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Immobilization of Trypsin onto Silk Fibroin Fiber via Spacer Arms

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

School of Biological Resources and Materials Engineering, Seoul National University, Seoul 151-742, Korea.
Department of Textile Engineerings, Sangju National University, Sangju 742-711, Korea.

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Trypsin can be immobilized on silk fibroin fiber (SFF) by introducing several spacer arms, such as ethylene diamine (ED), bovine serum albumin (BSA) and silk sericin (SS). Direct immobilization on silk fiber (SFF-GA) has low activity because of the steric hindrance between the trypsin and substrate. The introduction of spacer arms onto SFF-GA can enhance the activity of trypsin by reducing the steric hindrance. When ED is used as a spacer arm, the activity of trypsin has increased but its stability decreased due to the increased hydrophobicity of SFF. BSA and SS, as a spacer arm, have better results in both activity and stability. SFF-BSA shows some decrease in the specific activity due to improper immobilization. SFF-SS maintained 90% of its initial activity even after 12 hrs incubation at 50°C. In the case of repeated hydrolysis of silk sericin with immobilized trypsin, SFF-GA and SFF-ED lost 50% of their initial activity right after first run, whereas SFF-BSA and SFF-SS maintained 80% of their initial activities even after 5 runs. Higher operational stability is due to increased hydrophilicity of SFF by introducing hydrophilic spacer arms such as BSA and SS. The high content of serine in SS increases the hydrophilicity of SFF resulting the best results among other spacer arms.

Key words: Immobilization, Spacer arm, Silk fibroin fiber, Silk sericin, Trypsin, Ethylene

*To whom correspondence should be addressed.
School of Biological Resources and Materials Engineering, Seoul National University, San 56-1, Shillim, Kwanak, Seoul 151-742, Korea. Tel: +82-2-880-4627; FAX: +82-2-873-2285; E-mail: suhlee1229@hotmail.com

Introduction

Silk fibroin (SF) has been used as an immobilization matrix of several enzymes such as glucose oxidase (Kuzuhara *et al.*, 1987), peroxidase (Liu *et al.*, 1995) and uricase (Zhang *et al.*, 1998). The immobilized enzymes have higher stability against temperature, pH and time than those in free state and they can also be reused repeatedly. These enzymes are entrapped in the matrix of SF as a form of insoluble film (Asakura *et al.*, 1988). The advantage of entrapment is that it causes minimal stresses to the enzyme during immobilization by making them free in the matrix without any physical or chemical bonding. However to prevent the leakage of enzyme, the pore size of matrix must be smaller than enzyme itself. This limits its use for high molecular weight substrates (HMWS) because the matrix may act as a barrier to the mass transfer of substrates. Therefore, surface immobilization onto support via covalent bonding is recommended to avoid the leakage of enzyme and diffusional limitation (Tisher and Wedekind, 1999).

Immobilization supports could be prepared in various forms including bead, membrane and fiber. Although beads and membranes are favorable forms of immobilization support, the form of fiber has some advantages over these forms, especially when the HMWS are used. Because diffusional limitation is a critical factor in immobilized enzyme for the HMWS, high stirring speed and low enzyme loading per unit area reduce such limitation. The fiber has better mechanical properties than bead, which withstands the shear stress, and higher surface area than membrane, which enables more loading of enzyme. Application of the fiber for an immobilization support has been reported as a form of nonwoven fabrics (Chen and Lin, 2003).

Although surface immobilization is an adequate method for the HMWS, direct immobilization causes severe defects on the activity and stability of immobilized

enzyme. Besides diffusional limitation, the steric hindrance between enzyme and HMWS reduces the activity of immobilized enzyme. Because enzymes are attached randomly, there might be some enzymes whose active site is headed to support rather than to bulk solution, which prevents the HWMS from binding with the active site of enzyme (Guisán *et al.*, 1997). Another reason is the proximity of enzyme and support. The hydrophobicity of the support is an important factor on the stability of immobilized enzymes. If the surface of the support is hydrophobic, it will expel the water molecules away from the support and the microenvironment of immobilized enzyme will be changed. Because a ternary structure of enzyme is maintained by surrounding water molecules through hydrogen bonding, any change of surrounding water molecules leads to change of the ternary structure, which causes a denaturation of enzyme (Tisher and Wedekind, 1999).

Introducing a spacer arm between enzyme and support is one of the solutions that reduce the steric hindrance and the hydrophobicity of the support. The spacer arm gives higher mobility to enzyme, which can increase the affinity between enzyme and HMWS, and also moves the enzyme away from the surface of the support, which can reduce the effect of the support (Penzol *et al.*, 1998). The early studies of enzyme immobilization using silk were to immobilize the enzyme on fiber (fabrics) surface via covalent bonding or adsorption (Cordier *et al.*, 1982; Grasset *et al.*, 1982). In addition to the development of silk nonwoven fabrics (Kato *et al.*, 1998; Lee *et al.*, 1999) they can also be used for the enzyme immobilization. In the present study, we immobilized trypsin onto silk fiber and introduced some spacer arms to enhance the activity as well as the stability of immobilized trypsin and hydrolyzed silk sericin as HMWS.

Materials and Methods

Materials

Silk fibroin fiber (SFF) was obtained from Hung Jin Co., Ltd. Typsin (EC 3.4.21.4.), bovine serum albumin (BSA) and all other chemicals were purchased from Sigma-Aldrich Korea., LTD.

Activation of SFF

In order to immobilize trypsin on the SFF, the amine groups were activated with glutaraldehye (GA). Thirty milligrams of SFF was winded to a bundle and set in an Eppendorf tube. To increase the reactive site, it was partially hydrolyzed with 3.65 N HCl for 1 hr at 50°C. After washing 2 times with distilled water and 3 times with 0.2 M sodium bicarbonate buffer pH 9.2, 1 ml of 10%(v/v) GA, prepared in the same buffer as before, was added to activate the SFF. The reaction was continued for 1 hr at 25°C. The activated SFF (SFF-GA) was washed 2 times with distilled water and 3 times with 0.1 M sodium phosphate buffers pH 7.4. The SFF-GA was used for further coupling reactions with the spacer arms or the enzymes.

Introduction of ethylene diamine, BSA and silk sericin as spacer arms

Before introducing ethylene diamine (ED), SFF-GA was washed 3 times with 0.2 M sodium bicarbonate buffer pH 9.2. Coupling ED onto the SFF-GA was done by adding 1ml of 0.5 M ED in 0.2 M sodium bicarbonate buffer pH 9.2 for 2 hrs at 25°C and then washed 4 times with the same buffer solution. BSA was coupled onto the SFF-GA with 1.0% solution in 0.1 M sodium phosphate buffer pH 7.4. After 12 hrs incubation at 4°C, non-covalently bound BSA was removed by washing 2 times with 0.5 M NaCl in 0.1 M sodium phosphate buffer pH 7.4 and 3 times with 0.2 M sodium bicarbonate buffer pH 9.2.

Silk sericin (SS) was obtained by extracting from silkworm cocoons just before coupling. The cocoons were boiled with distilled water for 1 hr at 120°C and filtered through glass filter. Final concentration of SS solution was 1%. Appropriate amounts of sodium phosphate monobasic and dibasic were added to adjust the pH to 7.4. This solution was added to the SFF-GA and the coupling followed the same procedure as BSA.

Finally, each sample was reactivated with 10% GA for 1 hr at 25°C and washed 2 times with distilled water and 3 times with 0.1 M sodium phosphate buffer pH 7.4. Here, the SFF samples used for enzyme immobilization were designated as SFF-ED, SFF-BSA and SFF-SS after their name of the spacer arm, respectively.

Immobilization of trypsin

One percent (w/v) of trypsin solution was prepared in an ice-cold 0.1 M sodium phosphate buffer pH 7.4, and added to each SFF sample. After incubation at 4°C over night, non-covalently bound trypsin was removed by washing 2 times with ice-cold 0.1 M sodium phosphate buffer pH 7.4, containing 0.5 M NaCl. Finally, it was washed with 0.1 M sodium phosphate buffers pH 8.0 for 3 times and used for further experiments.

Quantification of bound trypsin and its activity test

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.

N-benzoyl-DL-arginine-p-nitroanilide hydrochloride

(BAPNA) was used as substrate. For the activity of immobilized trypsin, 250 μl of 10 mM BAPNA in DMSO were diluted with 1 ml of distilled water and 150 μl of 0.1 M sodium phosphate buffer pH 8. This mixture was added to trypsin immobilized SFF and incubated for 30 min at 25°C. The increase of absorbance at 405 nm was measured using UV spectrometer (UVICON 923, Kontron Instruments, USA). The activity was defined as mM BAPNA hydrolyzed within 1 min. In the case of free trypsin, the final amount of trypsin in the test tube was 15 μg.

Thermal and operational stability of immobilized trypsin was tested as follows. Thermal stability was measured at 50°C and each sample containing 0.1 M sodium phosphate buffer pH 8.0 was incubated for desirable times. On the other hand, operational stability was measured with 0.1% (w/v) SS solution as a substrate. The SS was hydrolyzed for 30 min at 25°C and the increase of N-terminal was measured by ninhydrin method. One hundred micro liters of hydrolyzed SS solution was taken from the test tube and the same amounts of ninhydrin reagent were added. After 15 min incubation in boiling water, it was cooled in ice-water bath and 500 μl of 50% (v/v) ethanol was added. Using UV spectrometer, the increase of absorbance at 570 nm was measured. The concentration of N-terminal was calculated from L-serine as a standard. After each reaction, the SFF 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.

Result and Discussion

The overall reaction schemes are shown in Fig. 1. The SFF has some functional groups, which can be activated by further reactions. These include hydroxyl, amino and carboxylic group on the side chain of amino acids and each terminal group of single protein molecule. Among these functional groups, amino groups are targeted to bind the enzyme because of its simple activation method. Although the SFF contains basic amino acids, such as lysine, arginine and histidine, their total contents are only about 1 mol% of total amino acids, which are insufficient to bind trypsin (Zhao and Asakura, 2001). Therefore, a partial hydrolysis is necessary to increase N-terminal groups on the surface of SFF before activation.

The amino groups of silk are first activated with GA at alkaline pH. Then the immobilization is completed by further reaction between the unreacted aldehyde groups of GA and the amino groups of enzyme. To enhance the activity and stability of immobilized trypsin, spacers are introduced.

Figure 2 shows the possible immobilization scheme how the spacer arms act between the support and the trypsin. ED was used as a low molecular weight spacer arm with a short chain length, while BSA and SS as high molecular weight spacer arms. In general, these spacer arms move trypsin away from the silk surface, thus reducing the steric hindrance and the effect of surface. Especially, in the case of BSA and SS, trypsin is attached via multiple bonds, which can exhibit the enhanced stability.

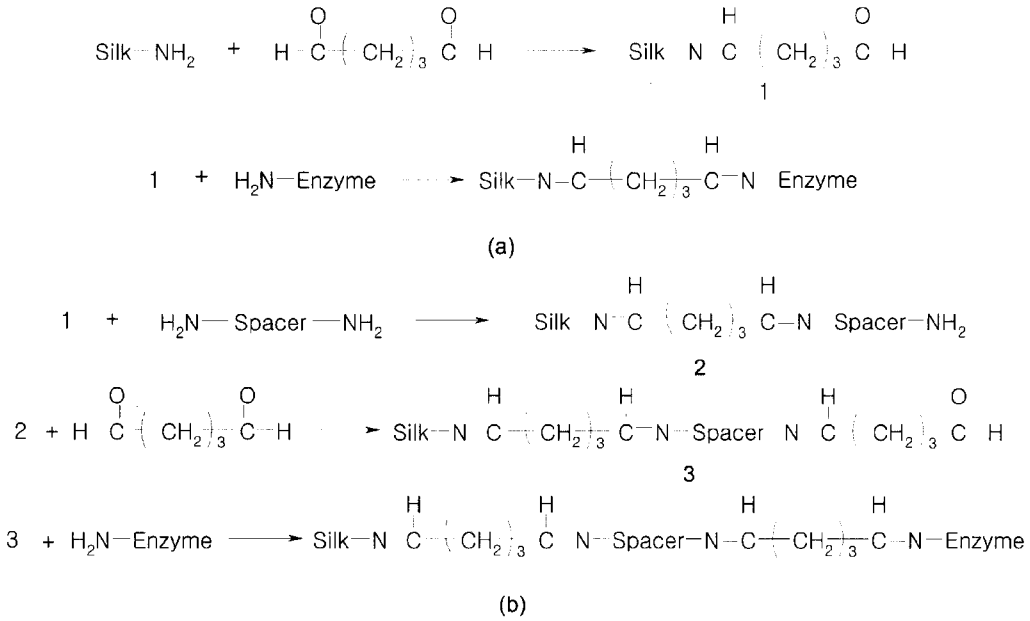

Fig. 1. The reaction scheme of trypsin immobilization onto SFF (a) directly and (b) through spacer arms. **1**: activated SFF with glutaraldehyde (SFF-GA), **2**: spacer arm introduced SFF, **3**: reactivated SFF with glutaraldehyde.

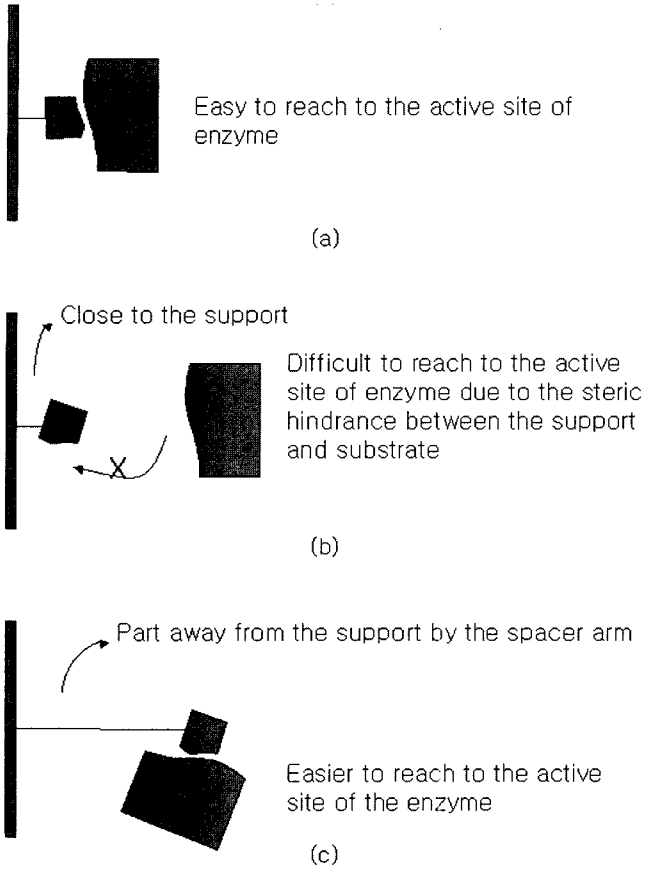

Fig. 2. The active site of enzyme could be faced to the bulk solution (a) or to the support (b). The spacer arm part the enzyme away from the support, thereby the substrate can easily bound to the active site of enzyme (c).

Table 1. Activity of immobilized trypsin on SFF

Support	Bound trypsin ($mg \cdot g^{-1}$)	Activity ($mM \cdot min^{-1}$)	Specific activity (100 $mM \cdot min^{-1} \cdot g^{-1}$)
Free Trypsin	–	0.39	25.87
SFF-GA	8.05	0.57	2.38
SFF-ED	6.17	0.59	3.19
SFF-BSA	10.8	0.63	1.96
SFF-SS	5.6	0.85	5.08

The amounts of bound trypsin and the activities were compared in Table 1. When trypsin was immobilized directly on SFF-GA, 8.05 mg/g of trypsin is immobilized. If ED was used as a spacer arm (SFF-ED), it reduced to 6.17 mg/g, due to reduced active functional groups. During the activation of SFF-ED, the color of SFF turns into yellow, caused by the reaction between GA and ED. Consequently, this reduces free aldehyde groups, which has to react with trypsin. The SFF-BSA showed the best results among the others while the SFF-SS showed the least. It could be explained if we consider the difference of amino acid composition between BSA and SS. GA reacts mainly with amino groups of lysine, arginine and histidine, and the total contents of these three amino acids are 16.79% (Hilger *et. al.*, 2001) and 7.70% (Komatsu, 1975) for BSA and SS, respectively, which result in more capability for BSA to react with GA further with trypsin.

Table 2. Kinetic parameters of immobilized trypsin

Support	Km (mM)	Vm (100 $mM \cdot min^{-1} \cdot g^{-1}$)
SFF-GA	66.31	64.18
SFF-ED	1.69	6.80
SFF-BSA	2.40	4.18
SFF-SS	4.05	16.28

The overall activity increased in the order of SFF-GA, SFF-ED, SFF-BSA and SFF-SS. The introduction of spacer arm has increased the activity of immobilized trypsin. But in the case of SFF-BSA, the specific activity decreases in spite of the increase of overall activity, which may be due to a high content of bound trypsin. Because trypsin is very susceptible to autolysis, there is a possibility of autolysis by a neighboring trypsin if they are bound too closer.

The kinetic parameters of immobilized trypsin was measured by Lineweaver-Burk plot and the results are shown in Table 2. As the spacer arms are introduced, the Michaelis-Menton constant, Km, and the maximum forward velocity of the reaction, Vm, are decreased. The decrease of Km value indicates that the affinity of enzyme with substrate has increased. By introducing the spacer arm, the mobility of immobilized trypsin has increased, while the steric hindrance has reduced. On the other hand, Vm decreases because of some improper immobilization such as covalent bonding near or at the active site of trypsin. Identically, the active site of trypsin should be protected during the immobilization step but it is hard to control, and the decrease of Vm is inevitable.

In the case of SFF-GA, Vm is extremely high, which means that a lot of trypsin are immobilized in a proper direction. But the surface characteristic of SFF prevents the proximity of substrates, thus resulting that overall activity is low. The SFF-ED showed the highest affinity due to its flexible linear chain structure, which allows higher mobility of immobilized trypsin. This gives more proximity between the active site of trypsin and substrate. But there may some improper immobilization, which could be known from a low Vm value. We have expected better results with hexamethylenediamine as a spacer arm, but the activity decreases due to a low bound trypsin as same reason as SFF-ED activation reaction (data not

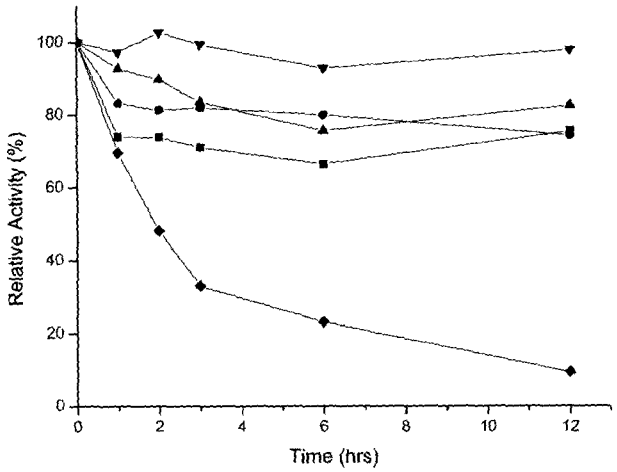

Fig. 3. Thermal stability of free (◆) and immobilized trypsin at 50°C; SFF-GA (■), SFF-ED (●), SFF-BSA (▲), and SFF-SS (▼). Relative activities were calculated from each initial activity as 100%.

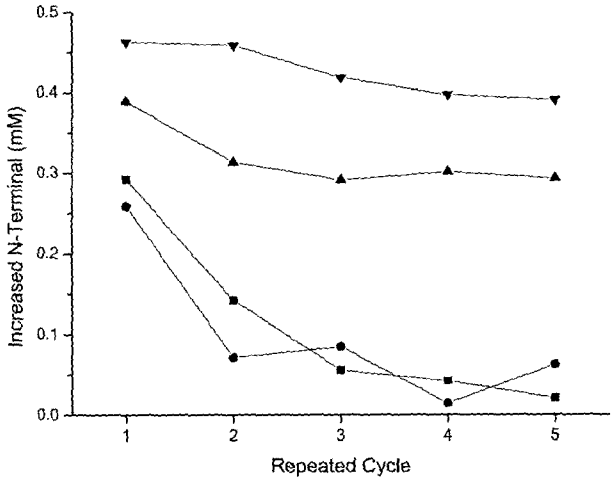

Fig. 4. Repeated hydrolysis of SS with immobilized trypsin; SFF-GA (■), SFF-ED (●), SFF-BSA (▲), and SFF-SS (▼).

shown). The SFF-BSA and SFF-SS show higher Km value even though they keep the trypsin long away from the support. Because these proteins have stable three dimensional structure, the mobility may be limited compared with ED. The difference between BSA and SS can also be explained by their structural difference. The BSA is a extremely soluble protein with globular structure whereas the SS is a slightly soluble protein with some α-sheet structure, which means SS has more restricted structure than BSA. The Vm value of SFF-BSA is lower than that of SFF-ED and, with the increase of Km value together, leads lower specific activity. The SFF-SS showed the highest Vm among the spacers, indicating that there may be less improper immobilization occurred.

Generally, immobilization of enzyme causes some loss of its activity, but in most cases, its stability increases, which compensates the former disadvantage. Figure 3 shows the thermal stability of immobilized trypsin at 50°C. Free trypsin lost 50% of its initial activity within 2 hrs while immobilized one kept 75% of its initial activity even after 12 hrs. The SFF-BSA and SFF-SS had better results than SFF-GA and SFF-ED, indicating that multipoint attachment of trypsin can occurs when the BSA and SS are used for spacer arms. 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 organic solvents (Mozhaev *et al.*, 1990).

The main reason of enzyme immobilization is to reuse the enzyme repeatedly. Thus, we investigated the operational stability with silk sericin as a high molecular substrate. As shown in Fig. 4, the SFF-GA and SFF-ED loose half of their activity right after a first run, whereas the SFF-BSA and SFF-SS still maintain over 80% of initial activity after 5 runs. Silk fiber is hydrophilic than commercial synthetic fibers but still hydrophobic compared with other soluble proteins. In the case of SFF-ED, even though there was a slight increase of specific activity, the operation stability was not improved. It may be because of the adsorption of sericin on the silk fiber. As mentioned before, during the activation of SFF-ED, excess amounts of GA and ED are added for a sufficient reaction and some of remained functional groups react each other, which may increase the hydrophobicity of SFF-ED. In both cases, SFF-GA and SFF-ED, hydrophobic interaction between SFF and sericin may pack the trypsin from each side, which cause a denaturation of trypsin.

On the other hand, the SFF-SS showed the best result, by remaining 90% of initial activity even after 5 runs. When the BSA and SS are used as a high molecular weight spacer arm, trypsin is far away from the silk fiber and the surface characteristic of silk fiber is diminished. It is interesting that BSA is more hydrophilic than SS in nature, expecting higher operational stability. Whether a protein is hydrophilic or not mainly depends on its amino acid composition. According to Fauchère-Pliška hydrophobicity scale, amino acids that are more hydrophilic than Gly are Arg, Lys, Asp, Glu, Asn, Gln and Ser in the order of decreased hydrophilicity (Fauchère and Pliška, 1983). Among these amino acids, Arg and Lys are coupled with GA during the activation step, as a result hydrophilic properties of these amino acids are diminished. Therefore the rest of amino acids are responsible for the hydrophilicity of BSA and SS. The contents of these amino acids are 31.61% (Hilger *et al.*, 2001) and 54.56% (Komatsu, 1975) for BSA and SS, respectively. We cannot predict precisely how much SS is more hydrophilic than

BSA, because we do not have exact information how many of these hydrophilic amino acids are actually participated in the accessible surface areas of protein. Nevertheless, the tendency can be explained. Even though Ser is less hydrophilic than other amino acids that mentioned above, its high content in SS, about 34% of its total amino acids, may play a role on the operational stability of trypsin. As a result, SFF-SS has better operational stability than SFF-BSA.

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