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Bioconjugation of Neutral Protease on Silk Fibroin Nanoparticles and Application in the Controllable Hydrolysis of Sericin

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ABSTRACT: *Bombyx mori* silk fibroin is a protein-based macromolecular biopolymer with remarkable biocompatibility. Silk fiber was degummed and subjected to a series of treatments, including dissolution and dialysis, to yield an aqueous solution of silk fibroin, which was introduced rapidly into excess acetone to produce crystalline silk fibroin nanoparticles (SFNs). The SFNs were conjugated covalently with a neutral protease (NP) using glutaraldehyde as the cross-linking reagent. The objective of this study was to determine the optimal conditions for biosynthesis of the SFN-NP bioconjugates. First, SFN-NP was obtained by covalent cross-linking of SFN and NP at an SFN/NP ratio of 5−8 mg:1 IU with 0.75% glutaraldehyde for 6 h at 25 °C. When adding 50 IU of the enzyme, the residual activity of biological conjugates was increased to 31.45%. Studies on the enzyme activity of SFN-NP and its kinetics showed that the stability of SFN-NP bioconjugates was greater than that of the free enzyme, the optimum reactive temperature range was increased by 5−10 °C, and the optimum pH value range was increased to 6.5−8.0. Furthermore, the thermal stability was improved to some extent. A controlled hydrolysis test using the poorly water-soluble protein sericin as a substrate and SFN-NP as the enzyme showed that the longer the reaction time (within 1 h), the smaller the molecular mass (<30 kDa) is of the sericin peptide produced. The SFN-NP bioconjugate is easily recovered by centrifugation and can be used repeatedly. The highly efficient processing technology and the use of SFN as a novel vector for a protease has great potential for research and the development of food processing.

KEYWORDS: Silk fibroin nanoparticles, cross-linker, neutral protease, bioconjugate, sericin, hydrolysis

■ INTRODUCTION

Neutral proteases (NPs) have attracted considerable interest because of the wide variety of possible applications, for example, in the production of functional food proteins by hydrolysis of mung bean (*Vigna radiatus*; formerly *Phaseolus aureus* or *Phaseolus radiatus*), cheese whey protein, corn gluten, tilapia (a cichlid fish), etc., in the improvement of the texture and sensory properties of dairy products, and in peptide synthesis in organic media.[1] Moreover, the chemical properties of soluble sericin peptides change as a function of the protease used; a mixture of peptides with molecular masses of 5−20 kDa, with a weight average molecular mass of ∼12 kDa, was obtained by hydrolysis with alkaline and NPs.[2] For this reason, enzymic hydrolysis of sericin is used in the production of antioxidants, [3] senior cosmetics, [4] antihyperlipidemic [5] and antitumor drugs,[6] and for serum-free cell culture,[7] etc. This technology has some disadvantages; for example, the free enzyme is difficult to refine and store and cannot be recycled, which restricts its use in large-scale industrial applications.

Enzyme immobilization technology can solve this problem and realize the recycle of enzymes. Several authors have described the effects of immobilized NP. Koneracka et al. immobilized Dispase, an NP, on magnetic particles using direct-binding procedures. Using a dye-binding procedure, they found that 90% of the protein was bound. They measured the proteolytic activity of Dispase and found that it retained 80% of its activity after immobilization.[8] Chellapandian and Sastry used vermiculite as an economic support for the immobilization of an NP and found that the pH and thermal stability of the immobilized NP were greater than those of the free enzyme.[9] Li et al. immobilized a protease on glutaraldehyde-treated *N*-succinyl chitosan hydrogel beads and used the biocatalyst obtained for the preparation of low molecular mass (1.9−23.5 kDa) chitosan and chito-oligomers from chitosan. This method allows repeated use of the immobilized enzyme and is suitable for the large-scale production of low molecular mass chitosan and chito-oligomers free of protein admixtures.[10] Ortega et al. showed that immobilization of a protease on alginate beads is simple and inexpensive, and the bioconjugate is active in extremely mild conditions; thus, the potential for industrial applications is considerable.[1]

Silk derived from the silkworm *Bombyx mori* is a protein-based macromolecular biopolymer that consists of two proteins, silk fibroin and sericin, which have excellent biocompatibility and are nontoxic.[11] After sericin, the "silk gum" that coats the fiber is removed, and the fibroin fiber is soluble in highly concentrated neutral salts such as $CaCl_2$. The regenerated liquid fibroin has good plasticity and can be made into various forms, including film, gel, particle, solution, and fiber. The silk protein films or membranes have good biocompatibility for the control-released carrier of enzymes/drugs.[12] We have developed a novel method for processing SFNs into a novel support or vector of silk fibroin for enzyme/drug bioconjugation or modification.[13] SFNs have a larger surface area and smaller grain diameters than the fiber,

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which makes it easy to couple some specific ligand with its surface and to regulate the structure and function of enzymes. The SFNs are globules with a fine crystallinity that offer various possibilities for surface modification and covalent drug attachment. More recently, we have used glutaraldehyde to conjugate insulin,[14] ASNase,[15] and β-glucosidase [16] with the SFN surface and found that covalent bonding with these SFNs improved the biological stability and activity in vitro. We suggest that SFNs have great potential for use in the study and development of new bioconjugates for enzyme/polypeptide drug delivery systems.

We report a highly efficient method for the production of fine crystalline SFN-NP bioconjugates in excess organic solvent. We describe in detail the biosynthesis, kinetics, in vitro activity, and biostability of a modified NP. We used the immobilized enzymes repeatedly to hydrolyze the sericin macromolecule to obtain sericin peptides with a range of molecular masses. The results of this study are a good foundation for research and development of SFNs as enzyme/drug carriers and provide new methods for inexpensive large-scale production of sericin peptides.

MATERIALS AND METHODS

Materials. Cocoons of the silkworm *B. mori* were provided by the Sericulture Department, Soochow University, China. The NP was purchased from the Naning Doing-Higher Biotech Co., Ltd.

Regenerated Liquid Silk Fibroin. Cocoon shells of *B. mori* were degummed by boiling twice in 0.2% (w/v) Na_2CO_3. The degummed fiber was subsequently dissolved in $CaCl_2$/ethanol/water at a molar ratio of 1:2:8 at 90 °C for 4 h in a constant temperature vibrator bath (120 rpm). The proportion of silk fiber to the solvent was 1:25−30 (w/v). After it was cooled and filtered, the filtrate was dialyzed continuously for 3 days against running water and then against deionized water for 1 day.

Preparation of SFNs. The regenerated 1.0% (w/v) silk fibroin solution was introduced rapidly into excess water-miscible acetone using a sample pipet and was heated at 40 °C. Milklike silk protein particles were formed immediately and were suspended in the mixture of water and organic solvent. These protein microparticles are insoluble in water and precipitate slowly due to aggregation. The precipitate was separated from the solvent and collected by repeated centrifugation at 16000 rpm (Avanti J-30I centrifuge; Beckman, United States) and then dispersed evenly in water and stored at 4 °C. The nanoparticles are insoluble but stably dispersed in aqueous solution and are globular particles with a diameter of 45−125 nm as determined by transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), and laser sizing.[17]

Preparation of SFN-NP. A 1.0 mL sample of SFN (10 mg/mL) was introduced into a plastic flask and mixed with a solution of NP at 4 °C. After mild homogenization, the required volume of 25% glutaraldehyde was added slowly to the mixture. Finally, phosphate-buffered saline (PBS, pH 7.0) was added to a final volume of 5.0 mL. The flask was stoppered tightly and then placed onto an orbital shaker for 30 min at 4 °C for the cross-linking reaction to proceed. The reaction was stopped by the addition of ~200 mg of glycine. The mixture was then centrifuged twice at 30000 rpm (Beckman Avanti J-30I) at 4 °C for 30 min to remove impurities. The supernatant was discarded, and the precipitate was concentrated to 10 IU/mL in PBS.

Preparation of Sericin. The poorly soluble sericin used as the reaction substrate in the section Hydrolysis of Sericin was prepared as described.[18] After they were washed repeatedly with large volumes of hot water, 1 kg of cocoon shells was immersed in 15−30 L of distilled water overnight and then heated in a high-pressure boiler (120 °C and 2 atm; AMA 240, Astell Co. Ltd., United Kingdom) for 1 h, and then, the degumming solution was filtered through activated carbon to remove impurities and/or precipitates. The resulting light yellow sericin solution agglomerated into a gel at room temperature because of the high molecular mass of the protein. The resulting sericin peptides were made into a powder with a spray dryer (PWGS-2, Shanghai Trustech Industrial Co. Ltd., People's Republic of China) at temperatures ranging from 70 to 120 °C. The sericin peptide powder is soluble in hot water and was used as described (see Hydrolysis of Sericin).

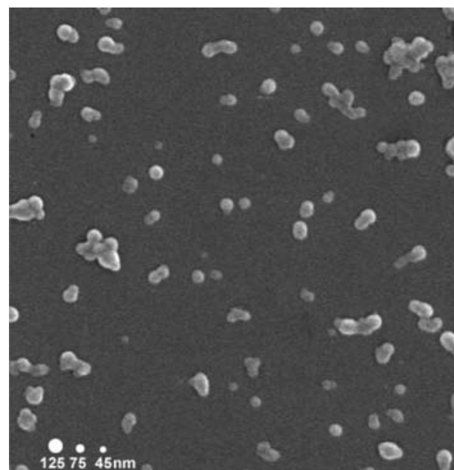

Figure 1. SEM images and size of SFN-NP conjugates.

Assay of Protease Activity. The enzyme activities of native NP and SFN-NP were determined by UV spectrophotometry (U-3000; Hitachi Co. Ltd., Japan).[19] The catalytic assay was measured in triplicate at constant temperature for 10 min by adding 1 mL of the solution of free NP or immobilized enzyme to 1.0 mL of casein (20 mg/mL in 0.2 mol/L PBS, pH 7.0); 2.0 mL of 10% trichloroacetic acid (TCA) was added to end the reaction. The control was a mixture of 2.0 mL of TCA and 1.0 mL of casein solution (20 mg/mL) for reaction. After 10 min, the mixture was filtered, and the absorbance of the filtrate at a wavelength of 275 nm (A_{275}) was monitored with a UV−Vis spectrophotometer. The activity of the immobilized enzyme was calculated as:

$$\text{immobilized activity} = (A_{275} \text{ of SFN-NP})/(A_{275} \text{ of free enzyme}) \times 100\%$$

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). The molecular mass range of sericin protein was determined by SDS-PAGE as described [20] with 12% (w/v) polyacrylamide running gel and 5% (w/v) polyacrylamide condensing gel.

RESULTS AND DISCUSSION

Size and Recovery of SFN-NP. The SFN-NP conjugate solution was subjected to two ultrasonic treatments of 2 min each to disperse the aggregated silk nanoparticles and then diluted for observation in a scanning electron microscope (SEM; Hitachi S-570). At a magnification of 10× K, the silk particles appeared to be globular granules with diameters of 45−125 nm (Figure 1). Nanoparticles in general, especially protein granules, aggregate readily in solution. SFN-NP in water or buffer subjected to five ultrasonic treatments of 2 min each was well dispersed and remained so for more than 2 days at room temperature. The recovery of SFN-NP activity ranged from 24.18 to 31.45% (Table 1). The greater the proportion of NP in the SFN-NP conjugates, the lower the percentage recovery of

NP activity is. The highest activity recovery of SFN-NP was 31.45% when 50 IU was added.

Effects of Cross-Linker Concentration on SFN-NP Bioconjugation. It is well-known that glutaraldehyde can act as a cross-linker, but it can act as a denaturant.[21] Thus, the concentration of glutaraldehyde will directly affect the activity of SFN-NP. As shown in Figure 2a, when the concentration of glutaraldehyde increased from 0 to 0.75%, the relative enzyme activity of the bioconjugates also increased, reaching a peak value at 0.75% glutaraldehyde, and then decreased gradually with increasing concentration of glutaraldehyde. This suggested that when the concentration of glutaraldehyde was less than optimal, it was inadequate as a cross-linking agent; that is, it cannot achieve sufficient cross-linking of the NP molecules and SFN to form a stable covalent structure. In addition, when the concentration was greater than optimal, although glutaraldehyde still acted as a cross-linking agent, it was also a protein denaturing agent, leading to decreased enzyme activity.[22]

Table 1. Effect of the Enzyme Concentration on the Immobilization of NP[a]

NPs in SFN-NP (IU/10 mg SFNs)	ABS (10 IU)	recovery (%)
50	0.134	31.45 ± 0.010
60	0.124	29.10 ± 0.023
70	0.118	27.70 ± 0.010
100	0.103	24.18 ± 0.028
free enzyme (10 IU)	0.426	100

[a] The data in the table were average values (±SDs) of three repeated measurements.

Effects of the Cross-Linking Proportion of NP and SFN on SFN-NP Bioconjugation. The relative costs of NP and silk fibroin suggest that we should choose the least amount of silk fibroin necessary to retain the maximum amount of NP. The results of the experiments described above show that the highest recovery of SFN-NP activity was obtained when 50 IU of NP was added. Therefore, we used different SFN/NP ratios to evaluate the effects of the cross-linking proportion of NP and SFN. The NP activity was constant at 50 IU, and the amount of SFNs was varied to give NP/SFN ratios of 4:1 (2.50 mg), 5:1 (10.00 mg), 8:1 (6.25 mg), 10:1 (5.00 mg), and 15:1 (3.33 mg) each in a total reaction volume of 5.0 mL. As shown in Figure 2b, the highest recovery of NP activity was obtained when 50 IU of NP was mixed with 10 mg of SFNs, an NP/SFN ratio of 5:1. Excess SFN limits the contact between enzyme and substrate, whereas insufficient SFNs do not achieve optimal cross-linking between enzyme and SFNs.

Effects of the Cross-Linking Reaction Temperature on SFN-NP Bioconjugation. As seen in Figure 2c, the optimal temperature for the immobilization process of SFN-NP conjugation was 25 °C. Cross-linking between NP and SFN with glutaraldehyde as the cross-linking agent is an exothermic chemical process. High temperature impairs the cross-linking reaction and causes enzyme deactivation, and the activity of SFN-NP declined rapidly with increased temperature.

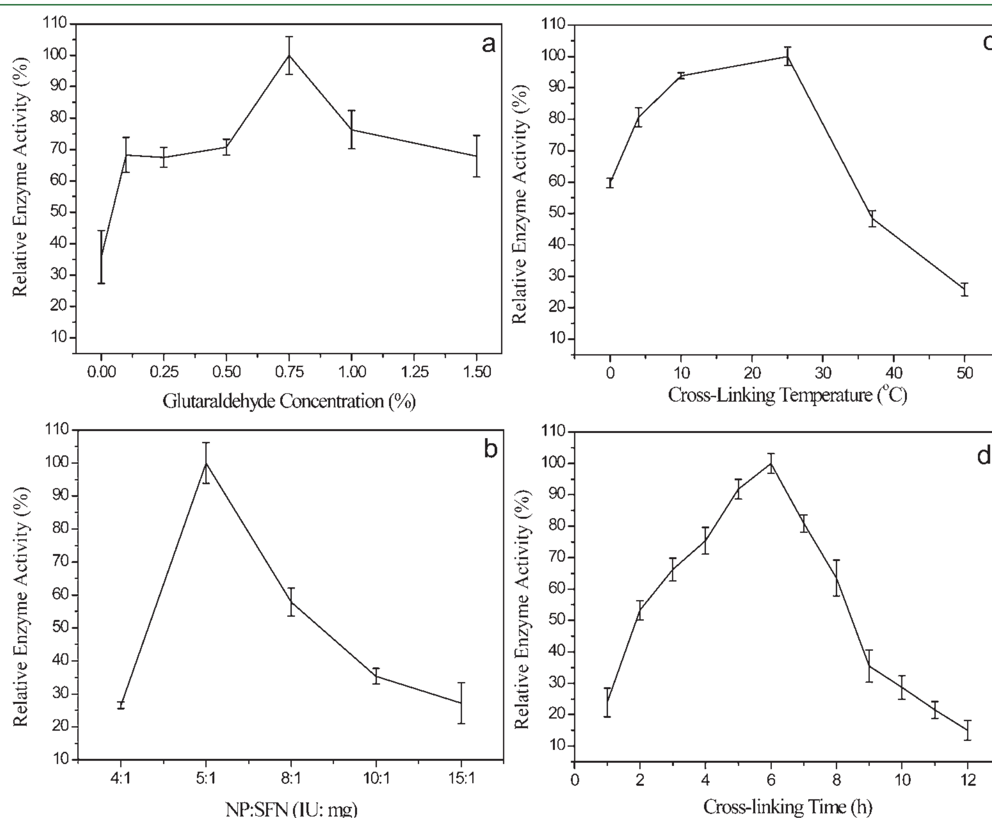

Figure 2. Effects of glutaraldehyde concentration (a), proportions of NP/SFN (b), and cross-linking temperature (c) and time (d) on SFN-NP bioconjugation. Proportions of NP/SFN in Figure 1b mean 4:1 (50 IU:12.50 mg), 5:1 (50 IU:10.00 mg), 8:1 (50 IU:6.25 mg), 10:1 (50 IU:5.00 mg), and 15:1 (50 IU:3.33 mg) in 5 mL of total reactive volume. The data in the figure were average values (±SDs) of three repeated measurements. Relative activities were calculated by using the highest ABS values as 100%.

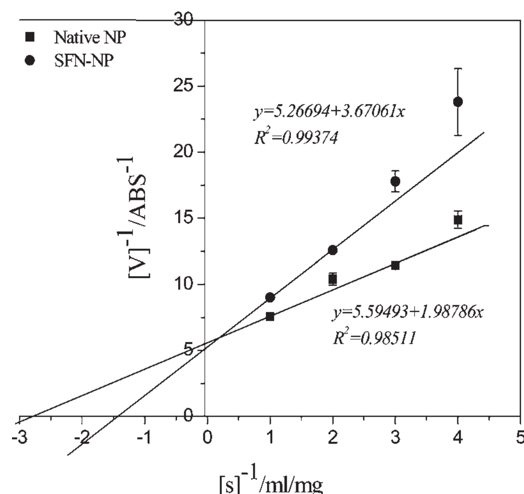

Figure 3. Lineweaver−Burk plots for the free and immobilized NP. The data in the figure were average values (±SDs) of three repeated measurements.

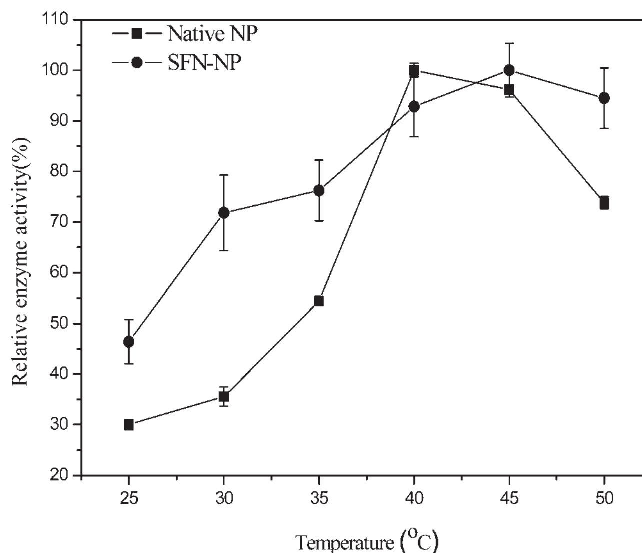

Figure 4. Effect of temperature on the activity of free and immobilized NP. The data in the figure were average values (±SDs) of three repeated measurements. Relative activities were calculated by using the highest ABS values as 100%.

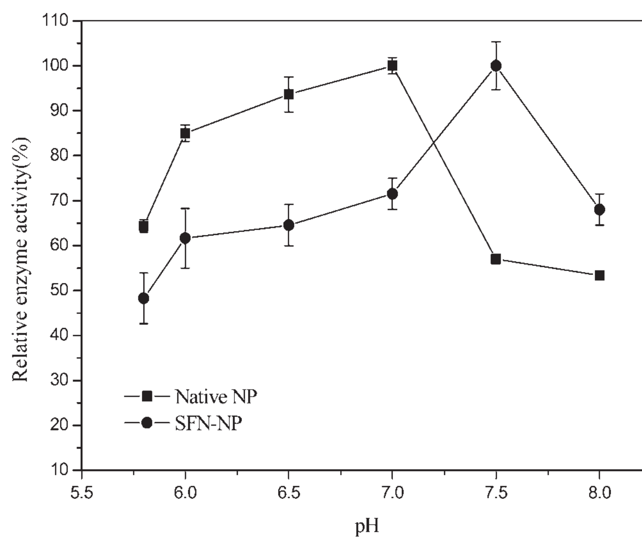

Figure 5. pH/activity curves for free and immobilized NP. The data in the figure were average values (±SDs) of three repeated measurements. Relative activities were calculated by using the highest ABS values as 100%.

Effects of the Cross-Linking Time on SFN-NP Bioconjugation. We found that the activity of NP bioconjugated with SFNs increased rapidly with increased length of cross-linking time (Figure 2d), reaching a peak value at 6 h. The activity decreased quickly during the period between 6 and 9 h and more slowly thereafter. The possible reason for this phenomenon is the influence of the glutaraldehyde.

Enzyme Kinetic Properties. The Michaelis constant for native and modified NP was determined (Figure 3). The apparent Michaelis constant, $K_{m(app)}$, of SFN-NP derivatives (6.97×10^{-4} mg/mL) was higher than that of the free enzyme (3.55×10^{-4} mg/mL), suggesting that affinity between the enzyme and its substrate casein was decreased considerably when NP was cross-linked with SFNs. The reason for this phenomenon might be that the SFNs are bulky and slightly retard the reaction with the substrate and thus increase the K_m value.

Temperature Activity Profile. The effect of temperature on the free NP or SFN-NP was investigated in the range 25−50 °C. The optimal temperature for SFN-NP activity (45 °C) was a little higher than that of the free enzyme (40 °C) (Figure 4), suggesting that SFN-NP bioconjugation is less sensitive to temperature than that with the free enzyme and had greater heat resistance.

Optimal pH. The optimum pH for the activity of free NP and SFN-NP was investigated, and the results are shown in Figure 5. Immobilization on a support can change the microenvironment of the enzyme, leading to a change of optimal pH. In the present study, the optimal pH of the SFN-NP was displaced toward more alkaline values. It is thus clear that the SFN-NP bioconjugate was less sensitive to pH than the free NP.

Thermal Stability. The thermal stabilities of both free and modified NP were investigated. The effect of heat treatment on the activity of the two enzymes is shown in Figure 6. The thermal stability of SFN-NP activity was higher than that of the free enzyme at temperatures of 60−70 °C. Bioconjugates lost their activity more slowly than the free enzyme, perhaps because SFNs in the bioconjugates abates the thermal motion of the enzyme molecules, especially at higher temperatures. We found no significant difference between the thermal stability of SFN-NP activity and that of the free enzyme, similar to the results that we found when L-asparaginase was cross-linked to SFNs.[23]

Hydrolysis of Sericin. Figure 7 shows the SDS-PAGE pattern of the products of sericin hydrolysis by SFN-NPs. It can be seen that the first electrophoresis belt (0 min) expressed the poorly soluble sericin and its molecular mass ranged between 30 and >200 kDa. After enzymatic hydrolysis (10−120 min), the range of molecular mass was decreased, and the range became shorter with increased time. When sericin was hydrolyzed by SFN-NP for 30 min, the range of the molecular mass of the resulting sericin peptides was <50 kDa, and at 60 min of hydrolysis, the range was decreased to <30 kDa. After hydrolyssis for >120 min, the sericin protein was degraded into smaller polypeptides. So, it is feasible that low molecular mass powder could be produced on an industrial scale by using SFN-NP hydrolysis of sericin for 1 h or longer.

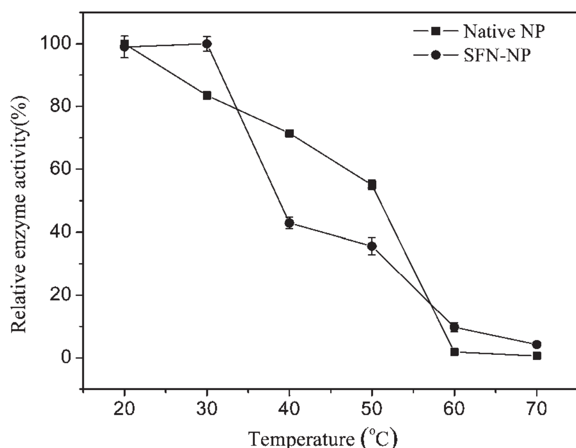

Figure 6. Thermal stability of free and immobilized NP. The data in the figure were average values (±SDs) of three repeated measurements. Relative activities were calculated by using the highest ABS values as 100%.

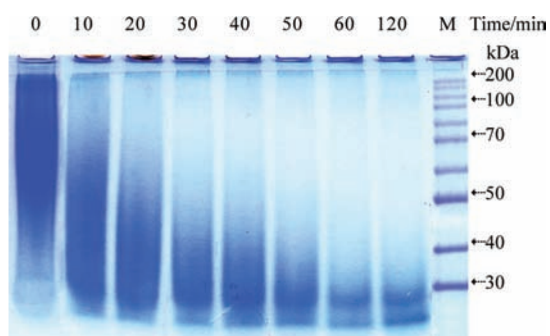

Figure 7. SDS-PAGE of the hydrolyzates of the poorly soluble sericin protein. The range of molecular weight is the sericin hydrolyzed by SFN-NP for 0, 10, 20, 30, 40, 50, 60, and 120 min, respectively. M means standard ladder protein.

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Author Contributions

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