

Study on immobilization of papain with magnetic porous alginate microspheres

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Abstract. Magnetic porous polymer microspheres were prepared by using biomacromolecule sodium alginate and magnetic Fe_3O_4 particles. Polyethylene glycol was involved in preparation procedure as a pore-forming agent. The microspheres were applied to immobilize papain via cross-linking method for catalysing the hydrolysis of protein. The formation of the porous structure improved the papain immobilization yield. In comparison to the free papain, the immobilized papain exhibited enhanced optimum pH, higher thermal stability and activity over the studied temperature 30°C - 70°C in casein hydrolysis. The immobilized papain retained nearly 72% of residual activity after repeating the casein hydrolysis reaction seven times. The apparent K_m increased after papain immobilization. Magnetic porous alginate microspheres can be potentially used as a carrier for enzyme immobilization for constructing efficient catalysts for industrial biocatalysis.

1. Introduction

The immobilized enzymes refer to enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities and being able to use them repeatedly and continuously [1,2]. Magnetic polymer microspheres are a functional polymer material that has been developed in recent years. Magnetic polymer microspheres have been widely investigated not only because they possess favourable superparamagnetic properties facilitating its recovery from the reaction mixture, but also because they have good biocompatibility and various surface functional groups which are suitable to couple enzymes. In recent years, great efforts have been put into the studies of using magnetic polymer microspheres for enzyme immobilization [3-5]. The basic composition of magnetic polymer microspheres is magnetic material and macromolecule material, of which Fe_3O_4 is the most used magnetic material. Polysaccharides and proteins with biocompatibility and biodegradability are widely used as a carrier immobilized enzyme in the biological, pharmaceutical, food and other industries. The efficiency of the immobilized enzyme is affected by the active sites and pore structure of the microspheres in addition to the shape and size of the microspheres. The porous structure of the immobilized material can greatly improve the efficiency of immobilized enzymes. The porous are prepared in the presence of a pore-forming agent, such as fructose, sucrose, polyethylene glycol (PEG), which are substances soluble in water or acid but not insoluble in organic solvents. They could be easily removed from the silica pores by simply washing the samples in excess water [6,7].

The aim of this study was to immobilize papain in magnetic porous alginate microspheres by using PEG4000 as pore-forming agent. The effect of PEG on immobilization yield were determined. Then, the enzymatic properties of immobilized papain were compared with free papain.



2. Materials and Methods

2.1. Materials

Papain (800 U/mg) was obtained from Guangzhou Huaqi Biotechnology Co., Ltd. (Guangdong, China); L-lysine, ferrous oxide, sodium alginate and casein were from Sinopharm Group Chemical Reagent Co., Ltd. (Beijing, China); calcium chloride, glutaraldehyde, trichloroacetic acid, sodium tetraborate, folin phenol reagent, and other reagents are all analytically pure. All the reagents and materials were used as received without any further purification.

2.2. Experimentals

2.2.1. Preparation of Magnetic Porous Alginate Microspheres. PEG4000 (1%~5% of the volume of sodium alginate solution) and Fe₃O₄ magnetic powder with the mass ratio of sodium alginate and magnetic powder of 5:1 were added into 2% sodium alginate solution under mild stirring at room temperature. The mixture was slowly injected into a 1% CaCl₂ solution with a syringe, and left for 2 h to obtain uniform particles and regular shape. The final beads were removed from the calcium solution and washed thoroughly with deionized water, then added to a 3% glutaraldehyde solution. After shaken for 2 hours at 30°C, they were repeatedly washed with water and acetone, and dried in vacuum at 40°C to obtain cross-linked magnetic porous sodium alginate microsphere carrier.

2.2.2. Preparation of Immobilized Papain. Magnetic alginate microspheres (0.40 g) were added into pH 7 sodium phosphate buffer solution with 2.5 mg/mL papain. The solution was shaken for 2 h at 40°C. After filtration, the collected beads were washed with a buffer solution, dried in vacuum at 30°C. The resulting immobilized papain was stored at 4°C.

2.2.3. Determination of Enzyme Activity. The enzyme assay of free and immobilized papain was estimated by using a reaction mixture of 1.5% (w/v) water soluble casein in Tris-HCl buffer as a substrate, and enzyme solution or weighed sample of the immobilized enzyme [8]. The reaction mixture was incubated for 30 min in a water bath at 37°C and stopped by adding trichloroacetic acid and centrifuged. The solubilized proteins in the supernatant were assayed using the phenol method. The enzymatic activity was calculated by hydrolysing casein per minute at 37°C to produce 1 µg tyrosine as a vitality unit.

The immobilization yield and relative activity were expressed by the following equations:

Immobilization yield (%) = (Activity of immobilized enzyme)/(Activity of free enzyme) × 100%

Relative activity (%) = (Enzyme activity measured under a certain condition)/(Highest enzyme activity of the series) × 100%

All the results are the means of at least three separate experiments.

2.2.4. Effect of pH and Temperature on the Activity in Immobilized and Free Papain. The effect of pH and activity of immobilized and free papain was determined by inducing papain activity using different buffers (pH6-10). The study of the optimum temperature of the activity was undertaken at temperatures ranging from 30°C to 70°C. The thermal stability was measured by incubating immobilized and free papain at 60°C for different time intervals. Aliquots were rapidly cooled to ambient temperature so as to refold the reversibly inactivated enzyme molecules.

2.2.5. Reusability of Immobilized Papain. The reusability of the immobilized papain is related to its operational stability and was determined by conducting repeated cycles of casein hydrolysis reaction.

2.2.6. Kinetic Parameters for Immobilized and Free Papain. The apparent Michaelis-Menten constant (K_m) was estimated using a Lineweaver-Burk double reciprocal plot by measuring reaction rates at different casein substrate concentrations according to the eq. [9]: $\frac{1}{v_0} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$, where v_0 is the rate of enzyme activity or the reaction rate of hydrolysis at substrate concentration, $[S]$ is the substrate

concentration, V_{max} is the maximum rate of hydrolysis and K_m is the substrate concentration at which the rate of hydrolysis is half of V_{max} .

3. Results and Discussion

3.1. Effects of Different PEG Concentration on Immobilization Yield

PEG4000 as a pore-forming agent was used in preparing immobilized papain. The effects of different PEG concentration on immobilization yield are shown in Figure 1. The immobilization yield appeared to increase with the increase of PEG concentration, and reached the maximum at 3% of PEG concentration, then decreased with the increase of PEG concentration. Immobilization yield was raised by about 35% at 3% of PEG concentration compared to the control group, which was significantly more favourable for the reaction. The reason for this result is that the formation of the porous structure improves the affinity of the immobilized enzyme to the substrate, and more enzymes attach to the substrate, thus the immobilization yield is higher than that of sample without adding PEG. However, on the other hand, as the concentration of PEG increases, the pores on the substrate are too dense, which results in the loss of papain during the immobilized enzyme preparation. Therefore, the immobilization yield decreases when the concentration of PEG is too high.

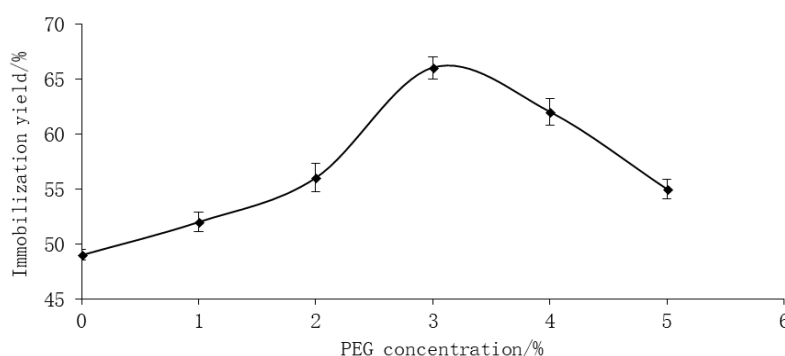


Figure 1. The effects of different PEG concentration on immobilization yield.

3.2. Optimum pH of Immobilized and Free Papain

Figure 2 shows the relative activity of immobilized and free papain at different pH values. The results showed that the optimum pH of the immobilized papain was shifted to a more alkaline range (pH 8) from 7, which is the optimum for free papain. Immobilized papain had 95.9% and 93.8% of its activity at pH 9 and 10 respectively, which means that the immobilization process made the papain more stable in this pH range. The higher activity of immobilized papain in pH 8-10 facilitates application in an alkaline environment. These effects might be dependent on the ionic environment around the enzyme active site. The charged surface of the beads and enzyme producing a charged microenvironment, which might affect the nature of the active enzyme and change the pH of the immobilized enzyme. Quirogai et al [10] report a similar shift in optimum pH towards an acid direction for immobilized araujiain in alginate beads.

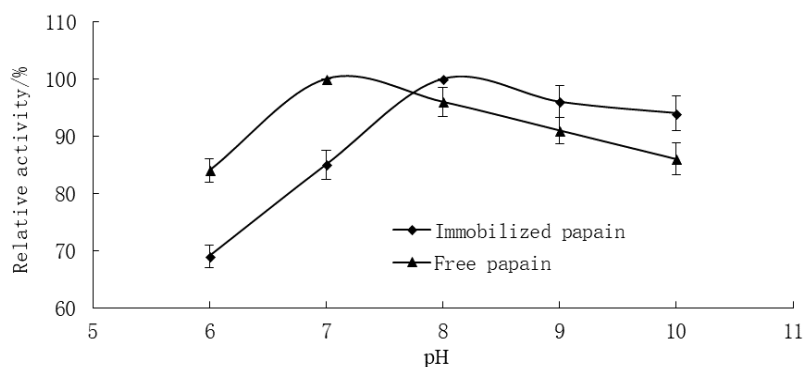


Figure 2. The relative activity of immobilized and free papain at different pH values.

3.3. Optimum Temperature and Thermal Stability of Immobilized and Free Papain

Figure 3 shows the relative activity of immobilized and free papain at different temperature. The designed enzyme hydrolyzation reaction temperatures were 30°C, 40°C, 50°C, 60°C and 70°C and absorbances were measured after 30 minutes of reaction. The results showed that the optimum temperature of immobilized papain was around 50°C, which was the same as that of free papain. However, the relative activity of immobilized papain was higher than that of free papain at the tested temperatures, which indicated that the applied immobilization procedure contributed to improvement of the enzyme stability.

Enzyme activities of the immobilized and free papain after incubation at 60°C at different times, were measured. Figure 4 shows thermal stability of immobilized and free papain. With the increase of treatment time, the relative enzymatic activity of the immobilized and free papain showed a decreasing trend. The thermal inactivation of two papain occurred at a similar rate and the activities were largely lost after 4 h treatment at 60°C. However, the relative activity of immobilized papain was higher than that of the free papain at the same treatment time. The immobilized papain retained 32% of the original activity after heating for 10 h. The free papain, however, retained only 17% of the original activity after the same treatment. Immobilized papain shows better thermo-stability than the free papain. This may be due to changes in the microenvironment where the enzyme is located after immobilization. The active centre of the enzyme is affected by the carrier, which changes the dissociation of the active group.

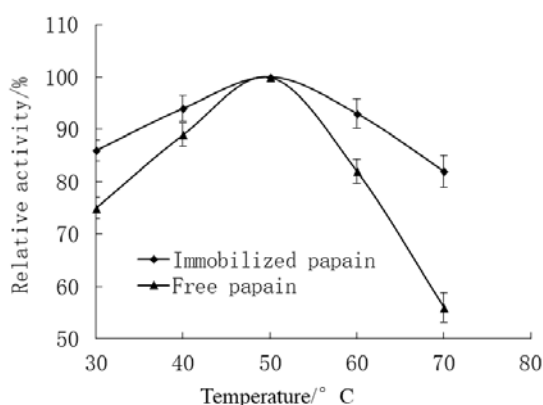


Figure 3. The relative activity of immobilized and free papain at different temperature.

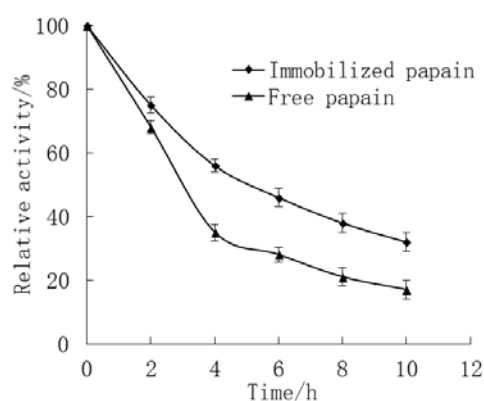


Figure 4. Thermal stability of immobilized and free papain.

3.4. Reusability of Immobilized Papain

The reusability of an enzyme is a key factor for its cost-effective industrial use due to there being a parameter which determines the financial viability of a bioprocess. Figure 5 shows reusability of the

immobilized papain. The immobilized papain had a certain degree of reusability. They retained nearly 80% residual activity after repeating the casein hydrolysis reaction in the fourth cycle and nearly 50% of residual activity in the seventh cycle. The reduction of the immobilized papain activity is due to the loss of carriers during the repeated recovery process, and at the same time, the leakage of the papain from the carriers.

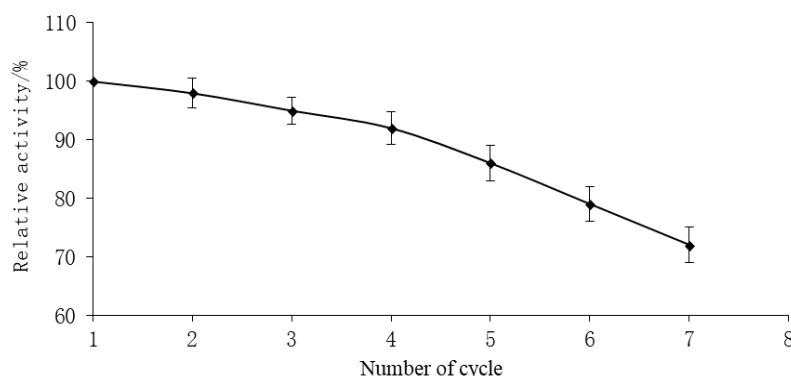


Figure 5. Reusability of the immobilized papain.

3.5. Kinetics of Hydrolysis of Immobilized and Free Papain

The physical meaning of the Michaelis constant K_m is the concentration of the substrate when the enzyme catalyses the reaction rate to reach half of the maximum reaction rate. It can be used as a marker to measure the affinity of the enzyme and the substrate. The smaller the K_m , the greater the affinity of the enzyme for the substrate.

Immobilized and free papain were taken, under optimal reaction conditions, with different concentrations (0.10%, 0.1%, 0.2%, 0.4%, 0.6%) of casein solution. The reaction was measured after 5 min. The enzyme activity was plotted using the double reciprocal method to obtain the Michaelis constant K_m . The results are shown in Figure 6. The calculated K_m (free papain) is 20.7 mg/mL and K_m (immobilized papain) is 31.5 mg/mL. The experimental results show that the apparent K_m increased after papain immobilization, indicating that the affinity of papain with the substrate is decreased after immobilization. The binding ability of the immobilized papain to the substrate is smaller than that of the free papain, which is explained that the apparent K_m decreases by diffusion limitation when the enzyme binds to the carrier. The velocity of the enzyme-catalysed reaction is limited by the rate of breakdown of the enzyme-substrate complex. Then with increasing K_m value more products are formed.

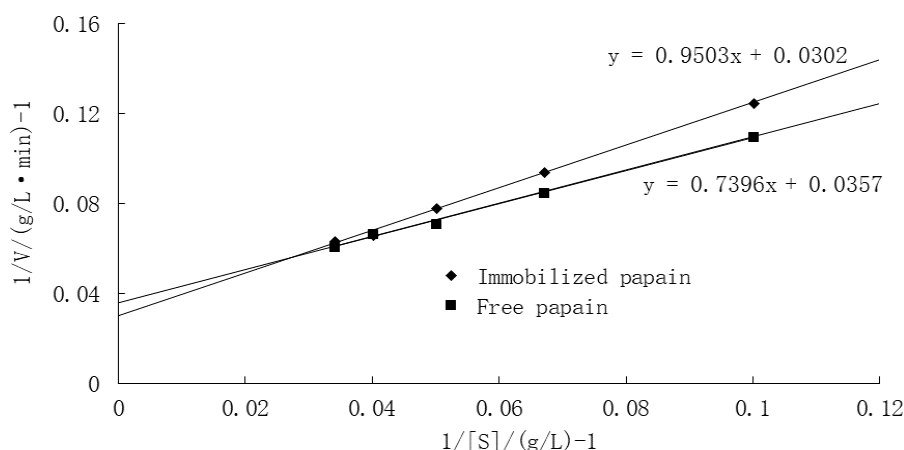


Figure 6. Lineweaver-Burk method of immobilized and free papain.

4. Conclusions

Fe₃O₄ was used as carrier and polyethylene glycol as a pore-forming agent to prepare Magnetic porous sodium alginate microspheres. The maximum immobilization yield of papain cross-linked in magnetic porous alginate microsphere was raised by about 35% with 3% PEG concentration compared to those without adding PEG. The immobilization was shown to be a promising technique for papain immobilization with good immobilization yield, an increase in thermal stability and alkaline environment, high reusability, and retained 72% residual activity after the seventh cycle. These results indicate a further improvement in the catalytic potential of papain due to the immobilization process.

Acknowledgments

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