Iranian Polymer Journal,
12 (6), 2003, 491-495

Synthesis of Macroporous Polymer Carrier and Immobilization of Papain

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Received 5 January 2003; accepted 21 September 2003

ABSTRACT

A macroporous resin microbeads of methyl methacrylate-divinylbenzene copolymer was synthesized by radical suspension polymerization with divinylbenzene in the presence of a pore-creating agent, petroleum ether. They have big specific surface area and a large degree of porosity was covered on all surface of the resin. This macroporous polymer carrier was aminated by hydrazine hydrate, to produce a large number of amino groups onto the carrier. Papain was immobilized on the porous polymer carrier by a glutaraldehyde cross-linking method or a diazo-coupling method. The determining factors involved with 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 a substrate. The results show that the activity recovery of immobilized papain can reach 60.1%. At the same time, the stability, resistance against inhibition, the Michaelis constant K_m, and reusability of the immobilized enzyme were also investigated. The results indicated that the immobilized papain by this percentage reached had not only higher activity recovery, but also is had remarkable stability, better reusability and environmental adaptability than free papain.

Key Words:
immobilization;
papain;
immobilized enzyme;
macroporous polymer carrier;
enzyme activity.

INTRODUCTION

Interest in immobilized enzymes and their applications in bioprocessing, analytical systems, and enzyme therapy have been exploited for many year. Immobilized enzymes can often be used more advantageously than the corresponding free enzymes. With immobilized enzymes, the process can be run continuously because of the resulting stabilization of enzymes when subjected to high temperature, pH, and inhibitor concentration. Papain (EC 3.4.22.2) is one of the thiol pro-

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teases, 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, and it is applied extensively in the fields of food and medicine [1-2]. The reverse reaction of hydrolysis of papain also can be employed in the synthesis of peptides and oligomers based on amino acids [2-3], especially immobilized papain, which has been employed in the enzymic syntheses of peptides and their derivatives in organic solvents [4]. Therefore, great attention has been focused on the investigation of immobilized papain recently [5]. In this paper, we report the immobilization of papain on poly(methyl methacrylate-divinylbenzene), macroporous resin microbeads carrier, in which glutaraldehyde and casein were used as coupling agent and substrate, respectively. The preparation and structure of this special carrier microbeads were also investigated by means of elemental analysis, Fourier-transform (FTIR), FTS-40, Bio-Rad and SEM methods.

EXPERIMENTAL

Materials, Instrumentation and Methods

Papain (Sigma,17 units/mg), glutaraldehyde (E. Merck Co.; 25%, w/w), Tris, l-cysteine and casein were biochemical reagents; trichloroacetic acid, EDTA and diethylenetriamine were Analar reagents. A Perkin-Elmer 2400CHN elemental anlayzer was used to carry out elemental analysis. A SEM was used for examination of the surface and inside of the carrier as well. The absorbency was assayed by a 721-spectrophotometer. FTIR Spectra were used to analyze the structure of the carriers.

Synthesis of the Carrier

A 7g methyl methacrylate, 1g divinylbenzene, 0.3g BPO, 4g pore-creating agent petroleum ether and a 40 mL 0.1% PVA solution (dispersed phase) were added into a three-necked flask equipped with a stirrer, a reflux condenser and a thermometer. The mixture was heated to 70°C with the stirring speed of 400-500 r/min for 8-10 h. The product was filtered, washed with distilled hot water and exracted by an extractor with ethanol for 24 h, and then it was vacuum dried. This macroporous polymer carrier and hydrazine hydrate solution was added into a three-necked flask, heating at 80°C for 24 h and sonicated for 20 min every 4 h. Then the aminated carrier was washed by filter press with distill water, and then it was dried.

Preparation of the Immobilized Papain

Carrier (1g) and 20 mL of 0.1M PBS buffer (including 0.05M *l*-cystein and 0.02 M EDTA, pH 7.2) were added to a 50 mL beaker flask immersed in ice water for 3 h, and then 10 mL of 0.4mg/mL papain solution and 0.2 mL of 5% glutaraldehyde were added to the beaker flask over night in refrigerator. Next morning the carriers were stirred for 3 h with a magnetic stirrer at 0° temperature. Then the reaction solution was removed by filtering, and the resulting immobilized papain was washed with distilled water and 0.1M 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 [10] using casein as a substrate. The determination was finished by the following procedures:

Free Papain Activity

Papain solution (0.5 mg/mL) was obtained by papain dissolved in 0.1M PBS buffer. Then 1 mL papain solution(equilibrium for 3-5 min at 37°C) and 2 mL 0.5% casein solution were mixed in glass tube for 15 min at 37°C. A 3mL of 10% trichloroacetic acid was added to this mixture for stopping the reaction. The precipitate was filtered off. The reaction consisted of the filtrate, 1 mL; 5 mL 0.55M Na_2CO_3 and 1 mL (1,2-naphthoquinone-4-sulphonic acid), then it was incubated for 15 min at 37°C and the absorbance of the coloured solution at 680 nm was determined. Enzyme activity unit produced 1 μg tyrosine acid every minute.

Immobilized Papain Activity

Papain solution was replaced by 50 mg immobilized papain. The other experimental procedures were similar was above. First, 1 mL 0.1M PBS buffer solution was added into 50 mg immobilized papain (equilibrium 3-5 min at 37°C) and 2 mL 0.5% casein solution were mixed in a glass tube for 15 min at 37°C. Then the immobilized papain was filtered off. A 3 mL 10%

trichloroacetic acid was added. The precipitate was filtered off again. The reaction consisted of the 1 mL filtrate, 5 mL 0.55 M Na_2CO_3 and 1 mL (1,2-naphthoquinone-4-sulphonic acid), then it was incubated for 15 min at 37°C and the absorbance of the coloured solution at 680 nm was determined.

RESULTS AND DISCUSSION

Structure of the Carrier

In this study, polymer resins were synthesized as described above by radical suspension polymerization. In controlled polymerization conditions, the porous polymer carrier with special structure was prepared. Figure 1 shows SEM photographs of the polymer resin.

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

$$P\text{—}COOCH_3 \xrightarrow{H_2NNH_2} P\text{—}COHNNH_2 \xrightarrow[E_n]{OHC(CH_2)_3CHO} P\text{—}COHNN{=}CH(CH_2)_3CH{=}N\text{-}E_n$$

For the resulting carrier, the nitrogen content was 19.1%, (with the Perkin-Elmer 2400 CHN elemental analyzer) which is also an evidence for the formation of hydrophilic acrylamide and amino groups. What is more important, the reactive primary group formed would be suitable for the immobilization of enzymes.

Properties of the Immobilized Papain

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

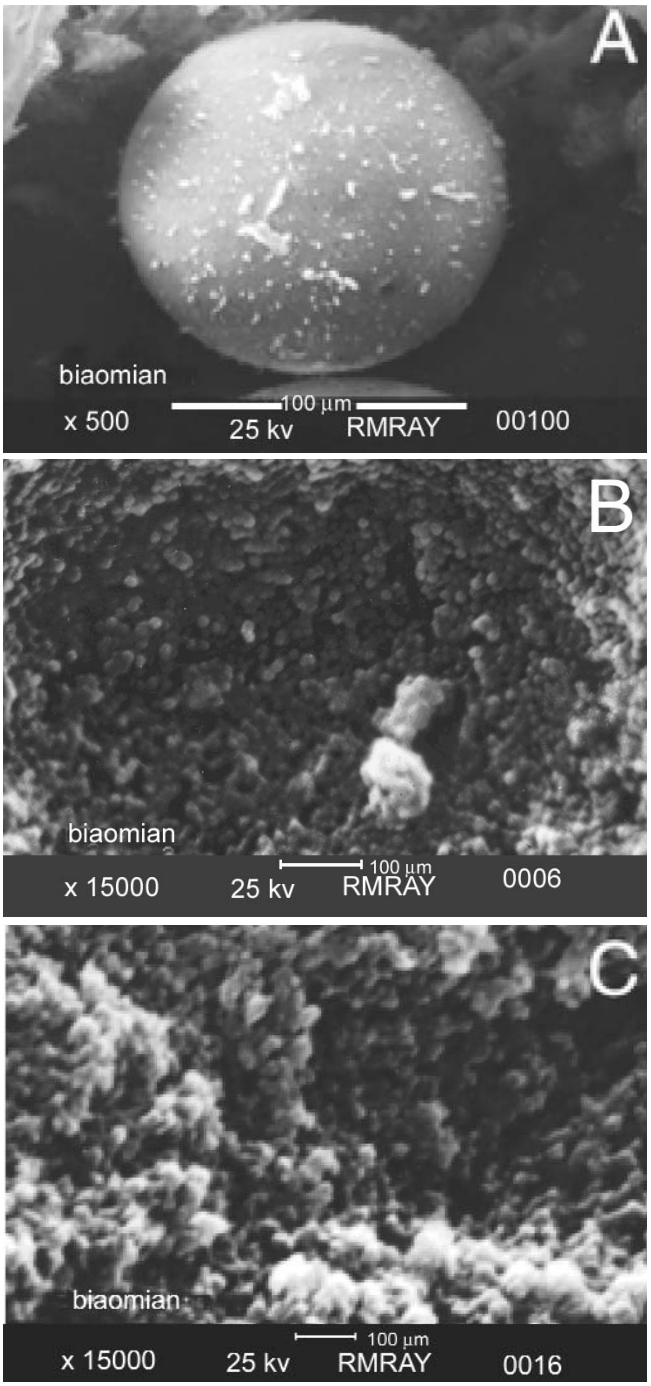

Figure 1. SEM Photographs of the porous polymer resin; (A) appearance, (B) surface, and (C) inside.

The Optimum Temperature

Immobilized and free papain were allowed to react with casein at various temperatures (Figure 3). The optimum activity temperature of the resulting immobilized papain was 30°C higher than the free papain. So,

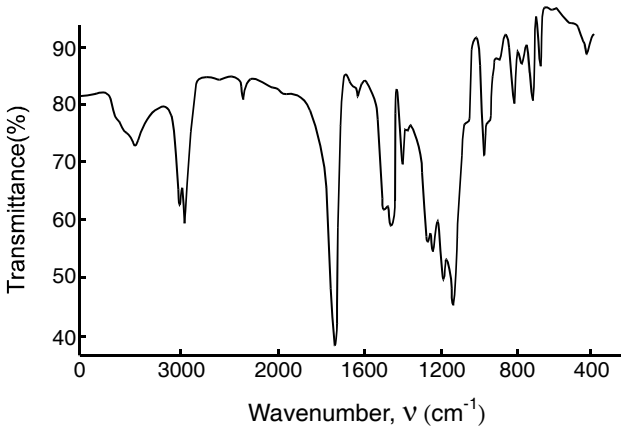

Figure 2. FTIR Spectrum of the carrier by amide.

the immobilized papain obtained shows good heat resistance.

Stability in 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 4. The activity of the immobilized papain changed slowly with pH variation and exhibited a better stability for medium acidity than free papain.

Inhibition Resistance

Immobilized and free papain, were stored for 1 h in urea solutions of different concentrations at 70°C, and then their activity curves were obtained as they are shown in Figure 5. With increasing urea concentration, the activity of free papain was lost quickly, while that of the immobilized papain changed very slowly. The

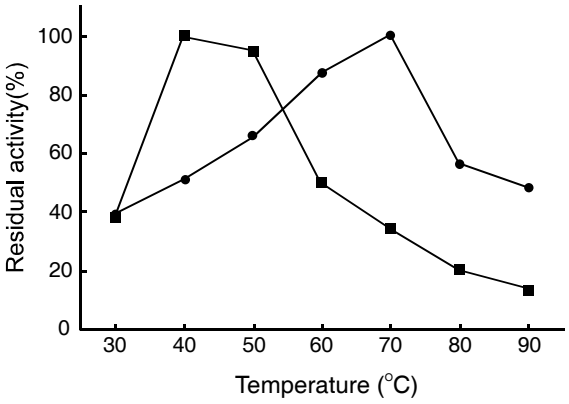

Figure 3. Effect of temperature on the residual activity of casein hydrolysis at pH 7.5 (●,immobilized enzyme;■, free enzyme).

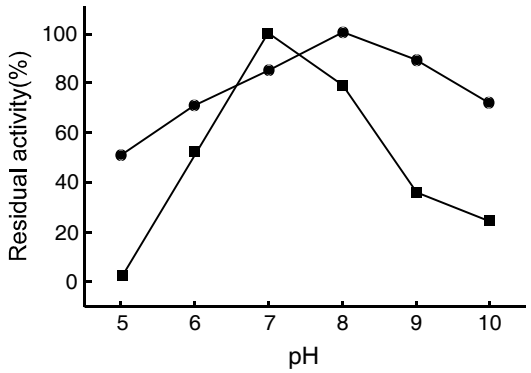

Figure 4. Stability of enzymes at different pH values at 70°C for 2 h (●,immobilized enzyme;■,free enzyme).

result proves that the immobilized papain has a better inhibition resistance to urea.

Re-usability

The reusability of the immobilized enzymes was determined at 37°C, the used immobilized enzyme was washed twice with 0.01M PBS buffer (pH 7.5), and then supplied again to the fresh reaction solution to determine the enzymatic activity. This cycle was repeated. Residual activity of immobilized papain is shown in Figure 6. The immobilized papain had a better re-usability, as it retained 45% residual activity after 10 times usage. Due to the thermal deactivation of enzyme, however, the residual activity of the immobilized enzyme decreased gradually again when it was reused more than ten times, as shown in Figure 6.

Constant K_m

Utilizing casein of different concentrations as a substrate, the activities of immobilized and free papain

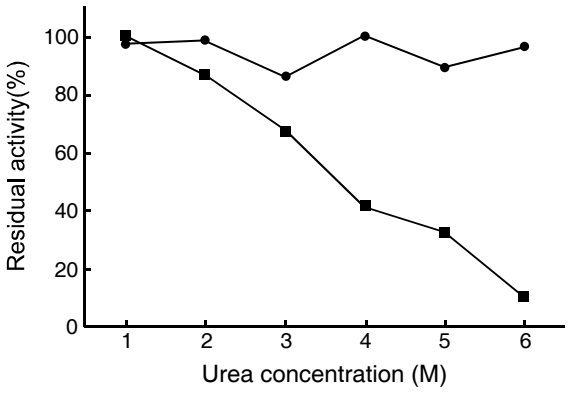

Figure 5. Stability of enzymes in the presence of urea at 70°C for 1 h (●,immobilized enzyme; ■,free enzyme).

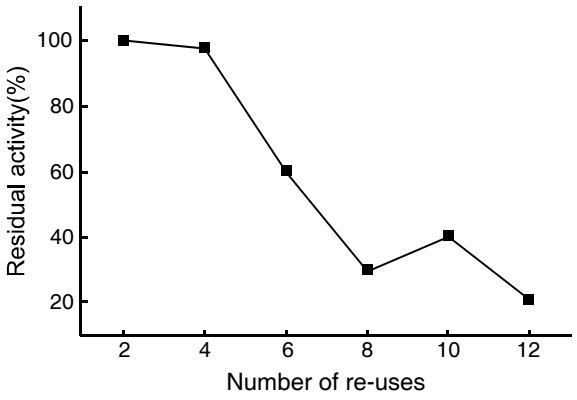

Figure 6. Effect of repeated use on the activity of immobilized enzyme for casein hydrolysis at pH 7.5 and 37°C for 15 min.

were assayed at 37°C in pH 7.5 medium and the results were shown in a Lineweaver-Burk curve. The k_m values of immobilized and free papain obtained from Lineweaver-Burk curve of enzymes for casein hydrolysis at pH 7.5 and 37°C are 1.01×10^{-3}g/mL and 0.93×10^{-3}g/mL, respectively. The data demonstrate that the concentration of the substrate casein needed for immobilized papain is greater than that needed for free papain under identical conditions.

CONCLUSION

This research has shown that papain could be immobilized successfully on the resulting macroporous polymer carrier using glutaraldehyde as a coupling agent, and that the immobilized papain exhibits remarkable stability for medium acidity, reaction and storage temperature, as well as good re-usability. The activity recovery of the immobilizes papain is basically similar to that obtained by silica-bead and the chitosan microspheres carriers, but higher than that of the chitin carriers 10%. Meanwhile, the inhibition resistance of the resulting immobilized papain for urea solution is better than that of the immobilized papain obtained by nylon and a nitrile on fibre carriers.

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