a high concentration of SF is continued longer than that in a low concentration.

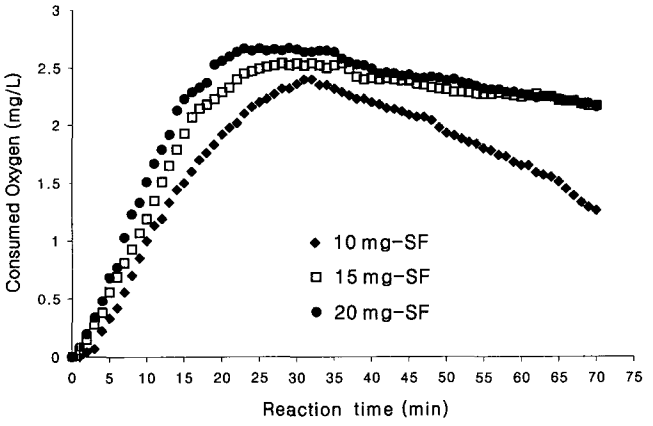


Figure 1. Oxygen consumption during the reaction catalyzed by tyrosinase. 10 mg-SF (♦), 15 mg-SF (□) and 20 mg-SF (•) represent the contents of SF in solution when 2 mg of tyrosinase was added in each solution.

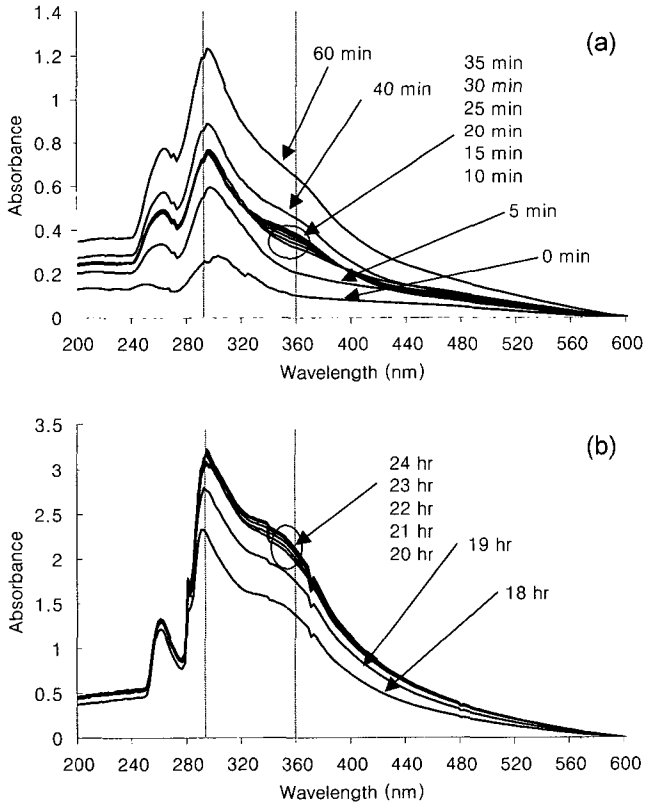


Figure 2. Spectroscopic observation of the oxidation of tyrosyl residues in SF and the self-crosslinking reaction of *o*-quinone residues with various reaction times; (a) 0-60 mins and (b) 18-24 hrs.

Figure 2 shows the UV/visible spectra monitored during the enzymatic and nonenzymatic reaction of SF solution. As shown in Figure 2(a), a gradual increase of absorption intensity at 300 nm was observed after an initiation of the reaction by tyrosinase. Interestingly, there were little changes of the intensity at 300 nm while a new peak at around 360 nm appeared and its intensity was increased with an increase of reaction time during 10-35 min reaction time. Moreover, the absorption intensities of both wavelengths (300 and 360 nm) were increased continuously at above 35 minutes. It has been reported that the absorption peaks at 300 and around 360 nm exhibit *o*-quinone residues and *p*-crosslinked quinone by Michael-addition reaction, respectively [15]. It can be concluded that, at the initial stage, the *o*-qunone residues are formed progressively until the maximum oxygen consumption reaches due to the oxidation of tyrosyl residues in SF. The oxidation occurs very fast and this result is in good agreement with the result observed in Figure 1. For the second stage (here, 10-35 min reaction time), *p*-crosslinked quinone can be formed via Michael-addition reaction. As proposed in Scheme 1, it seems that the *N*-terminal amino groups of adjacent molecular chains in SF are reacted to the *p*-position of *o*-quinones. The reason why any change of the intensity at 300 nm was not observed for 10-35 min might be due to a reach for an equilibrium state of *o*-quinone between the production by the oxidation of tyrosyl residues and the consumption by *p*-crosslinked quinone. When the reaction continues to much longer time (above 20 hrs), there were little changes of absorption peaks and intensities, indicating

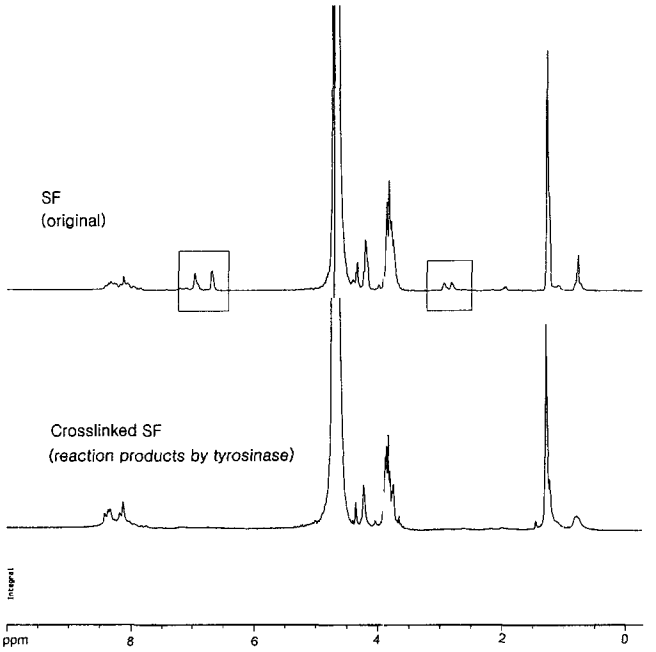


Figure 3. The solution phase ^{1}H-NMR spectra for D_2O-exchanged SF and crosslinked SF.

that both reactions were nearly terminated at around 20 hrs as observed in Figure 2(b).

^{1}H-NMR spectra for D_2O-exchanged SF and crosslinked SF by tyrosinase were compared in Figure 3. The peaks for aromatic group and *N*-terminal amino group were clearly observed at the range of 6.5-7.0 and 2.5-3.0 ppm, respectively, for the SF samples [16]. However, these peaks disappeared after the reaction occurred. This result strongly supports that most aromatic groups in SF, tyrosyl residues, are oxidized to *o*-quinones via dihydroxyphenyl residues and *N*-terminal amino groups are then reacted to *o*-quinones by means of Michael-addition mechanism.

It should notify that the quinone-quinone coupling reaction [2] and Maillard reaction [17] can also be occurred. Even though there is no direct evidence for these reactions in this study, some reactions may occur from the fact that the absorbance at the range of 280-500 nm increases continuously with a reaction time. For the quinone-quinone coupling reaction, the radicals are generated during the enzymatic oxidation [18] and phenoxy radicals are reacted by self-condensation to produce coupled forms [19]. And also, carbonyl group can be reacted with amino group to form Schiff base via Maillard reaction mechanism [17]. The possible reaction pathways are proposed in Scheme 1. In order to find the accurate mechanism of SF by enzymatic and, subsequently, nonenzymatic process, further research must be carried out in the future.

According to the measurement of molecular weight distribution of SF and crosslinked SF from gel filtration chromatography (GFC) (Figure 4), an apparent peak at low molecular weight ranges disappeared after the crosslinking reaction. Additionally, the peak-intensity of high molecular weight region in crosslinked SF curve was higher than that in original SF curve. This means that the *N*-terminal amino groups in molecules having low molecular weights are

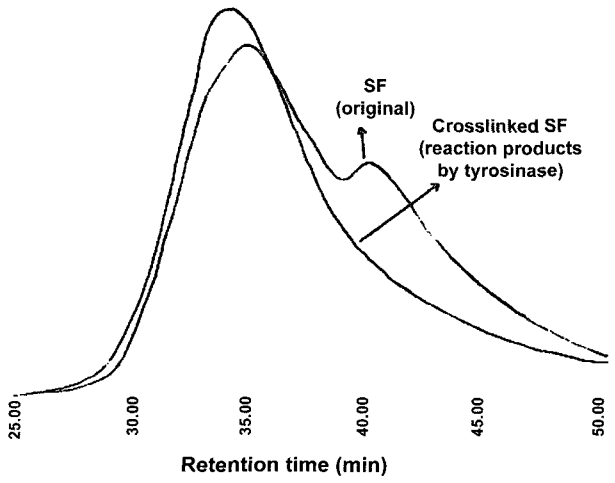


Figure 4. Molecular weight distributions of original SF and crosslinked SF from gel filtration chromatography.

participated in the reaction with *o*-quinone more easily. It is because that the mobilities of molecules brisk as the molecular weight is low, resulting in more possibilities of attachment between the reactive groups.

Conclusions

The SF is capable of self-crosslinking reaction by tyrosinase. The enzyme catalyzes the oxidation of tyrosyl residues in SF to *o*-quinones and the oxidation products, subsequently, leading to the Michael-addition reaction with *N*-terminal amino groups of SF molecules at the *p*-position of *o*-quinone residues. The reaction mechanism was proposed and confirmed by the measurement of oxygen consumption as well as the measurements of UV, ^{1}H-NMR and GFC analysis. Based on our results for the self-crosslinking reaction of SF and tyrosinase, it is useful for further research to prepare the water-resistant adhesives using SF (rich in tyrosyl residues) and chitosan (abundant in amino residues). In addition, the specific enzymes may be immobilized to biocompatible SF for preparing the substances to have high bioactivities using the reaction.

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