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Application of Electrospun Silk Fibroin Nanofibers as an Immobilization Support of Enzyme

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Abstract: Silk fibroin (SF) nanofibers were prepared by electrospinning and their application as an enzyme immobilization support was attempted. By varying the concentration of SF dope solution the diameter of SF nanofiber was controlled. The SF nanofiber web had high capacity of enzyme loading, which reached to 5.6 wt%. The activity of immobilized α-chymotrypsin (CT) on SF nanofiber was 8 times higher than that on silk fiber and it increased as the fiber diameter decreased. Sample SF8 (ca. 205 nm fiber diameter) has excellent stability at 25 °C by retaining more than 90 % of initial activity after 24 hours, while sample SF11 (ca. 320 nm fiber diameter) shows higher stability in ethanol, retaining more than 45 % of initial activity. The formation of multipoint attachment between enzyme and support might increase the stability of enzyme. From these results, it is expected that the electrospun SF nanofibers can be used as an excellent support for enzyme immobilization.

Keywords: Electrospinning, Nanofiber, Silk fibroin, Enzyme immobilization

Introduction

Enzymes have several advantages over conventional inorganic catalysts, such as stereo- and regio-selectivity, reduced side reactions and mild reaction conditions. However, some drawbacks limit their use in large industrial scales. Since enzymes are proteins, any changes in reaction conditions may lead to the deformation of their structure, resulting in the loss of activity. The cost of enzymes is also another barrier, because it cannot be used repeatedly. The idea of immobilization of an enzyme on insoluble supports provides a wide challenge to use the enzymatic reaction in industrial scale. The immobilized enzyme can be reused throughout several reactions and easily separated from the product, which decreases the cost problem and the contamination of product, respectively. Moreover, the stability of enzymes is also increased after immobilization [1].

However, there are still several problems to be overcome. For example, a high loading efficiency of enzyme is a critical problem in enzyme immobilization. Because there is some loss of enzyme activity after immobilization, it can be compensated by increasing the amount of enzyme that immobilized. For this reason, porous materials are used as a support in most of the cases. In this case, however, the diffusional limitation of substrate is being a critical problem especially when the substrate has a high molecular weight. Therefore, it would be ideal if nonporous materials with large surface area could be used as a support [1].

Various forms of support have been used in enzyme immobilization including beads, particles, hydrogels, membranes, films and fibers. Among these forms, the fiber is a non-porous material with high surface areas, which enables a low diffusional limitation and a high loading of enzyme. Recently, electrospinning technique has been highlighted because of the possibility to produce a fiber in nanoscale diameter [2]. Due to its fine diameter, it has extremely high specific surface areas than any other supports, which makes it capable to bind more enzymes. However, there are only a few reports of its use as an immobilization support of enzyme [3-5].

Many researchers have successfully prepared regenerated silk fibroin (SF) nanofibers by electrospinning technology [6-9], but their applications are in the beginnings and only limited to the scaffolds for tissue engineerings until now [10, 11]. However, since SF has been successfully used as an immobilization support of enzyme [12], we expect that the SF nanofibers could be also utilized in the same purposes. In our previous studies, we utilized both natural silk fiber and sericin-fixed silk fiber as an immobilization support of enzyme, but the loading efficiency was less than 1.2 %, which has to be improved [13,14].

In this study, we prepared regenerated SF nanofiber in a form of nonwoven webs and attempted to evaluate a novel use of SF nanofiber as an immobilization support of enzyme. Alpha-chymotrypsin (CT) was used as model enzyme, and its activity and stability were measured for the evaluation.

Experimental

Materials

Regenerated SF sponge was prepared by following procedure. Silkworm cocoons were degummed twice with 0.5 % (o.w.f.) Marseilles soap and 0.3 % (o.w.f.) sodium carbonate solution at 100 °C for 1 hour, and rinsed thoroughly with warm distilled water. Degummed silk cocoons were first dissolved in a

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ternary solvent system of $CaCl_2/H_2O/EtOH$ solution (1/8/2 mole ratio) for 30 min at 85 °C. Aqueous SF solution is obtained by dialysis of dissolved SF solution in a cellulose tube (molecular weight cutoff = 12,000-14,000) against distilled water for 4 days at room temperature. Then, aqueous SF solution was freeze-dried to obtain regenerated SF sponge.

Preparation of Electrospun Silk Fibroin Nanofiber Webs

The regenerated SF sponge was dissolved in formic acid (98-100 %) having different concentrations (8, 9, 10 and 11 %). The silk-formic acid solution was placed in a 3-m*l* syringe (G-22). The tip-to-collection plate distance was 10 cm and, a voltage of 12 kV was applied to the collection drum. The electrospun SF nanofibers were collected on an aluminum foil, which covers the collection drum, in a form of nonwoven webs. After spinning, it was removed from the foil and immersed in methanol (99 %) for 1 hour. Finally, it was dried with tension to prevent shrinkage and cut into 1 × 1 cm square shapes.

Activation of SF Nanofiber

In order to immobilize CT on the SF nanofiber, the amine groups of SF were activated with glutaraldehye (GA). To one piece of SF nanofiber web, 1 m*l* of 10 % (v/v) GA in 0.2 M sodium carbonate buffer, pH 9.2, was added to activate the SF. The reaction was continued for 1 hour at 25 °C. The activated SF was washed 2 times with distilled water and 3 times with 0.1 M sodium phosphate buffers, pH 7.4.

Immobilization and Quantification of Enzyme

One percent (w/v) of CT in 0.1 M sodium phosphate buffer, pH 7.4, was prepared and added to the activated SF nanofiber web. After overnight incubation at 4 °C, unreacted enzyme was washed out with ice-cold 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5 M of NaCl. Further it was washed 3 times with the same buffer but without NaCl. The bound CT was measured by bicinchoninic acid (BCA) assay methods. The amount of bound CT was calculated by subtracting the amount of remaining CT from the initial amount of CT. The enzyme loading efficiency was defined as a percentage of the amount of bound CT to the weight of SF nanofiber web.

Activity Test of Enzyme

N-benzoyl-DL-tyrosine-p-nitroanilide hydrochloride (BTPNA) was used as substrate of CT. Five hundred μl of 10 mM BTPNA in DMSO were diluted with 750 μl of distilled water and 150 μl of 0.1 M sodium phosphate buffer, pH 7.4. This mixture was added to enzyme immobilized SF nanofiber web and incubated for 30 min at 25 °C. The increase of absorbance at 400 nm was measured using UV spectrometer (UVICON 923, Kontron Instruments, USA). The activity was defined as μmol BTPNA hydrolyzed within 1 min. In the case of free enzyme, the final amount of enzyme in the test tube was 10 μg.

Stability Test of CT

The stability of immobilized CT was tested as follows. Thermal stability was measured at 25 °C and 50 °C and each sample, containing 0.1 M sodium phosphate buffer pH 7.4, was incubated for desirable times. On the other hand, the stability against ethanol was measured by incubating each sample for 1 hour at 25 °C. After each incubations, the SF was washed 2 times with ice-cold 0.1 M sodium phosphate buffer pH 7.4, containing 0.5 M NaCl and 3 times with cold distilled water.

Results and Discussion

Preparation of Electrospun SF Nanofiber Webs

SF nanofibers could be prepared successfully by electrospinning. The diameter and morphology of the fibers are affected by various factors such as dope solution (molecular weight of polymer and concentration), spinning conditions (electric field strength and tip-to-collector plate distance) and solvent properties [15,16]. In this study, we changed only the concentration of the dope solution for electrospinning conditions, and Figure 1 shows the relationship between fiber diameters and dope concentrations. The mean diameter of nanofiber increased with the concentration of the dope solution but the variation increased at the same time. At the concentration of 6 %, lots of beads were formed in the middle of fiber even though it had smallest fiber diameter near 100 nm. The beads disappeared when the concentration of dope solution was above 8 %, but the concentration was limited to 13 % for proper electrospinning of SF. Four different kinds of concentration, 8, 9, 10 and 11 %, were chosen for enzyme immobilization and referred as to SF8 (205 ± 30 nm), SF9 (235 ± 10 nm), SF10 (305 ± 90 nm) and SF11 (320 ± 110 nm fiber diameter), respectively.

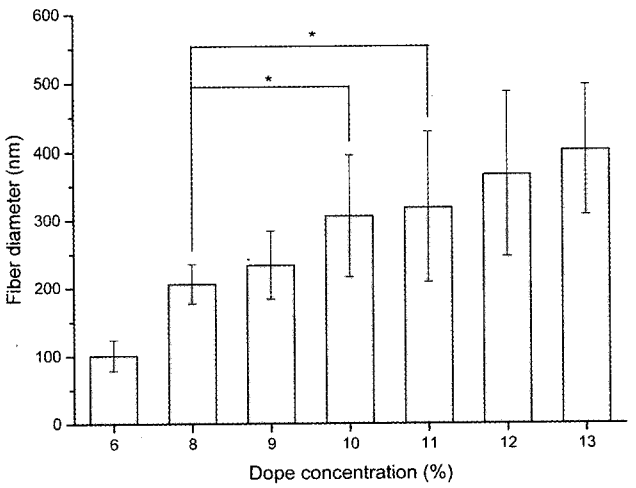

Figure 1. Fiber diameter of electrospun SF nanofibers prepared from different concentration of dope solution. *Significant at $p<0.01$ according to student *t*-test ($n = 15$).

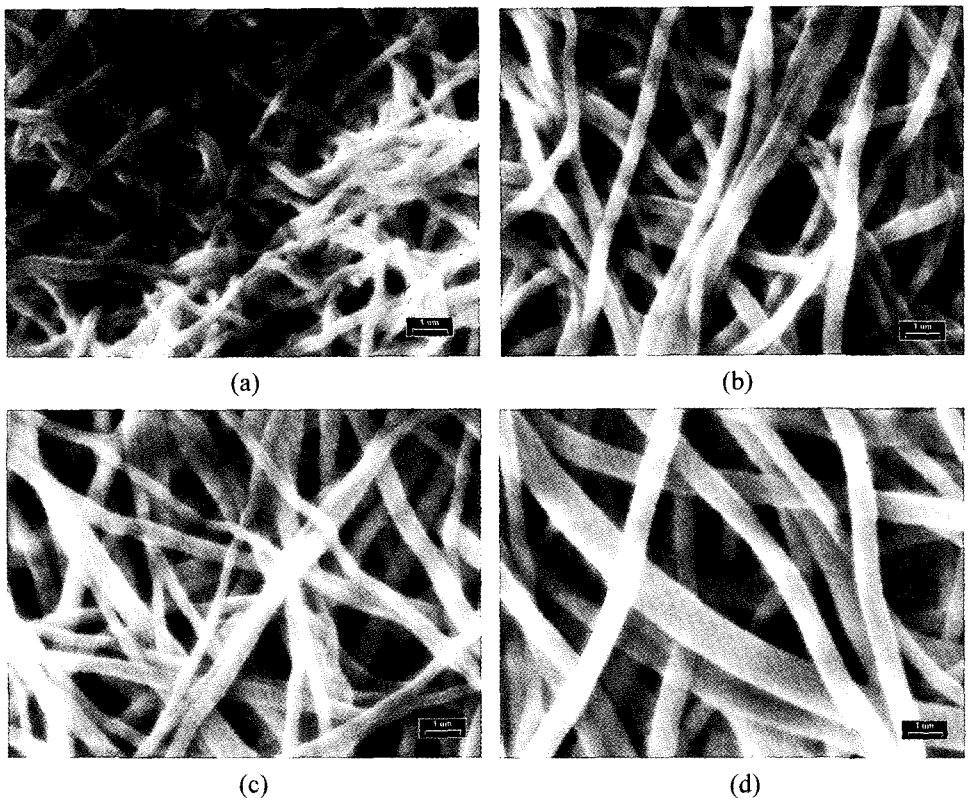

Figure 2. SEM image of the electrospun SF nanofibers after the activity test of immobilized CT; (a) 8 %, (b) 9 %, (c) 10 %, and (d) 11 % solution.

Figure 2 shows the SEM image of SF nanofiber webs after the activity test of immobilized CT. The fiber diameter was slightly increased due to the swelling during the experiment. Though the methanol treatment made the SF nanofiber webs insoluble in aqueous solutions, tearing was occurred into pieces during the vigorous stirring. However, after the GA treatment, which was originally adopted for the activation of SF nanofiber, the SF nanofiber webs were stable throughout the experiments, even after several steps of vigorous washing and exposure to proteolytic enzyme, CT. Since GA is a powerful crosslinking agent, crosslinks between SF molecular chains might be occurred additionally to the activation of SF.

Effectiveness of SF Nanofiber as an Immobilization Support of Enzyme

The effectiveness of SF nanofiber as an immobilization support of enzyme is shown in Table 1, by comparing with our former results with silk fiber. The amount of CT that immobilized onto the SF8 was 56.6 μg/mg, which corresponds to 5.6 wt% in enzyme loading efficiency. This is higher than our previous results with degummed silk fiber and sericin-fixed silk fiber (SFx), which were 8.05 and 11.1 μg/mg, respectively [13,14]. Even though, in the previous study with the degummed silk fiber, trypsin was immobilized instead of CT, we confirmed that there were no significant differences between the amount of trypsin and CT that bound onto the support. The enzyme loading efficiency of SF8 is also higher than previous reports with electrospun fiber, where 1.8 % was the highest with cellulose fibers [5]. In general, the enzyme loading efficiency of supports that are commonly used in enzyme immobilization is in the range of 0.1-10 % of the support weight [1].

Table 1. Comparison of overall property of immobilized CT on different supports

Sample	*Fiber diameter* (nm)	Bound protein (μg/mg)	Activity per mg of support (μmol/min/mg)	Specific activity[a] (μmol/min/mg)
SF 8	205 ± 30	56.6	3.78	66.78
Degummed silk fiber	20700 ± 2000	8.05[b]	-	-
Sericin-fixed silk fiber (SFx)	42500 ± 540	11.1[c]	0.48	43.81[c]

[a]Activity per mg of CT, [b]data from ref. 13, [c]data from ref. 14.

The activity of immobilized CT onto SF8 was almost 8 times higher than the same enzyme onto SFx. However, the increase of activity is not only due to the increase of immobilized enzyme but also some favorable characteristics of SF nanofiber. The specific activities of immobilized CT on SF8 and SFx were 66.78 and 43.81 μmol/min/mg, respectively. It means that the immobilized CT on SF8 retains more activity of free CT than that on SFx. In other words, SF8 provides better microenvironment to CT than SFx.

Activity and Stability of Immobilized CT on SF Nanofiber

Figure 3 shows the activities of immobilized CT on SF nanofiber webs prepared from different dope concentrations. The activity decreased with the increase of fiber diameter, but, according to the statistical analysis, the activity of immobilized CT was significantly different only between SF8 (3.58 ± 0.19 μmol/min/cm^2) and SF11 (2.76 ± 0.78 μmol/min/cm^2). As mentioned previously, the variation of fiber diameter increases with the concentration of dope solution and it caused several problems throughout the experiments. For example, a reliable determination of the amount of immobilized CT on SFs was only available with the sample SF8. Though the exact amount of immobilized CT could not be obtained with the other SFs, there was a decreasing tendency of the amount of immobilized CT as the fiber diameter increases. This is probably due to the decrease of the surface area of SF nanofiber web. Therefore, the relationship between the fiber diameter and the activity of immobilized CT may be due to the difference of the amounts of immobilized CT. For further stability tests, SF8 and SF11 were chosen because they were significant according to student *t*-test in both activity and fiber diameter.

Figure 4 shows the thermal stability of the immobilized CT on SF8 and SF11. At both 25 °C and 50 °C, the immobilized CT has higher thermal stability than free CT except in the case of SF 11 at 25 °C. At 25 °C, the immobilized CT onto SF8 retains more than 90 % of its initial activity after 24

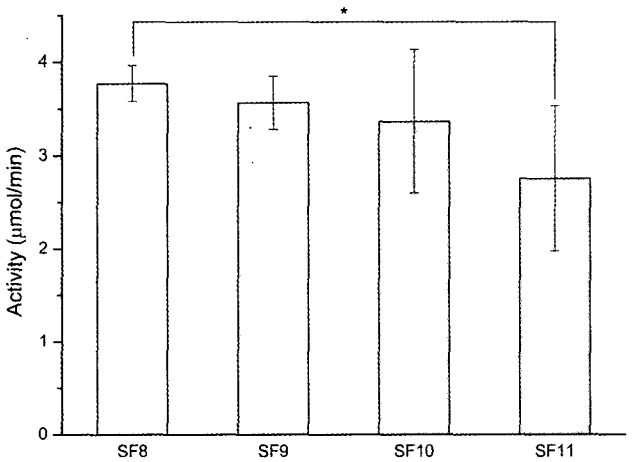

Figure 3. Activity of immobilized CT on different SF nanofiber webs. *Significant at $p<0.05$ according to student *t*-test ($n = 4$).

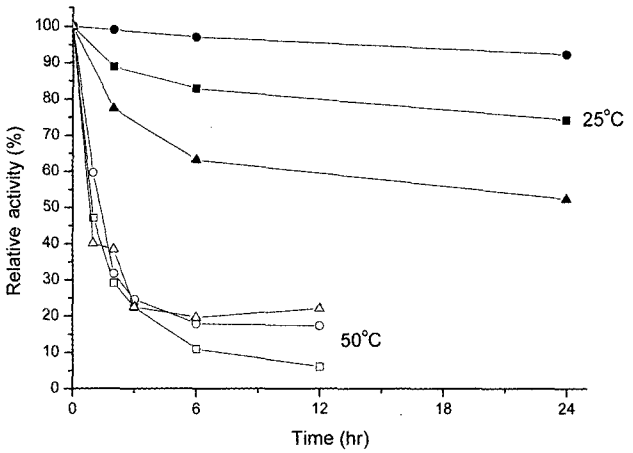

Figure 4. Thermal stability of free CT (square) and immobilized CT on SF 8 (circle) and SF 11 (triangle) at different temperatures; 25 °C (solid) and 50 °C (open).

hours. Generally, in the case of proteolytic enzymes, autolysis can be prevented upon immobilization and, as a result, the stability of enzymes increases. However, the activity of immobilized CT onto SF 11 decreased much more than free CT. There might be another reason that affected the stability of immobilized CT onto SF11. Since we did not convert the free aldehyde groups into hydroxyl groups after immobilization, there is still a chance for further reaction between some amine groups of enzyme and free aldehyde groups of support. This secondary reaction is far slower than the formation of first enzyme-support link, and finally, it provides multipoint attachment of enzyme on support [17]. In most of the cases, the multipoint attachment restricts the conformational changes of enzyme, and thereby increases the stability of enzyme against harsh conditions [18]. But, at the same time, there is a loss of activity of enzyme by the conformational changes induced by multipoint attachment. Thus, in the case of SF11, multipoint attachment might be occurred during the incubation time and, as a result, it lost more activity than free CT.

The stability of CT at 50 °C, a temperature which induces the conformational changes of enzyme, was not good enough than expected. Although it is more stable than free CT, it lost too much activity compared with natural silk fibers [13,14]. Since the fiber diameter of SF nanofiber is one hundredth to that of silk fiber, there might be less attachment between CT and SF nanofiber than between CT and silk fiber and, as a result, the conformational change of CT would occur easily like free CT. In addition, it was found that SF11 was slightly more stable than SF8. Since SF11 had larger fiber diameter than SF8, there might be more attachment between CT and SF11 than between CT and SF8, even though it was not enough to prevent the conformational change of CT.

Figure 5 shows the stability of CT against ethanol. Free CT retained only about 5 % of initial activity after exposure to ethanol for 1 hour, whereas immobilized CT showed much

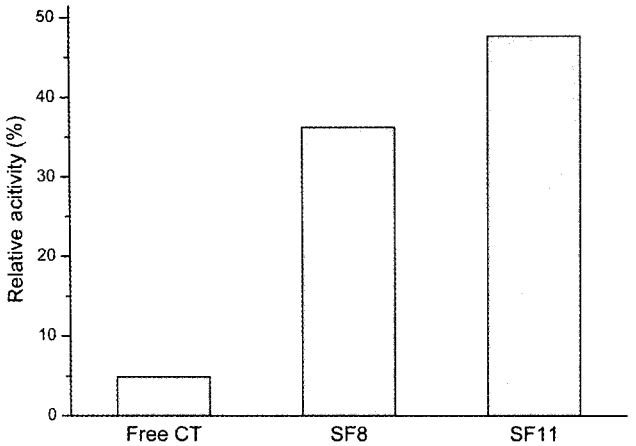

Figure 5. Stability of free CT and immobilized CT onto SF nanofiber webs in ethanol.

higher stability, retaining more than 45 % of their initial activity, due to the multipoint attachment. Hydrophilicity of SF might be also playing a role here. From the thermal stability test, the multipoint attachment was not sufficient to prevent deformation of CT. Thus it is hard to believe that only the multipoint attachment of enzyme contributes to the stability against ethanol. Since the complex structure of enzyme is maintained in an aqueous condition, hydrophilic support, in this case SF, retains more water molecules nearby enzyme, which enables the enzyme to maintain its native state. Therefore, the immobilized CT could retain much more activity than free CT. The difference between SF8 and SF11, however, could be elucidated by the effect of multipoint attachment of enzyme. Since these two supports differ only in fiber diameter, more covalent bonding might be formed between the enzyme and SF11 compared with SF8.

Conclusion

Electrospun SF nanofibers, in the range of ca. 205-320 nm fiber diameter, were prepared for their uses in enzyme immobilization supports. The effectiveness of SF nanofiber as an immobilization support of enzyme was excellent with high capacity of enzyme loading. Also, the activity of the immobilized enzyme on SF nanofiber was much higher compared with silk fiber and the activity was affected by nanosize of SF. As a result of enzyme stability test, CT immobilized on SF8 had excellent stability at ambient temperature but was inferior at evaluated temperature. On the other hand, CT immobilized on S11 was tolerant to the exposure of ethanol. This can be explained by that multipoint attachment may be formed between CT and support. In conclusion, SF nanofiber web can be an excellent candidate for enzyme immobilization with high enzyme loading capacity.

Acknowledgements

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