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Research Article

Coimmobilization of Naringinases on Silk Fibroin Nanoparticles and Its Application in Food Packaging

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Bombyx mori silk fibroin is a macromolecular biopolymer with remarkable biocompatibility. It 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), which were conjugated covalently with naringinase using glutaraldehyde as the cross-linking reagent. The SFN naringinases are easily recovered by centrifugation and can be used repeatedly. Naringinase is a bienzyme consisting of α-L-rhamnosidase and flavonoid-β-glucosidase. The enzyme activity and its kinetics were similar to those of the native form, and the optimum reactive temperature for both is 55°C. In our study, centrifugation allowed the separation of enzyme and substrate; after eight cycles the SFN naringinases retained >70% residual activity. The highly efficient processing technology and the use of SFN as a novel vector for a bienzyme have great potential for research and the development of food processing such as the debittering of naringin-containing juices.

1. Introduction

Naringinase is a bienzyme consisting of α-rhamnosidase (EC 3. 2.1.40) and flavonoid-β-glycosidase (EC 3.2.1.21) [1]. α-L-Rhamnosidase hydrolyses naringin into the flavonoid prunin, which is converted to naringenin (4,5,7-trihydroxyflavonone) by β-D-glucosidase. Naringinase catalyses the hydrolysis of naringin to naringenin, glucose, and rhamnose. On the one hand, the removal of naringin (the bitter orange substance) with specific debittering of the strong flavour caused no detriment to the nutritional value of orange juice. On the other hand, the enzyme-extracted single glycosides of naringenin degradation products can be better absorbed by the body and can be used as pharmaceutical raw materials. Many immobilized preparations of naringinase have been used successfully for debittering and for producing biochemicals such as rhamnose and prunin. Several authors have described the effects of immobilised naringinase. In early 1985, Manjón et al. covalently immobilized naringinase to glycophase-coated, controlled-pore glass. The efficient kinetic parameters shown by the most active and stable derivative enabled it to be used for the debittering of naringin-containing juices [2]. In another case naringinase of *Penicillium sp.* when entrapped in cellulose triacetate fibers and when it showed it had a higher *Km* values than in its soluble form [3]. In the third case naringinase was immobilizated in packaging films that had an increased catalytic efficiency for reducing the concentration of naringin in grapefruit juice [4]. Moreover, in an attempt to use naringinase effectively for food packaging applications effectively, a food-grade active film that was able to reduce the naringin content of grapefruit juices during storage was obtained by a direct interaction with the product. The active film consists of a crosslinked matrix in which naringinase was completely immobilized [5]. The naringinase immobilized by adsorption onto diatomaceous earth [6] and glutaraldehyde-coated woodchips [7] had been studied. The efficiency and repetitive use of immobilized enzyme were also examined. Recently naringinase was bound to mesoporous silica MCM-41 via adsorption with glutaraldehyde and used it to debitter white grapefruit. The immobilized catalysts showed excellent thermal stability and storage stability, could be recycled 6 times, and retained ~44.57% activities [8]. In summary, immobilized enzyme

technology can enhance the utilization of naringinase, its production, and characterization.

Silk fibroin is a polymer protein biomaterial with useful biocompatibility and mechanical properties. When the silk protein is dissolved in highly concentrated solutions of neutral salts, the resulting liquid silk fibroin can be made into various forms of fibroin including film, gel, powder, and fibre. Silk fibroin had been used as a natural enzyme immobilization carrier [9]. Zhang et al. studied glucose oxidase, peroxidase, and urease immobilized in silk fibroin membrane [10, 11]. In addition, β-glucosidase has not only been fixed in film made from liquid silk fibroin, but also has very high level of activity [12, 13]. Recently, we prepared both water-soluble silk sericin and fibroin as the modifiers or bioconjugates for insulin and L-asparaginase modifications [14–17]. The results indicated that silk fibroin was a useful polymer for drug delivery, due to the controllable level of crystallinity and the ability to process the biomaterial in biocompatible fashion under ambient conditions in water. More recently, we also developed a novel method for processing silk protein nanoparticles [18], which are globules with fine crystallinity which have various possibilities for surface modification and covalent drug attachment. They have been used as the modifiers or bioconjugates for modifications of insulin, L-asparaginase, and β-glucosidase [19–21]. However, SFNs have been immobilized or modified to a monomeric enzyme but not to a bienzyme.

In the present study we used a novel and highly efficient method [22] to process fine crystalline SFN-naringinases in excess organic solvents by the configuration transition mentioned above. High-performance liquid chromatography (HPLC) was used to analyze the biological activities of the free enzyme and SFN naringinases.

2. Materials and Methods

2.1. Materials and Compounds. Cocoons of silkworm *Bombyx mori* were provided by the Department of Agriculture Science and Technology, Medical College of Soochow University, Suzhou, China. The pupa and its covering in the cocoon were removed, and the cocoon shells were cut into small pieces of ~1.0 cm^2 for the following experiments.

The naringinase (500 U/g·solid), naringin, and glutaraldehyde were purchased from Sigma Co. (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3), calcium chloride ($CaCl_2$), ethanol, acetonitrile, and acetone were all analytical-grade reagents (Shanghai Chemicals Factory, China).

2.2. Regenerated Liquid Silk Fibroin. Cocoon shells of *B. mori* were degummed twice in boiling solution of 0.5% (w/v) Na_2CO_3 for 0.5 h, and the degummed fibre was subsequently dissolved in $CaCl_2$/ethanol/water at a molar ratio of 1 : 2 : 8 at 70°C for 2 h in a constant temperature vibrator bath (120 rpm). After centrifuged at 8000 rpm for 10 min, the supernatant was dialyzed continuously for 48 h against running pure water to remove $CaCl_2$, smaller molecules and some impurities, using a cellulose semipermeable membrane with molecular weight cutoff of 10 kDa. The liquid silk fibroin was stored at 4°C.

2.3. Preparation of SFN Naringinases. A given volume of silk nanoparticles (40 mg/mL) was introduced into a plastic flask and mixed with naringinase solution at 4°C (silk nanoparticles/naringinases: 100 mg/75 U). After mild homogenization, the required volume of 25% glutaraldehyde solution was gently added into the mixture. The flask was stoppered tightly and placed in an orbital shaker at 4°C for cross-linking reaction. The reaction was stopped by the addition of ~100 mg of glycine. The reaction mixture was then centrifuged repeatedly at 30,000 rpm (Beckman Avanti J30I) to remove impurities, unreacted reagents, and even uncross-linked naringinase. The precipitate was then subjected to supersonic treatment (Output Watts: 16) for 2 min × 5 (Ultrasonic Processor; Sonics & Materials Inc, Newtown, CT, USA). Finally the nanoparticles were dispersed evenly in phosphate buffer and stored at 4°C in refrigerator for the following experiments.

2.4. Chromatographic Conditions. The mobile phase consisted of solvent A (acetonitrile) and solvent B (water), which were passed through a 0.2 μm pore size hydrophilic polypropylene filter and degassed in an ultrasonic bath before being used. The gradient was as follows: 0–8 min, 23% (v/v) A; 8–25 min, 23–65% A; 25–30 min, 23% A. The chromatographic column was Pursuit XRS C18 (150 × 4.6 mm, 5 μm). The photodiode array detector (PAD) was set at 200–400 nm and the chromatogram detected at 282 nm. The analysis was performed at 25°C (column oven temperature), with a flow rate of 0.8 mL/min and the injection volume was 20 μL.

2.5. Hydrolysis Reaction. Naringin solution with a certain concentration was mixed with 0.1 mL of the solution of free enzyme or SFN naringinases. The ethanol was added to end the reaction. At last, the mixture of SFN naringinases was centrifuged at 14,000 rpm for 10 min and then filtered through a 0.45 μm pore size hydrophilic polypropylene filter and stored at 4°C. The mixture of free enzyme was directly filtered. After the reaction, the content of residual naringin was measured as described above. The amount of naringin hydrolyzed was determined from the initial and residual naringin contents.

3. Results and Discussion

3.1. Standard Curve of Naringin by HPLC. The linear range for naringin (50–200 μg/mL) concentration was evaluated in Figure 1. The linearity equation was calculated by using linear regression analysis, and typical calibration curve was defined by the following equations: $y = 69988.3 + 24233.8x$, with a determination factor of 0.9983.

The reaction that SFN naringinases hydrolyzed naringin was the main work in this paper. The HPLC method was used to follow the enzymatic reaction at different reaction times. The results were summarized in Figure 2. Before hydrolysis reaction, the HPLC chromatographic pattern of the naringin (substrate) was shown as red solid line. We could

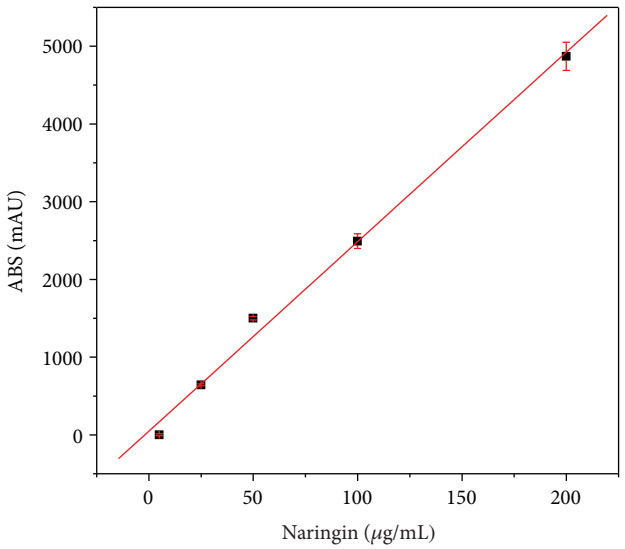

Figure 1: Standard curve of naringin by HPLC.

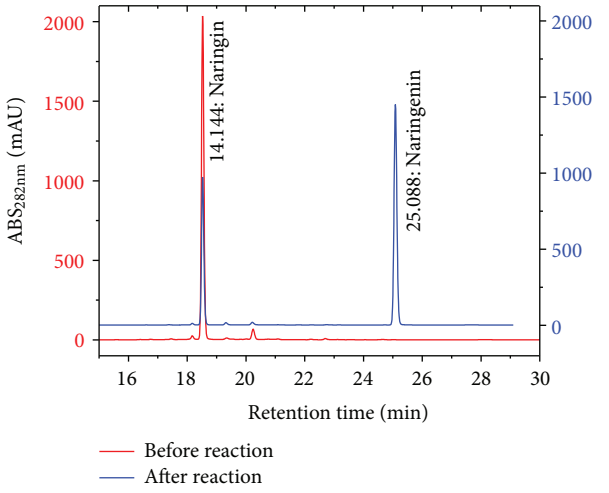

Figure 2: The HPLC chromatograms of the naringin and the naringin hydrolysis by SFN naringinase.

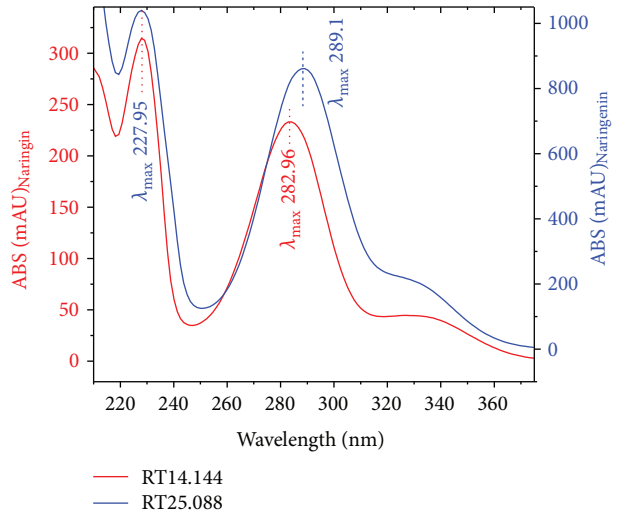

Figure 3: UV spectra of the naringin and the product naringenin the naringin hydrolysis by naringinase on PAD.

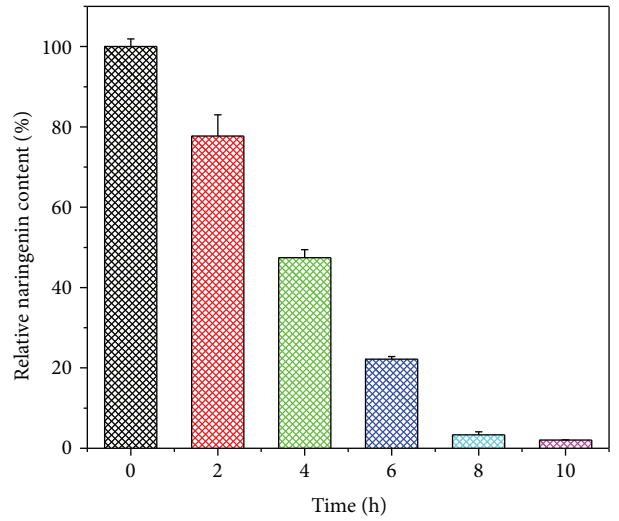

Figure 4: The residual naringin content in hydrolyzate: the initial naringin content was set as 100%.

observe that a main peak at the retention time of 14.144 min was the naringin with bitterness. When hydrolyzed by SFN-naringinases, it was found that the main peak of naringin disappeared but a new peak which is naringenin (reactive product) without bitterness appeared at the retention time of 25.088 min. In addition the longer the reactive time, the higher the peak of naringenin and the lower the peak of naringin (blue solid line). The decrease in content of naringin at the retention time of 14.144 min can be directly correlated with the reduction in bitterness. The UV spectra of the naringin peak (RT 14.144 min) and the naringenin peak (RT 25.088 min) could be extracted from the 3D data of PAD. We could see that the UV spectrum of the naringenin (blue solid line in Figure 3) without rhamnose and glucose was very similar to that of naringin (red solid line in Figure 3). The spectra peak shift between the two was about 6 nm at a band range of 250~320 nm. They had the same absorption peak of λ_{max} 227.95 nm at the absorption band of 220~240 nm.

3.2. The Hydrolysis of Naringin Catalyzed by SFN Naringinases. Standard assays of the SFN naringinases were carried out as follows: 1 mL of SFN-naringinases solution was introduced into 1 mL of 0.2% naringin which dissolved in McIlvaine buffer (pH 4.0) and then incubated at 40°C with shaking, sampling every 2 hours by adding ethanol to terminate the reaction. The mixture was centrifuged at 14,000 rpm for 10 min. Then, the supernate was filtered through a 0.45 μm pore size hydrophilic polypropylene filter and stored at 4°C for the following HPLC experiments to measure the residual naringin content. As shown in Figure 4, along with the extension of time the content of naringin

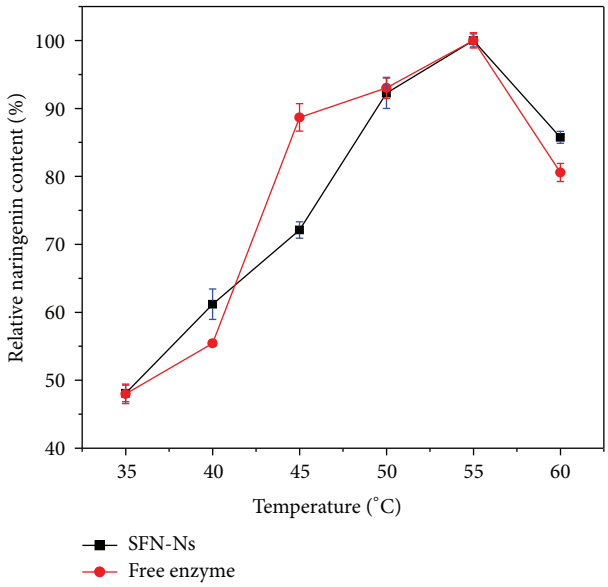

Figure 5: Effect of temperature on the hydrolysis degree of naringin by free and SFN naringinases.

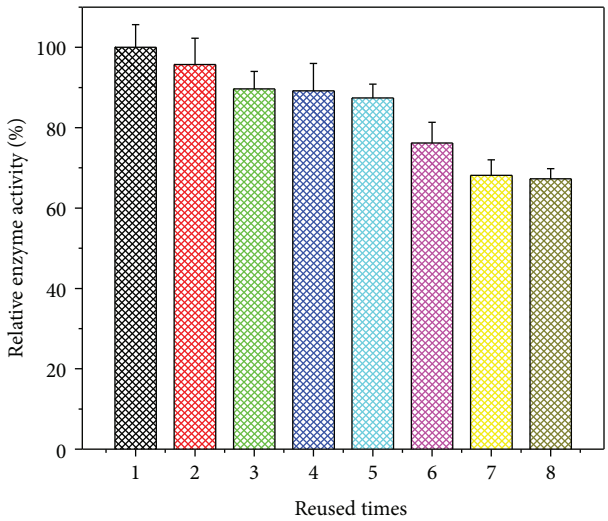

Figure 6: Operational stability of the SFN naringinases: the first value in the figure was set as 100%.

decreased obviously. After 8 hours, the naringin was completely hydrolysed. This suggested that the SFN naringinases were effective on the reaction and had great potential in the development of food processing such as the debittering of citrus fruit, grapes, and other fruits.

3.3. Optimal Reaction Temperature. The naringinase was sensitive to temperature. The dependence of the free or SFN naringinases on temperature was investigated across the range 30–80°C in acetate buffer (pH 4.0). Compared with free enzyme, the optimal temperature of SFN naringinases was the same and was 55°C; above or below it, the enzyme activity decreased rapidly (Figure 5). In the range 35–40°C and 55–60°C the activity of SFN naringinases was higher than the free enzyme. Suggesting that SFN naringinases were less sensitive to temperature than that with the free enzyme and had greater heat resistance, but the optimal reaction temperature of the enzyme was not changed.

3.4. Operational Stability. The mixture of naringin and SFN-naringinases was incubated in 55°C, pH 4.0-acetated buffer, 2 hours for reacting, and then centrifugation was allowed to separate the enzyme and substrate; after eight cycles the residual activity of SFN naringinases retained 67% (Figure 6). This indicated that SFN naringinases had a good operational stability. The relative decline in enzyme activity may be due to ultrasonic treatment for each batch of experiments, and the slight loss of the enzyme during centrifugation. Thus, the stability of the enzyme may be better in industrial processes than the experimental results reported here.

4. Conclusion

In this study, silk fibroin nanoparticles as carriers with glutaraldehyde as crosslinking agent were first carried out bienzyme immobilization to prepare SFN naringinases. Studies show that SFN naringinases have good hydrolysis capacity of naringin. Enzymatic reaction was at 55°C for 8–10 h, and then the enzyme and the product can be separated by a simple centrifugation. After 8 repeated enzymatic reactions it can maintain its enzymatic activity about 70% of the original activity. Therefore, the technology of this method to produce SFN naringinases is low-cost, simple process and has potential value in industrial debitter citrus juice processing research and development.

Conflict of Interests

The authors declare that they have no financial or personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in paper.

Authors' Contribution

M.-H. Wu and L. Zhu contributed equally to this study.

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