0273-2289/1559-0291 (Online)/07/136/0119/$30.00

Characterization of Immobilized Nuclease P1

Quo-Qing Ying,[1] Lu-E Shi,*,[2] Xiao-Ying Zhang,[3] Wei Chen,[3] and Yu Yi[1]

[1]College of Chemical Engineering and Materials Science and [2]College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310014, Zhejiang, China, E-mail: shilue@126.com; and [3]College of Pharmacy, South Dakota State University, Brookings, SD 57007

Received March 1, 2006; Accepted June 2, 2006

Abstract

To improve the efficiency of the use of nuclease P1, enzyme immobilization technology was applied using nuclease P1. Characterization of immobilized nuclease P1 on different supports was studied. The results showed that the optimum pH and temperature of nuclease P1 immobilized via different supports were enhanced. The immobilized enzyme was obviously stable when stored for long periods and was reusable. The best results were obtained when nuclease P1 was immobilized on chitosan nanoparticles. The nanoparticles were applied to protect the activity of nuclease P1 and improved enzyme activity by 13.17% over that of free nuclease P1 at the same conditions. The Michaelis constant *Km* and V_{max} were determined for free and immobilized enzyme as well.

Index Entries: Chitosan; nanoparticles; cellulose; nuclease P1; immobilization.

Introduction

Chitosan, poly (β-[1-4]-linked-2-amino-2-deoxy-d-glucose), is the *N*-deacetylated product of chitin, which is a major component of arthropod and crustacean shells such as lobsters, crabs, shrimps, and cuttlefishes *(1)*. Chitosan has several significant biologic and chemical properties such as biodegradability, biocompatibility, bioactivity, and polycationic properties *(2)*. Thus, it has been widely used in industrial and biomedical applications including in wastewater treatment, as chromatographic support, in enzyme immobilization, and as carrier for controlled drug delivery *(3–6)*.

*Author to whom all correspondence and reprint requests should be addressed.

In addition, chitosan is economically attractive, because it is an abundant natural polymer, second in abundance to cellulose.

Nuclease P1 (EC 3.1.30.1), an extracellular enzyme, was first identified by Kuninaka et al. *(7)*. The enzyme cleaves 5N-nucleotides (e.g., 5N-AMP, 5N-GMP, 5N-CMP, and 5N-UMP) successively from the 3N-hydroxy termini of 3N-hydroxy-terminated oligonucleotides originating from RNA *(8)*. 5N-Nucleotides are known to exhibit flavor-enhanced properties in food. The yeast extract is used in the following foods: noodle soups, sauces, gravy, ready to consume meals, processed flavorings, and processed cheese *(9)*. Moreover, 5N-nucleotides have been widely used in the pharmaceutical industry *(10)*. They can be used to synthesize antiviral and anticancer medicaments as the acridine *(11)*. For these reasons, nuclease P1 was chosen as the enzyme material for immobilization. To improve the efficiency of the use of nuclease P1 and decrease the cost of production, enzyme immobilization technology is applied using nuclease P1, which is an effective means of reusing enzyme and improving its stability. Chitosan is known as an ideal support material for enzyme immobilization because of its excellent characteristics, such as improved resistance to chemical degradation, lack of disturbance of metal ions to enzyme, and antibacterial property *(12,13)*. In recent years, nanotechnology has received significant attention in the preparation of immobilized enzymes. Under the nanoscale, nanomaterials have characteristics such as magnetism and large surface area. These characteristics are in favor of immobilized enzymes.

The literature provides numerous studies about the preparation of chitosan nanoparticles and their applications in the carrier of drugs *(14–16)*, but there are few studies regarding applications in the immobilization of enzymes. To the best of our knowledge, studies of nuclease P1 immobilized on chitosan nanoparticles have never been reported. In the present study, chitosan nanoparticles were prepared by ionization gelation methodology. Nuclease P1 was immobilized on chitosan nanoparticles and cellulose. Characterization of immobilized enzyme was studied systematically. In addition, chitosan nanoparticles acted as protector of nuclease P1.

Materials and Methods

Chemicals

Chitosan was purchased from Yuhuan Ocean Biochemical (Zhejiang, China). The molecular weight of chitosan was 91,000. TPP abbreviated from sodium polyphosphate was purchased from Dongsheng Chemical Preparation Factory (Zhejiang, China). Cellulose was purchased from Sigma. All other chemicals were of analytical grade.

Culture Conditions for NucleaseP1 Production

For nuclease P1 production, the bacteria was grown at 30°C in a medium (pH 7.0) consisting of (w/v) 3.0% sucrose, 0.10% potassium

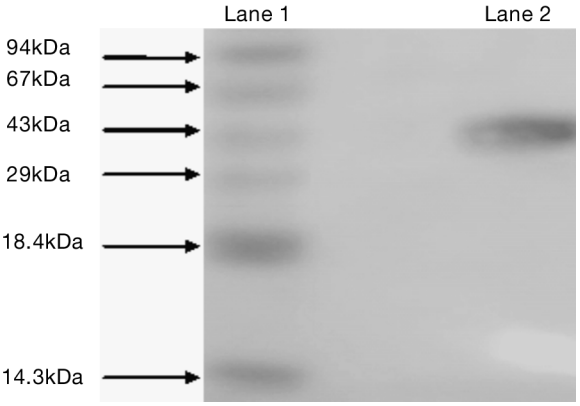

Fig. 1. Electrophoretogram of protein after various steps of purification on SDS-PAGE. Electrophoresis was carried out using a 15% crosslinked polyacrylamide. Lane 1, standard molecular weight markers; lane 2, purified enzyme.

dihydrogen phosphate, 0.010% ferrous sulfate, 0.30% sodium nitrate, 0.050% magnesium sulfate, 0.050% potassium chloride, and 20% potato extract. This 24-h grown mother culture (10 mL) was used to inoculate 50 mL of production medium containing (w/v) 6.0% glucose, 0.20% peptone, 0.50% groundnut meal, 0.030% zinc sulfate, 0.040% calcium carbonate, and 0.10% potassium dihydrogen phosphate. The pH of the medium was adjusted to 5.4 with HCl. Erlenmeyer flasks (500 mL) containing 50 mL of medium were incubated at 28°C in an orbital shaker at 240 rpm for 49 h. The 5N-phosphodiesterase solution was harvested by centrifuging at 4000 rpm at 4°C for 10 min, and the supernatant thus obtained was used as the crude enzyme preparation. The enzyme was purified to homogeneity according to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by thermal deactivation, ultrafiltration, $(NH_4)_2SO_4$ precipitation, phenyl Sepharose chromatography, ion-exchange chromatography, and gel filtration. Figure 1 presents the results.

Preparation of Chitosan Nanoparticles

Twenty milligrams of chitosan was dissolved in 40 mL of 2.0% (v/v) acetic acid. Twenty milliliters of 0.75 mg/mL TPP was dropped into the beaker. Chitosan nanoparticles could be stored in distilled water stably. The morphologic characterization of chitosan nanoparticles was evaluated by scanning electron microscope. Figure 2 presents the results.

Determination of Activity of Free and Immobilized Nuclease P1

Enzyme activity was measured in terms of the amount of acid-soluble nucleotides produced by RNA hydrolysis catalyzed by nuclease P1 *(17,18)*. Enzyme solution (0.10 mL) or immobilized enzyme (0.1 mg) was incubated with substrate solution (1.0% RNA; 0.125 *M* acetate buffer, pH 5.4; 3.0 m*M* Zn^{2+}) at 69°C for 15 min. The reaction was stopped by the addition of 2.0 mL

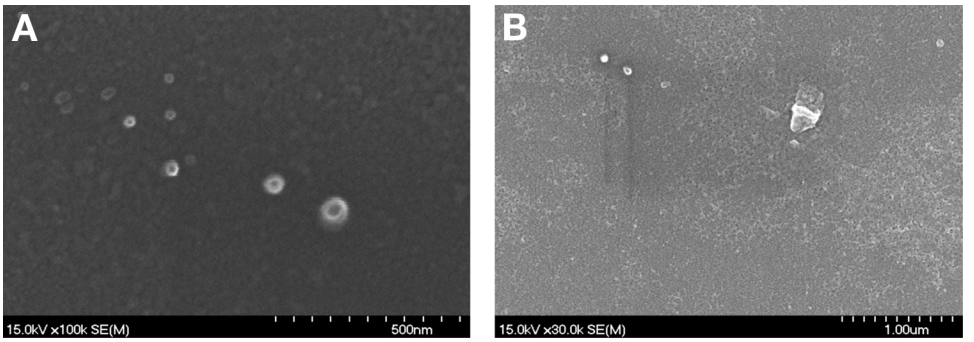

Fig. 2. Scanning electron micrograph of chitosan nanoparticles. The size of the chitosan nanoparticles can be measured with a ruler.

of ice-cold nucleic acid precipitator (0.25% ammonium molybdate dissolved in 2.5% perchloric acid). The mixture was settled in an ice bath for more than 10 min. The precipitated RNA was removed by centrifuging at 4000 rpm at 4°C for 10 min. The supernatant fluid was diluted 50-fold with distilled water. The absorbance at 260 nm of the diluted solution was read with a blank incubation without enzyme. The activity (U) of the enzyme was calculated according to the following formula:

$$\text{enzyme activity (U/mL)} = \text{OD}_{260} \times 4.0 \times 50/0.1 \times 15 = \text{OD}_{260} \times a \times 133.3$$

in which *a:A* is the dilution factor of the enzyme before the enzyme activity assay.

One unit of enzyme activity was defined as the amount of enzyme that produced an increase in the optical density of 1.0 in 1 min at 260 nm.

Optimum pH and Temperature

To determine the optimal pH of the enzyme, the enzyme assay was carried out at pH values of 3.0–8.0 at 40°C. Optimal temperature was determined by performing a standard activity assay in a temperature range of 40–80°C.

Stability of Free and Immobilized Enzyme

The stability of free and immobilized nuclease P1 was measured by calculating the residual activity after a long storage time. One milliliter of nuclease P1 (0.1 mg/mL) or 1.0 mL of phosphate buffer containing 0.1 mg of immobilized enzyme was stored at 4°C. Enzyme activity was assayed at regular intervals.

Reuse of Immobilized Nuclease P1

The activity of immobilized enzyme was determined under standard assay conditions. The same immobilized enzyme was reused after it was thoroughly washed with buffer.

Application of Chitosan Nanoparticles as Protector for Free Enzyme

Five milliliters of nuclease P1 (1.0 mg/mL) in tubes was incubated in a water bath for 20 min. Next the nuclease P1 was added to 1.0 mL of different concentrations of chitosan nanoparticle solution. Then the mixture was stored at 40°C for 2 h. The remaining activity was determined under standard assay conditions.

Determination of Michaelis Constant

The kinetic constant was determined using a Lineweaver Burk plot by initial reaction rates of free or immobilized enzyme. Immobilized enzyme was reacted using different concentrations of RNA as substrate in 0.020 *M* phosphate buffer (pH 7.5) at 40°C.

Determination of Protein Concentration

Protein was measured by the Bradford *(19)* method with bovine serum albumin as standard. The concentration of protein during purification studies was calculated from the standard curve.

Results and Discussion

Thermal Stability of Immobilized Enzyme

Thermal stability was investigated by incubating free enzyme and enzyme immobilized on cellulose and chitosan nanoparticles, respectively, at 70°C for 2 h. As shown in Fig. 3, after 2 h the activity of immobilized enzyme on chitosan nanoparticles and cellulose remained at about 92.07 and 77.20%, respectively, whereas the free enzyme activity decreased 45.65%. The conformational flexibility of enzyme was affected by immobilization. Immobilization of nuclease P1 caused an increase in enzyme rigidity and protected it from unfolding; thus, immobilized enzyme showed higher thermal stability than that of free enzyme.

Optimum pH and Temperature

pH is one of the most influential parameters altering enzyme activity in an aqueous medium. Immobilization is likely to result in a conformational change of enzyme, which causes inactivity of the enzyme. Free enzyme remained stable in a pH range of 4.0–6.0, and immobilized enzyme on chitosan nanoparticles and cellulose was found to be stable up to pH 7.0. The activity of immobilized enzyme was determined at different temperatures ranging from 40 to 80°C. The optimum temperature of free enzyme was lower than that of immobilized enzyme. The optimum immobilized temperature on chitosan nanoparticles and cellulose was 75 and 72°C, respectively, and that of free enzyme was 68°C.

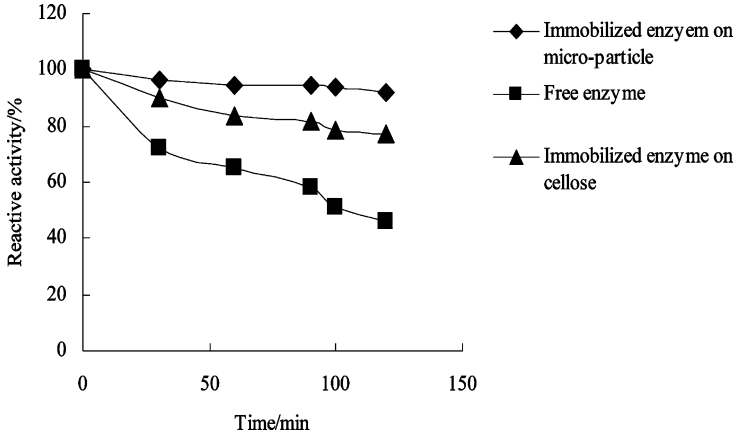

Fig. 3. Thermal stability of nuclease P1. The immobilized enzyme and free enzyme were incubated at 70°C in a water bath. At different time intervals, the activity of the enzyme was determined.

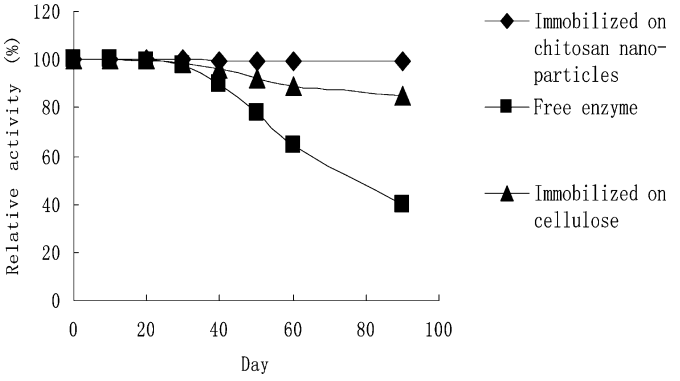

Fig. 4. Storage stability of enzyme. The free and immobilized enzyme was stored at 4°C for long periods of time. The enzyme activity was measured at certain intervals.

Storage Stability of Immobilized Enzyme

Figure 4 illustrates the stability of free and immobilized enzyme at 4°C for long periods of storage. Enzyme activity was measured after certain intervals. The activity recovery of immobilized enzyme on chitosan nanoparticles was >99% after 90 d. The activity of enzyme immobilized on cellulose remained about 99.2% after 30 d. From then on, the remaining activity decreased slowly until reaching 83% after 90 d. However, the activity recovery of free enzyme was 40% after 90 d. The results indicated that immobilized enzyme had good storage stability.

Reuse of Immobilized Enzyme

Figure 5 shows that immobilized enzyme could be reused. Experiments were carried out at 70°C, and the enzyme immobilized on chitosan

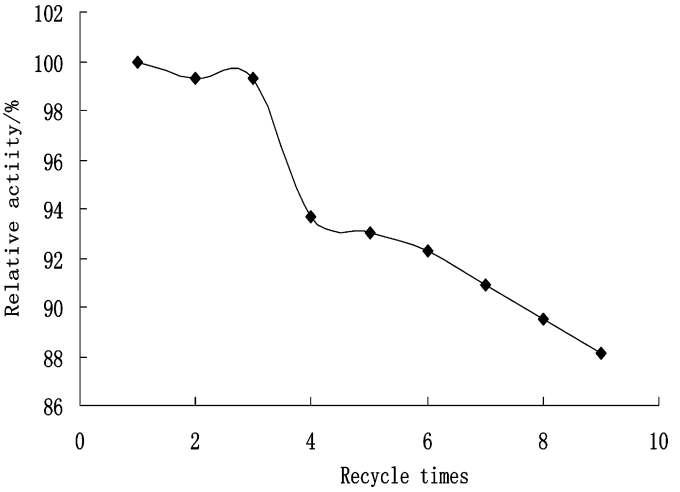

Fig. 5. Operation stability of immobilized enzyme. The activity of immobilized enzyme was determined at 70°C.

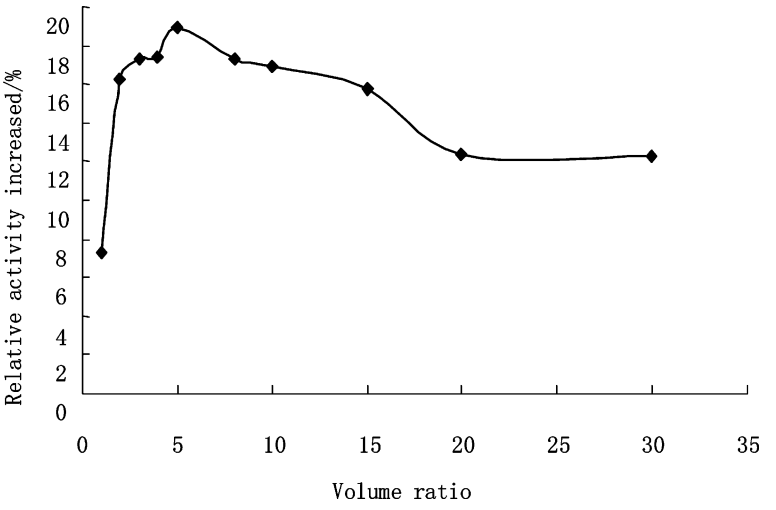

Fig. 6. Application of chitosan nanoparticles as protector of enzyme. Enzyme activity was determined at 70°C.

nanoparticles was reused nine times with most of the activity retained. The results confirmed that the stability of immobilized enzyme was good.

Application of Chitosan Nanoparticles as Protector of Free Enzyme

Figure 6 shows that the chitosan nanoparticles could be used as protector for free enzyme. Experiments were carried out at 70°C for 2 h. As shown in Fig. 5, chitosan nanoparticles could improve the stabilization of free enzyme. The results confirmed that chitosan nanoparticles could be used as protector for free enzyme.

Determination of Michaelis Constant

The *Km* value of immobilized enzyme on chitosan nanoparticles, cellulose, and free enzyme was found to be 8.06, 9.14, and 35.44 mg/mL by Lineweaver Burk plots, respectively. The *Km* value of immobilized enzyme was lower than that of free enzyme, which means that immobilized enzyme has a strong affinity to substrate.

Conclusion

Nuclease P1 could be immobilized on chitosan nanoparticles and cellulose. The optimal temperature of immobilized enzymes using chitosan nanoparticles and cellulose was 75 and 72°C, respectively. The immobilized enzyme using chitosan nanoparticles and cellulose exhibited maximal activity at pH 7.0. The *Km* value of immobilized nuclease P1 using chitosan nanoparticles and cellulose was 8.06 and 9.14 mg/mL, respectively, lower than that of free nuclease P1 (35.44 mg/mL). Greater than 99% of the immobilized enzyme activity using chitosan nanoparticles could still be discerned after being kept at 4°C for 90 d, whereas most of the free enzyme became inactive after 90 d. Immobilized enzyme using chitosan nanoparticles retained a residual activity of 88.13% after nine hydrolysis cycles. Thus, chitosan nanoparticles proved to be good as an immobilized enzyme carrier.

References

1. Juang, R. S., Wu, F. C., and Tseng, R. L. (2001), *Bioresour. Technol.* **80,** 187–193.
2. Denkbas, E. B., Kilicay, E., Birlikseven, C., and Ozturk, E. (2002), *React. Funct. Polym.* **50,** 225–232.
3. Selmer-Olsen, E., Ratnaweera, H. C., and Pehrson, R. (1996), *Water Sci. Technol.* **34,** 33–40.
4. Kucera, J. (2004), *J. Chromatogr. B* **808,** 69–73.
5. Chiou, S. H. and Wu, W. T. (2004), *Biomaterials* **25,** 197–204.
6. Mi, F. L., Kuan, C. Y., Shyu, S. S., Lee, S. T., and Chang, S. F. (2000), *Carbohydr. Polym.* **41,** 389–396.
7. Kuninaka, A., Kibi, M., Yoshino, H., and Sagaguchi, K. (1961), *Agric. Biol. Chem.* **25,** 693–701.
8. Steensma, A., van Dijck, P. W. M., and Hempenius, R. A. (2004), *Food Chem. Toxicol.* **42,** 935–944.
9. Kuninaka, A. (1996), in *Biotechnology*, 2nd ed., vol. 6., Rehm, H. J. and Reed, G.., eds., VCH, Germany, pp. 561–612.
10. Gerald, R. and Tilak, W. (1991), in *Yeast Technology*, 2nd ed., AVI, New York, pp. 369–412.
11. Ying, G. Q., Shi, L.-E., Tang, Z. X., and Shan, J. F. (2004), *Food Res. Dev.* **25,** 120–123.
12. Duran, N., Rosa, M. A., D'Annibale, A., and Gianfreda, L. (2002), *Enzyme Microb. Technol.* **31,** 907–931.
13. Juang, R. S., Wu, F. C., and Tseng, R. L. (2002), *Adv. Environ. Res.* **6,** 171–177.
14. Berthold, A., Cremer, K., and Kreuter, J. (1996), *J. Control. Release* **39,** 17–25.
15. Tian, X. X. and Groves, M. J. (1999), *J. Pharm. Pharmacol.* **51,** 151–157.
16. Cui, Z. and Mumper, R. J. (2001), *J. Control. Release* **75,** 409–419.
17. Fujishima, T., Uchida, K., and Yushino, H. (1972), *J. Ferment. Technol.* **50,** 724–730.
18. Fujimoto, M., Kuninaka, A., and Yoshino H. (1974), *Agric. Biol. Chem.* **38,** 777–783.