Fibers and Polymers 2005, Vol.6, No.1, 1-5

Sericin-Fixed Silk Fiber as an Immobilization Support of Enzyme

Ki Hoon Lee*, Gyung Don Kang, Bong Seob Shin[1], and Young Hwan Park

School of Biological Resources and Materials Engineering, Seoul National University, Seoul 151-742, Korea
[1]Department of Textile Engineerings, Sangju National University, Sangju 742-711, Korea
(Received September 30, 2004; Revised February 11, 2005; Accepted February 18, 2005)

Abstract: In this study, we attempted to evaluate a novel use of sericin-fixed silk fiber (SFx) as an immobilization support of enzyme. Sericin was fixed on the silk fiber using glutaraldehyde as a fixation reagent. After 6 hours of fixation, the degree of fixation increases linearly with linear decrease of the amount of bound α-chymotrypsin (CT). This suggests that the increase of the degree of fixation is due to the further crosslinking of free aldehyde groups on the surface of sericin-fixed silk fiber (SFx). Even though perfect fixation was not achieved, sericin did not dissolve seriously and could be removed by further washing. The specific activity did not differ significantly after 6 hours of fixation. The activity of immobilized CT on SFx decreased to its half after 6 hours of incubation at 50 °C. However, it retained 78 % of initial activity even after 1 hour of treatment with 100 % ethanol. As a result, the SFx could be used as an immobilization support of enzyme in non-aqueous media at ambient temperature.

Keywords: Silk, Sericin, Sericin-fixed silk fiber, Enzyme immobilization, Non-aqueous media

Introduction

Silk fiber consists of two proteins, fibroin and sericin. Two strands of fibroin are embedded in the sericin matrix. The inherent luster and touch of silk appears after the sericin is removed by the degumming process. On the other hand, there have been some attempts to fix the sericin on the silk fiber for summer apparels, even though these were not successful due to several reasons [1-3]. The toxicity of crosslinking agent may cause severe defects on the skin of human and the change of color may cause some problems during the dyeing process. In industrial application, however, these problems are less serious than in apparels.

Recently, the application of enzyme in a non-aqueous media is getting interest in polymer sciences. Although the inherent characteristics of enzyme are based on aqueous media, the advantages of enzyme-catalyzed reaction, such as regio- and stereoselectivity, reduced side reactions, etc., continuously lead the scientists to expand its use in non-aqueous media [4]. In such applications, the maintenance of the enzyme activity is a critical point, because non-aqueous media causes changes in the environment of enzyme, followed by the conformational transition of 3D structure of enzyme. One of the strategies to stabilize the enzyme is the immobilization of enzyme on a support. During the immobilization on a support, the characteristics of support are important factors e.g. surface area, hydrophilicity/hydrophobicity, insolubility, mechanical stability, form and size of support, etc. [5].

Among many forms of support, fiber could satisfy most of the requirements. Fiber is a non-porous material with high surface area, which enables low diffusional limitation and high loading of enzyme. The hydrophilicity of support is also an important factor because it could retain water molecules nearby enzymes. Among commercially available fibers, silk fiber is more hydrophilic than others. It is also insoluble in most organic solvents and has good mechanical stability. Thereby, silk fiber could be used as an immobilization support of enzyme.

In our recent study, trypsin was immobilized on silk fiber, but the activity was low because of low loading efficiency [6]. However, we found that the activity and stability of immobilized enzyme was improved using sericin as a spacer arm.

In this study, we attempted to evaluate a novel use of sericin-fixed fiber (SFx) as an immobilization support of enzyme. Glutaraldehyde (GA) was used as a fixation agent, and the enzyme loading efficiency and stability of SFx was observed by varying the fixation time. Trypsin and α-chymotrypsin (CT) were used as model enzymes, and its activity and stability were observed.

Experimental

Materials

Raw silk was obtained from Heung Jin Co., Ltd. (Korea). All other chemicals were purchased from Sigma-Aldrich Co., Ltd. (Korea).

Preparation of Sericin-Fixed Silk Fiber

The raw silk (30 mg) was wound to a bundle and set in an Eppendorf tube. Glutaraldhyde (GA) was used as fixation agent of sericin. One milliliter of 10 % (v/v) GA in 0.2 M sodium bicarbonate buffer, pH 9.2, was added and kept for 2 (SFx2), 6 (SFx6), 12 (SFx12), and 24 (SFx24) hours at 25 °C. After fixation, the SFx was washed 5 times with 0.1 M sodium phosphate buffer, pH 7.4, and used for further experiments. The degree of fixation was calculated after boiling the SFx for 1 hour at 120 °C as follows:

*Corresponding author: prolee@snu.ac.kr

$$Degree\ of\ fixation\ (\%) = \left(1 - \frac{Degumming\ rate\ of\ SFx}{Degumming\ rate\ of\ raw\ silk}\right) \times 100$$

Activation of SFx

The amino groups and carboxyl groups of sericin were activated to immobilize the enzyme via covalent bonding. Since GA is frequently used to activate the amino groups and there might be some unreacted aldehyde groups, the SFx was used directly without any further reactions.

The carboxyl groups were activated with N-hydroxy-succinimide (NHS). The SFx was first washed 4 times with dioxane, and 1ml of 0.2 M dicyclohexylcarbodiimide (DCC) in dioxane was added. Adequate amount of NHS was added to reach the final concentration of 0.2 M. After 70 min of incubation at 25 °C, it was washed 2 times with dioxane and 3 times with methanol. Finally, it was washed 3 times with 0.1 M of sodium phosphate buffer, pH 7.4.

Immobilization and Quantification of Enzyme

One percent (w/v) of trypsin or CT in 0.1 M sodium phosphate buffer, pH 7.4 was prepared and added to the activated SFx. 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 trypsin was measured by bicinchoninic acid (BCA) assay methods. The amount of bound trypsin was calculated by subtracting the amount of remaining trypsin from the initial amount of trypsin.

Activity Test of Enzyme

N-Benzoyl-DL-arginin-p-nitroanilide hydrochloride (BAPNA) and N-benzoyl-DL-tyrosine-p-nitroanilide hydrochloride (BTPNA) was used as substrate of trypsin and CT, respectively. For the activity of immobilized trypsin, 250 μl of 10 mM BAPNA in DMSO were diluted with 1ml of distilled water and 150 μl of 0.1 M sodium phosphate buffer pH 8. For the activity of immobilized α-chymotrypsin, 500 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 SFx 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 BAPNA or 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 α-chymotrypsin 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, stability against ethanol was measured by incubating each sample for 1 hour at 25 °C with different concentration of ethanol. After each incubation, the SFx was washed 2 times with ice-cold 0.1M sodium phosphate buffer pH 7.4, containing 0.5 M NaCl and 3 times with cold distilled water.

Results and Discussion

It has been reported that GA reacts with the amino groups of amino acids, especially lysine, histidine and arginine [2]. The total content of these amino acids in sericin is 7.70 % [7]. Since an excess amount of GA was added during the fixation process, there might be some free aldehyde groups remained unreacted. Thus, the unreacted free aldehyde groups could further react with the amino groups of trypsin. On the other hand, sericin also has carboxyl groups, which frequently targeted for immobilization of enzyme. The exact amount of aspartic acid and glutamic acid could not be obtained because asparagine and glutamine usually converted to acidic form during the amino acid analysis. However, according to the DNA sequencing, the contents of both amino acids are in the range of 6-9 % [8]. Therefore, the carboxyl groups were activated with NHS after the fixation of sericin for 12 hours. Figure 1 shows the overall reaction of SFx.

Table 1 shows the effect of different activation procedure of SFx on the amount of bound trypsin and its activity. At the beginning of this study, we expected better results with NHS-activated SFx (SFx12-NHS) because it was found previously that half of the amino acids containing amino group were consumed during the fixation step [2]. Thus, additional activation of carboxyl groups might increase the amounts of immobilized enzyme. However, there were no significant

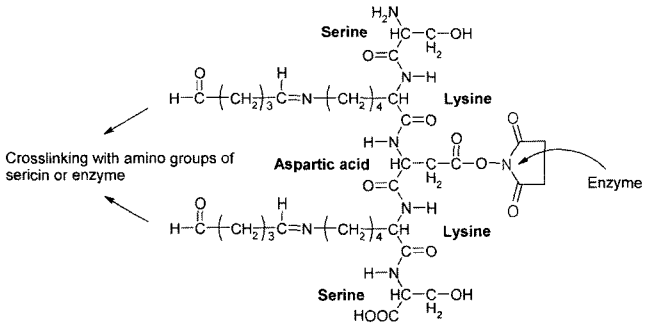

Figure 1. Schematic representation of reactions of sericin fixation and enzyme immobilization. The names of amino acids are in bold characters.

Table 1. Effects of immobilization procedure on trypsin activity

Sample ID	Bound protein (mg/g)	Activity (μmol/min)	Specific activity (μmol/min/mg)
SFx12	10.6	9.80	30.93
SFx12-NHS	11.1	10.28	30.77

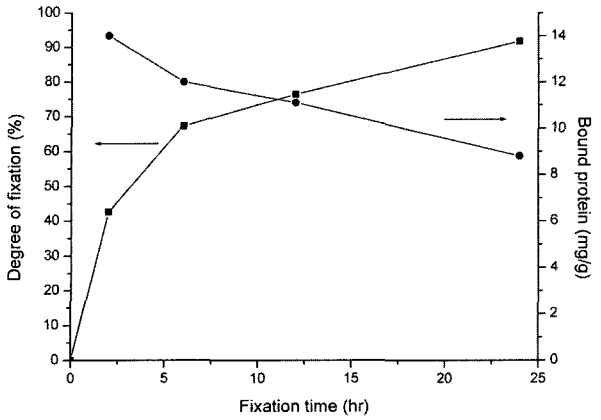

Figure 2. Effect of fixation time on the degree of fixation and CT bound on SFx.

differences between SFx12 and SFx12-NHS. The reason is not clear but it can be derived from the difference of amino acids content in the bulk phase and at the surface of sericin. In many polymers, the surface characteristics are much different from the bulk phase [9]. There might be less free carboxyl groups at the surface of SFx than in the bulk phase. Nevertheless, it is an advantage that the preparation and activation of SFx could be achieved at the same procedure. Thus, more detailed studies were done with SFx without further reactions.

Figure 2 shows that the degree of fixation of SFx increased with the reaction time. A rapid fixation occurs at the beginning of the reaction. And after certain times (6 hours in our study), the degree of fixation increases linearly and the fixation rate becomes constant. It can be said that GA reacts fast between two amino groups at the beginning, which are sufficiently close together to react. As times go on, swelling of sericin matrix occurs, which increases the mobility of the molecular chain, and enables further crosslinking between two amino groups. After 24 hrs, 91.8 % of sericin was fixed on the silk fiber.

The amount of CT on the SFx is also shown in Figure 2. If the degree of fixation increases, there might be less free aldehyde groups available for CT immobilization. As expected, the bound CT decreased as the degree of fixation increased. While the degree of fixation of SFx24 was about 2.2 times higher than that of SFx2, the bound CT on the SFx24 was 63 % of SFx2. The interesting thing is a linear behavior of the degree of fixation and the bound protein, after 6 hours of fixation.

We estimated the amounts of bound CT from the degree of fixation, assuming that the degree of fixation is inversely proportional to the amounts of bound CT. Table 2 shows that the estimated values of bound CT calculated from the degree of fixation accord with the observed values. In the case of SFx6 and SFx24, the degree of fixation has increased about 1.4 times, while the bound CT on the SFx12 has decreased about 0.7 times, 8.8 mg/g, which matches exactly with the estimated value. Since CT was immobilized just on the surface

Table 2. Observed and estimated value of bound CT on SFx after 6 hours of fixation time

Sample ID	Observed value of bound CT (mg/g)	Estimated value of bound CT (mg/g)
SFx6	12.0	-
SFx12	11.1	10.6
SFx24	8.8	8.8

of SFx, the amount of bound CT is strongly related to the amounts of free aldehyde groups on the surface of SFx. From these results, we can suggest that the amounts of free aldehyde groups decrease continuously with the fixation time, which means the degree of fixation increases by the further crosslinking occurred at the surface of SFx.

In our experiment, the complete fixation of sericin could not be achieved. Therefore, we checked the loss of sericin from SFx at 25 °C and 50 °C. As shown in Figure 3, the amount of sericin that dissolved from the SFx was less than 1.0 % of the total SFx, even though the fixation rate did not reach to 100 %. Furthermore, after washing sufficiently, there was no more loss of sericin from the SFx throughout further experiments.

Table 3 shows the activity of immobilized α-chymotrypsin on the SFx. The low specific activity of immobilized CT compared with free CT is due to some improper immobiliza-

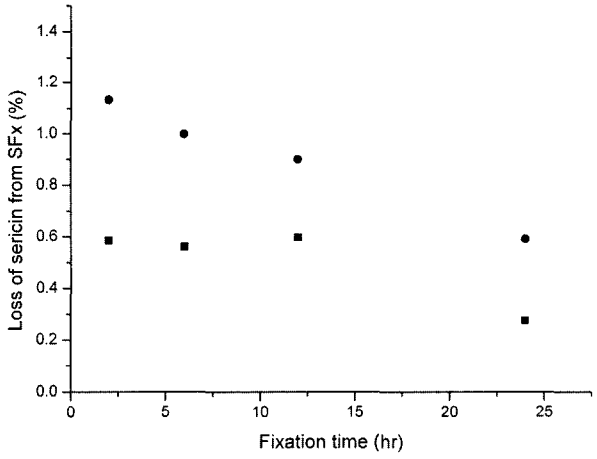

Figure 3. Loss of sericin from the SFx at 25 °C (■) and 50 °C (●).

Table 3. Activity of CT immobilized SFx

Sample ID	Activity (μmol/min)	Specific activity (μmol/min/mg)
Free CT[a]	2.83	282.89
SFx2	13.8	32.78
SFx6	15.0	41.67
SFx12	14.5	43.81
SFx24	11.6	43.93

[a]: 10 μg of CT was used.

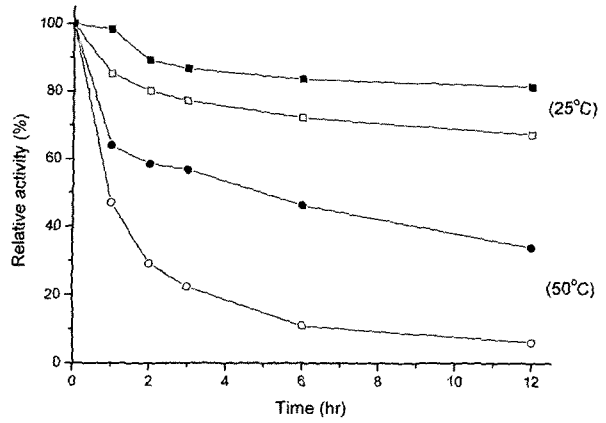

Figure 4. Thermal stability of free CT (open) and immobilized CT of SFx6 (solid) at 25 °C (□, ■) and 50 °C (○, ●).

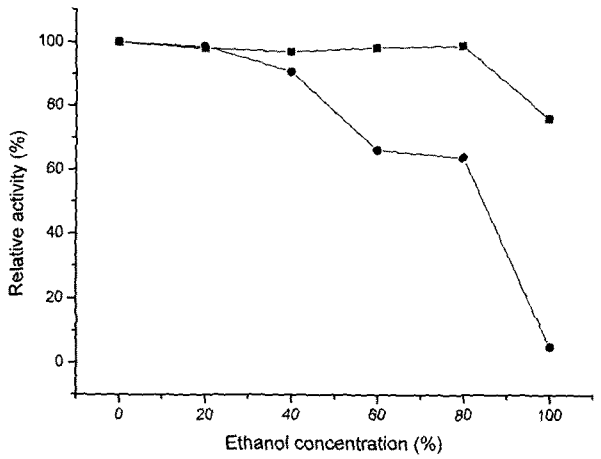

Figure 5. Stability of free CT (●) and immobilized CT on the SFx6 (■) against ethanol.

tion of CT. If the covalent bonding occurs near or at the active site, the substrate can not reach to the active site of CT because of the steric hindrance. In the case of SFx2, it showed lowest specific activity even though more CT was immobilized. After 6 hours of fixation time, however, there were no significant differences in the specific activity. Thus, SFx6 was chosen for further experiments because it has the highest activity and a specific activity with in an error range.

Figure 4 shows the thermal stability of immobilized CT on SFx6. At both 25 °C and 50 °C, the immobilized CT has higher thermal stability than free CT. At 50 °C, the immobilized CT retains half of its initial activity, whereas free CT looses almost of its initial activity after 6 hours. However, the thermal stability of immobilized CT was not good enough comparing with the results with degummed silk fiber previously reported by us [6]. The relative activity for immobilized enzyme showed more than 80 % after 12 hours of incubation at 50 °C. This is probably due to the hydrophobicity of the SFx. Since the GA reacts mostly with lysine, which is the most hydrophilic amino acid, the content of free amino group decreases as the fixation time increases, and result in an increase of hydrophobicity of SFx. We measured the moisture regain of SFx as a probe of hydrophobicity [10]. The moisture regain of SFx24 was 7.5 %, where that of SFx2 was 8.3 %. Therefore, at evaluated temperature, the increased hydrophobicity of SFx facilitates the hydrophobic interactions between CT and SFx, which may cause the denaturation of CT.

Although the immobilized CT on SFx had low thermal stability, it showed better stability against severe environment (Figure 5). Different concentrations of ethanol were used to force the denaturation of CT. In the case of SFx6, there was no significant loss of activity up to 80 % of ethanol. Even at 100 % of ethanol, it retained 78% of its activity, whereas free CT was nearly deactivated in this solvent. As the denaturation of enzyme is caused by the conformational change of protein structure, the multipoint attachment restricts the chain mobility of protein and prevents its conformational change, even in non-aqueous media [11]. There was some indirect evidence of the multipoint attachment of CT on SFx. In Figure 2, we explained the linear increase and decrease of the degree of fixation and the amount of bound CT. Assuming that the degree of fixation is proportional to the consumed free aldehyde groups at the surface of SFx, the decrease of the free aldehyde groups is much greater than the decrease of the amount of bound CT. In other words, as the degree of fixation increases, the less free aldehyde groups are reacted with CT. Actually, the remained activity of immobilized CT on SFx24 was only 23 % of its initial activity after 1 hour incubation with 100 % ethanol.

Conclusions

From the results of this study, the SFx can be used as an immobilization support of enzyme in non-aqueous media. The fixation of sericin and the activation of SFx for enzyme immobilization was achieved the same procedure. After 6 hours of fixation, the increase of the degree of fixation was due to the further crosslinking of free aldehyde groups and free amine groups at the surface of SFx. Although the fixation was not completed, there was no significant loss of sericin from the SFx. There were no significant differences in both the amount of bound CT and the activity of immobilized CT after 6 hours of fixation. Though the thermal stability of immobilized CT was low, the stability against ethanol was outstanding because of the multipoint attachment of CT on SFx. Further studies are on progress to evaluate the activity of immobilized enzyme on SFx in non-aqueous media.

Acknowledgment

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

References

1. H. Kakinoki and H. Ishizaka, *J. Seric. Sci. Jpn.*, **41**, 99 (1972).
2. H. Shiozaki and A. Ozaki, *J. Seric. Sci. Jpn.*, **44**, 476 (1975).
3. M. Yano and K. Yasuda, *J. Seric. Sci. Jpn.*, **54**, 271 (1985).
4. M. N. Gupta and I. Roy, *Eur. J. Biochem.*, **271**, 2575 (2004).
5. W. Tischer and F. Wedekind, *Top. Curr. Chem.*, **200**, 95 (1999).
6. K. H. Lee, G. D. Kang, B. S. Shin, Y. H. Park, and J. H. Nahm, *Int. J. Indust. Entomol.*, **8**, 195 (2004).
7. K. Komatsu, *Bull. Sericult. Exp. Stat.*, **26**, 135 (1975).
8. A. Garel, G. Deleage, and J. Prudhomme, *Insect Biochem. Molec. Biol.*, **27**, 469 (1997).
9. B. D. Ratner in "Biomaterials Science : An Introduction to Materials in Medicine", (B. D. Ratner, A. S. Hoffman, F. J. Schoen and J. E. Lemons Eds.), p.12, Academic Press, San Diego, 1996.
10. J. S. Son and D. S. Ji, *Fibers and Polymers*, **4**, 156 (2003).
11. V. Mozhaev, M. Sergeeva, A. Belova, and Y. Khmelnitsky, *Biotech. Bioeng.*, **35**, 653 (1990).