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Study on Papain Immobilization on a Macroporous Polymer Carrier

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Macroporous resin microbeads of methyl methacrylate-divinyl benzene copolymer were synthesized by radical suspension polymerization of acrolein with divinylbenzene in the presence of a pore-creating agent, petroleum ether. The microbeads had a large specific surface area and large pores covered the entire surface of the resin. This macroporous polymer carrier was aminated by hydrazine hydrate that produced a large number of amino groups on the carrier. Papain was immobilized on the porous polymer carrier by glutaraldehyde cross-linking or diazo-coupling. The factors involved in the activity recovery of the immobilized papain and the enzymic properties of the resulting immobilized papain were studied in comparison with free papain, for which casein was chosen as the substrate. The results show that the activity recovery of immobilized papain can reach 60.1%. At the same time, the stability, resistance against inhibition, Michaelis constant Km, and reusability of the immobilized enzyme were also investigated. The proteolytic residual activity of free enzyme, using casein as substrate, was 100-50% at 37-60 °C. The proteolytic residual activity of immobilized papain, reached 100-50% although the temperature increased to 70-90 °C. The optimum range of pH was 5-10 for immobilized papain and 5-7.8 for free enzyme at 37 °C. Its residual activity was 98% when immobilized enzyme had been stored at 0-4 °C for 30 days. The results indicated that the immobilized papain by this method had not only higher activity recovery, but also remarkable stability, better reusability and environmental adaptability than free papain.

Key Words: Immobilized papain, macroporous polymer carrier, enzyme activity.

Introduction

Papain (EC 3.4.22.2) is a thiol protease, and its active site consists of Cys-25, His-159 and Asp-158. Papain shows extensive proteolytic activity towards proteins, short-chain peptides, amino acid esters and amide links [1], and is applied extensively in the fields of food and medicine [2,3]. The reverse reaction of hydrolysis of papain can also be employed in the synthesis of peptides and oligomers based on amino acids [3−5], especially immobilized papain, which has been employed in the enzymic synthesis of peptides and their derivatives in

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organic solvents[6]. Therefore, great attention has been focused on the investigation of immobilized papain recently[7−12,14,15,18]. In this paper, we report the immobilization of papain on poly(methyl methacrylate-divinyl benzene), macroporous resin microbeads, in which glutaraldehyde and casein were used as the coupling agent and substrate respectively. The preparation and structures of the these special carrier microbeads were also investigated by means of an elemental analyzer, Fourier-transform spectroscopy (FT-IR), SEM, specific surface area measurements and absorption capacity.

Materials and Methods

Materials

Papain (Sigma, 17 units/mg), glutaraldehyde (E. Merck Co.; 25% , W/W), Tris, L-cysteine and casein were the biochemical reagents; trichloroacetic acid, EDTA and diethylenetriamine were the AnalaR reagents.

Instrumentation

A SEM (KYKY-1000B, KYKY TECHNOLOGY DEVELOPMENT LTD.) was used to examine the surface and inner structure of the carrier. The Perkin-Elmer 2400CHN elemental anlayzer was used to carry out elemental analysis. The absorbency was measured by a 721-spectrophotometer (SX721, Gao Mi Analysis Instrument Lot., Shan Dong, China). FT-IR spectroscopy (FTS-40, BIO-RAD) was used to analyze the structure of carriers. The specific surface area of the beads was determined using nitrogen absorption (ST-08A specific surface area measuring instrument, Beijing Huapu Analysis Instrument Lot., China). The absorption capacity was measured by the static state method[15]. An ultrasonic instrument (KQ-250, Kuen Shan Analysis Instrument Lot., Jiang Su, China) was used to improve the velocity of the reaction.

Synthesis of the carrier

Seven grams of methyl methacrylate, 1 g of divinyl benzene, 0.3 g of BPO, 4 g of pore-creating agent petroleum ether and 40 mL of 0.1% PVA solution (dispersed phase) were added to a three-necked flask equipped with a stirrer, a reflux condenser and a thermometer. The mixture was heated to 70 °C with an stirring speed of 400-500 r/min for 8-10 h. The product was filtered, washed with distilled hot water and extracted by an extractor with ethanol for 24 h, and then vacuum dried. This macroporous polymer carrier (5 g) and hydrazine hydrate solution (50 mL) was added to a three-necked flask, which was heated at 80 °C for 24 h and sonicated for 20 min every 4 h. Then the aminated carrier was washed by vacuum filter with distilled water, and then dried.

Preparation of the immobilized papain

Carrier (1 g) and 20 mL of 0.1 M PBS buffer (including 0.05 M L-cystein and 0.02 M EDTA, pH 7.2 see Figure 4) were put into a 50 mL beaker flask and immersed in ice water for 3 h. Then 10 mL of 0.4 mg/mL papain solution in PBS and 0.2 mL of 0.5% glutaraldehyde in water were added to the beaker flask, which was put in a refrigerator overnight. The next morning the solution was stirred for 3 h with a magnetic stirrer at 0 °C. Then the reaction solution was removed by filtering, and the resulting immobilized papain was washed with distilled water and 0.1 M PBS buffer. Finally, it was dried in a desiccator with anhydrous $CaCl_2$ at 4-5 °C.

Assay for papain activity

The activity of free and immobilized papain was determined according to the published methods[13] using casein as substrate by the following procedures:

Free papain activity: Papain solution (0.5 mg/mL) was obtained by dissolving papain in 0.1 M PBS buffer. Then 1 mL of papain solution (equilibrium for 3-5 min at 37 °C) and 2 mL of 0.5% casein solution were mixed in a glass tube for 15 min at 37 °C. Three milliliters of 10% tirichyloroacetic acid was added to this mixture to stop the reaction. The precipitate was filtered off. The reaction consisted of the filtrate 1 mL, 5 mL of 0.55 M Na_2CO_3 and 1 mL of Folin agent, which was incubated for 15 min at 37 °C and the absorbance of the colored solution at 680 nm was determined. From one unit of enzyme 1 μg of tyrosine acid is produced every minute.

Immobilized papain activity: Papain solution was used instead of 50 mg of immobilized papain. The other experimental procedures were similar to those described above.

Results and Discussion

Composition and structure of the carrier

In this study, polymer resins were synthesized as described above by radical suspension polymerization. Under controlled polymerization conditions, a porous polymer carrier with a special structure was prepared. The specific surface area and absorption capacity are 400-500 m^2/g and 60-70 mg/g respectively. Figure 1 shows SEM photographs of the polymer resin.

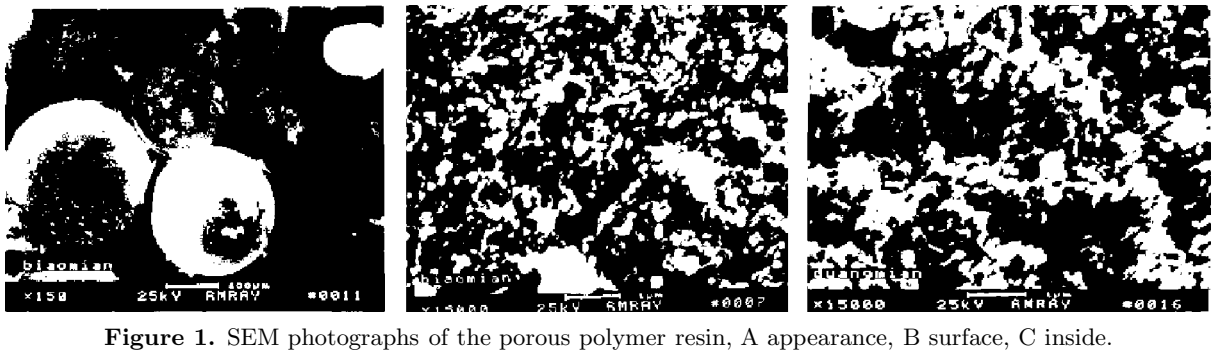

Figure 1. SEM photographs of the porous polymer resin, A appearance, B surface, C inside.

The FT-IR spectrum of the carrier and the activated carrier is illustrated in Figure 2. We observe the carbonyl band ($\nu_{C=O}$, 1730 cm^{-1}), the ester band ($\nu_{C=O}$, 1250 cm^{-1}), and the band of disubstituted benzene (δ_{C-H},835 and 802 cm^{-1}); at the same time, there exists IR characteristic absorption of the secondary amide link (1662, 1576, 1298 and 718 cm^{-1}), νNH_2 and νNH (3306 cm^{-1}), as well as the absorption peaks of the primary groups (δNH, 1607 cm^{-1}). The above IR analysis results confirm the formation of the (hydrazine hydrate, H_2N-NH_2) acrylamide group. Therefore, the resulting carrier should have the following structure and reaction equation:

$$\text{(p)—COOCH}_3 \xrightarrow{H_2NNH_2} \text{(p)—COHNNH}_2 \xrightarrow[\text{En}]{\text{OHC(CH}_2)_3\text{CHO}} \text{(p)—COHNN=CH(CH}_2)_3\text{CH=N–En}$$

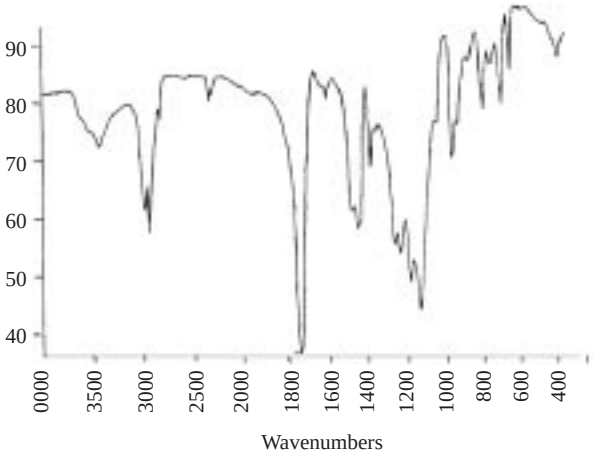

Figure 2. FT-IR spectrum of the carrier by amide.

For the resulting carrier, the nitrogen content was 19.1% (with the Perkin-Elmer 2400CHN elemental analyzer). which is also evidence of the formation of hydrophilic acrylaimide and amino groups. More importantly, the reactive primary group formed is suitable for the immobilization of enzymes.

Factors affecting activity recovery for papain immobilization

Effect of glutaraldehyde concentration: Figure 3 shows the effect of glutaraldehyde concentration on the recovery of activity. The highest activity recovery may be obtained using 0.5% glutaraldehyde as a cross-linking agent. Because glutaradehyde is both a cross-linking agent and a denaturant for the immobilization of papain, activity recovery will be decreased when the glutaraldehyde concentration is greater or less than 0.5%.

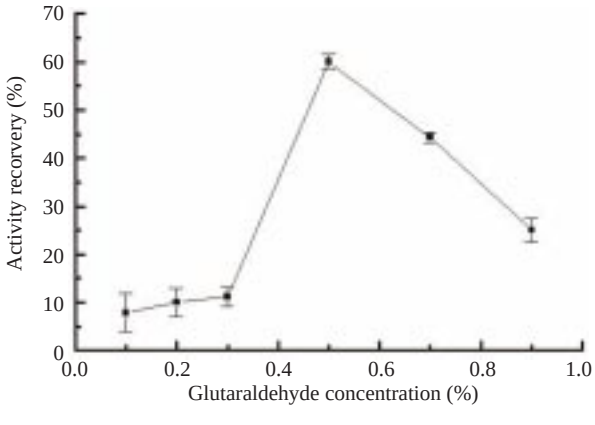

Figure 3. Effect of glutaraldehyde concentration on the activity recovery of immobilized enzyme at pH 7.5 and 0 °C for 15 h.

Effect of medium pH: The immobilized papain was prepared in 0.1 M PBS buffers of different pH values (5.0-10.0): the curve of activity recovery obtained is illustrated in Figure 4. The highest activity recovery was obtained at pH 7.5.

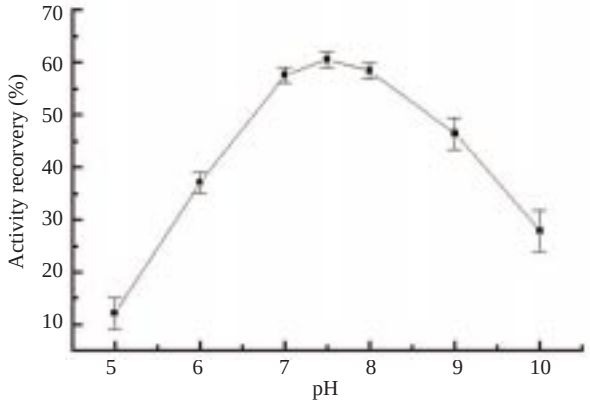

Figure 4. Effect of pH value on the activity recovery of immobilized enzyme at 0 °C for 15 h.

Effect of temperature: Figure 5 illustrates the effect of coupling temperature on activity recovery. In order to effectively facilitate the cross-linking reaction and prevent enzyme deactivation at higher or lower reaction temperatures, it is very important to choose the optimum coupling temperature. The highest activity recovery was obtained at 35 °C or 0 °C, as shown in Figure 5. Therefore, the coupling temperature should be between 30 °C and 40 °C or 0 °C. We chose 0 °C, because the lower temperature can prevent enzyme deactivation.

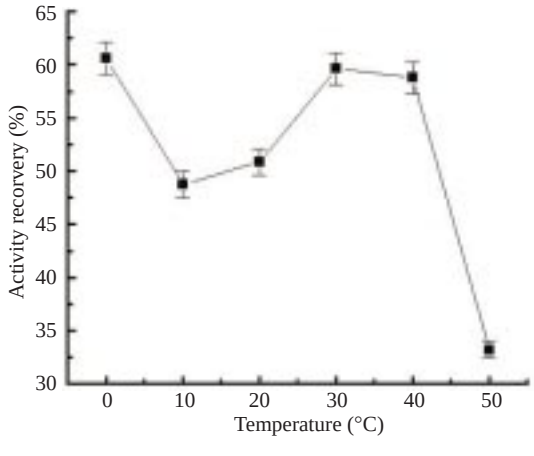

Figure 5. Effect of time on the activity recovery of immobilized enzyme at pH 7.5 and 0°C for 15 h.

Effect of time: Figure 6 illustrates the activity of the immobilized papain prepared at different reaction times. The activity recovery of the immobilized papain increased with prolonged reaction times and the highest activity recovery was obtained under immobilization allowed to proceed for 15 h. However, activity recovery decreased if the reaction time was longer because of the increasing process deactivation.

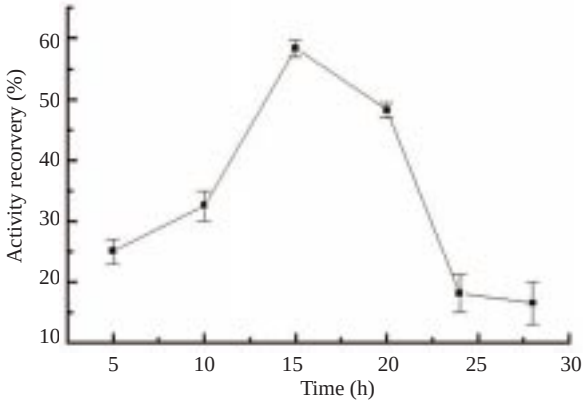

Figure 6. Effect of time on the activity recovery of immobilized enzyme at pH 7.5 and 0 °C.

Effect of papain added: Figure 7 illustrates the effect of different papain amounts on the activity recovery when 50 mg of carrier was used in immobilization. The highest activity recovery was obtained using 0.4 mg of papain. Because a higher amount of enzyme supported makes the enzyme form an intermolecular space hindrance, which restrains the diffusion of the substrate and product, the activity recovery decreased slowly above 0.4 mg of papain.

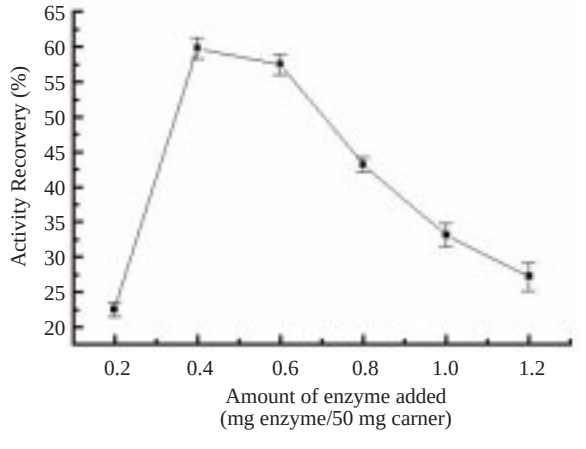

Figure 7. Effect of the amount of enzyme added on the activity recovery of immobilized enzyme at pH 7.5 and 0 °C for 15 h.

Properties of the immobilized papain

By taking the activity recovery of immobilized papain under optimal conditions as 100%, the activity values obtained from different enzymic reactions could be defined as the residual activity of the immobilized papain.

The optimum temperature: Immobilized and free papain were allowed to react with casein at various temperatures (Figure 8).The optimum activity temperature of the resulting immobilized papain was 30 °C higher than that of free papain. Therefore, the immobilized papain obtained shows good heat resistance.

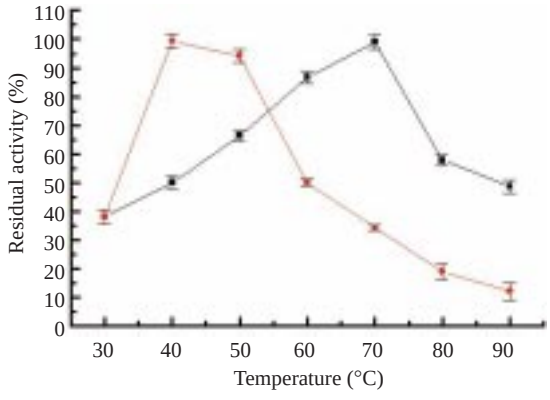

Figure 8. Effect of temperature on the residual activity of casein hydrolysis at pH 7.5 (■ immobilized enzyme; ● free enzyme).

The optimum medium pH value: Figure 9 illustrates variations in the relative activities of the immobilized and free papain between medium pH values of 5.0 and 10.0. With changing medium acidity, the activity of the immobilized papain varied slowly; even at pH 10.0, it retained 91% residual activity, whereas the residual activity of free papain was only 31%. In other words, the immobilized papain exhibits good adaptability to environmental acidity.

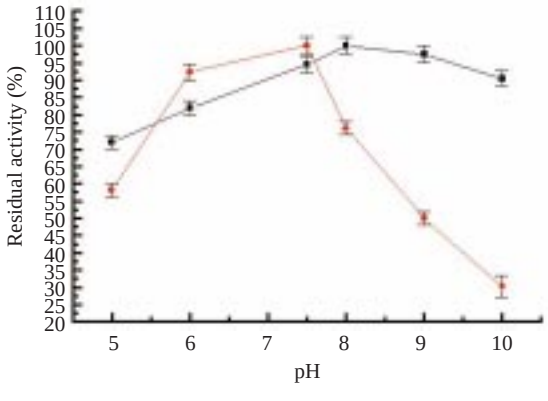

Figure 9. Effect of pH value on the residual activity of casein hydrolysis at 37 °C (■ immobilized enzyme; ● free enzyme).

Thermal stability: The immobilized and free papain were suspended in pH 7.5 PBS buffer without a substrate and stored at 70 °C for different times, and the activity variations assayed are shown in Figure 10. After 10 h, the immobilized and free enzyme activity drop to 40% and 10% of the original value respectively. The results demonstrate that the immobilized papain retained higher activity than free papain. However, after immobilized and free papain were stored for 1 h at various temperatures in the same buffer at pH 7.5 (Figure 11), immobilized papain showed the highest activity at 70 °C, but free papain was deactivated. The results in Figures 10 and 11 show that the immobilized papain has good thermostability.

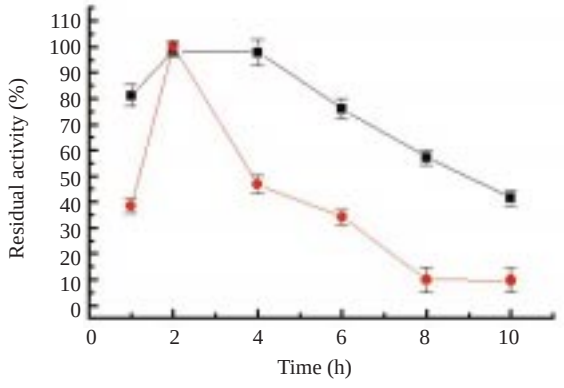

Figure 10. Thermostability of enzymes at pH 7.5 and 70°C for different times (■ immobilized enzyme; ● free enzyme).

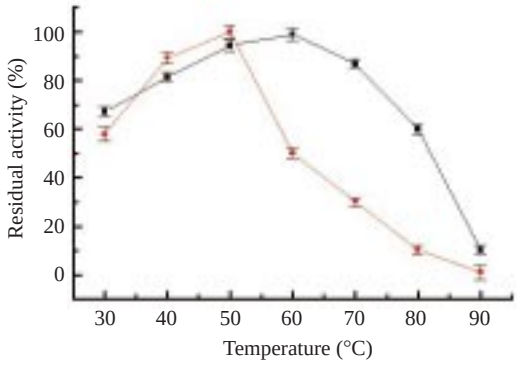

Figure 11. Thermostability of enzymes at pH 7.5 and different temperatures for 1 h (■ immobilized enzyme; ● free enzyme).

Stability for medium pH: The immobilized and free papain were stored for 2 h at 70 °C in different pH buffers, and their activity variations are shown in Figure 12. The activity of the immobilized papain changed slowly with pH variations and exhibited a better stability for medium acidity than free papain.

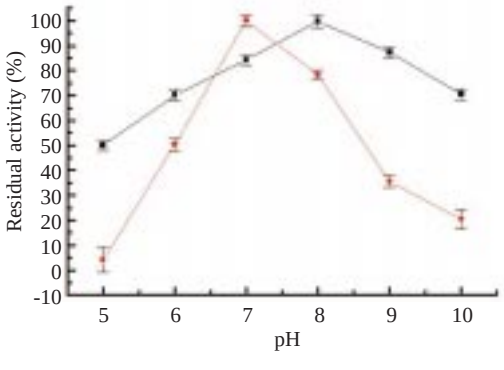

Figure 12. Stability of enzymes at different pH values at 70 °C for 2 h (■ immobilized enzyme; ● free enzyme).

Reusability: The reusability of the immobilized enzymes was determined at 37 °C; the used immobilized enzyme was washed twice with 0.01 M PBS buffer (pH 7.5), and then supplied again to the fresh

reaction solution to determine the enzymatic activity. This cycle was repeated. The residual activity of immobilized papain is shown in Figure 13. The immobilized papain had better reusability, as it retained 45% residual activity after being used 10 times. Due to the thermal deactivation of the enzyme, however, the residual activity of the immobilized enzyme decreased gradually again when it was reused more than 10 times, as shown in Figures 13. Figure 14 showns the storage stability of the immobilized enzyme. When the immobilized and free enzyme had been stored for 30 days in a refrigerator at 0-4 °C, their residual activity was 98% and 10% respectively. The immobilized enzyme storage stability was better than that of the free enzyme.

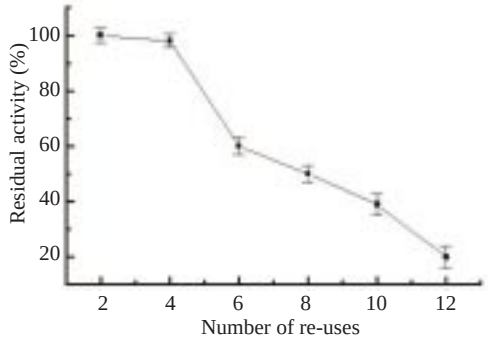

Figure 13. Effect of repeated use on the activity of immobilized enzyme for casein hydrolysis at pH 7.5 and 37 °C for 15 min.

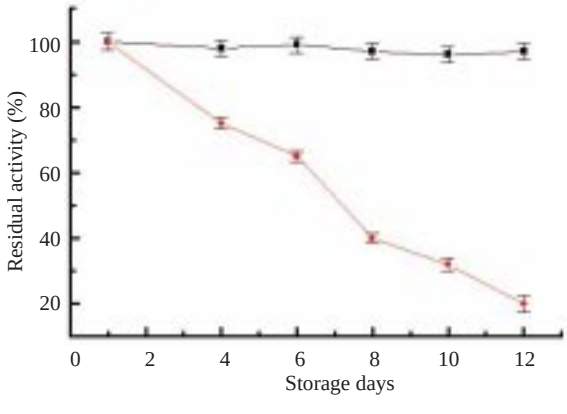

Figure 14. Stability of immobilized enzyme storage at 0-4 °C for different days (■ immobilized enzyme; ● free enzyme).

Constant Km: Utilizing casein of different concentrations as a substrate, the activities of immobilized and free papain were assayed at 37 °C in pH 7.5 medium and the results are shown in a Lineweaver-Burk curve (Figure 15). The k_m values of immobilized and free papain obtained from Figure 15 are 1.01×10^{-3} g/ml and 0.93×10^{-3} g/ml respectively. The data demonstrate that the concentration of substrate casein needed for immobilized papain is greater than that needed for free papain under identical conditions.

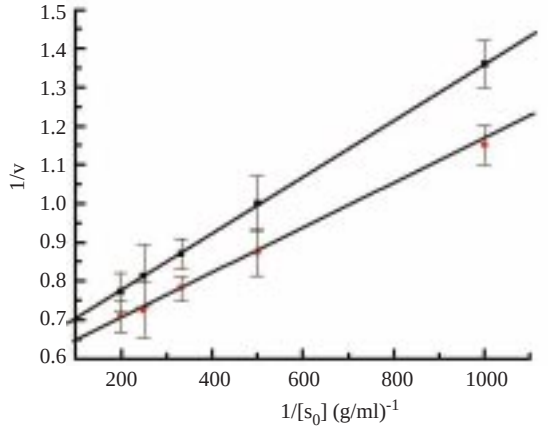

Figure 15. Lineweaver-Burk curve of enzymes for casein hydrolysis at pH 7.5 and 37 °C (■ immobilized enzyme; ● free enzyme).

Inhibition resistance: Immobilized and free papain were stored for 1 h in urea solutions of different concentrations at 70 °C; their activity curves are shown in Figure 16. With increasing urea concentration, the activity of free papain was lost quickly, while that of immobilized papain changed very slowly, proving that immobilized papain has a better inhibition resistance to urea.

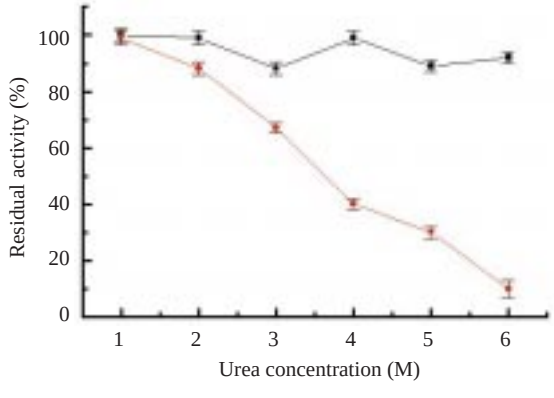

Figure 16. Stability of enzymes in the presence of urea at 70°C for 1 h (■ immobilized enzyme; ● free enzyme).

Conclusion

This research has shown that papain an be immobilized successfully on the resulting macroporous porous polymer carrier using glutaraldehyde as a coupling agent, and that immobilized papain exhibits remarkable stability for medium acidity, reaction and storage temperature, as well as good reusability. The activity recovery of immobilized papain is basically similar to that obtained by silica-bead and chitosan microsphere carriers[14−15], but higher than that of chitin carriers (10%)[16]. Meanwhile, the inhibition resistance of the resulting immobilized papain for a urea solution is better than that of immobilized papain obtained by nylon and nitrilon fiber carriers[17−18].

Acknowledgments

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