Immobilization of Chymotrypsin with Interpolymer Complexes of P(TM-*co*-AAm)/PAA

HONGZUO WANG, SHIYONG LIU, YING WANG

Department of Chemistry, Wuhan University, Wuhan 430072, People's Republic of China

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ABSTRACT: Chymotrypsin was immobilized with interpolymer complexes formed by the cationic polymer poly(allyltrimethyl ammonium chloride-*co*-acrylamide) [P(TM-*co*-AAm)] and poly(acrylic acid) (PAA). The introduction of a small amount of cationic groups led to a much stronger polymer–polymer interaction between P(TM-*co*-AAm) and PAA. The characteristic pH sensitivity of this kind of complex provided the possibilities of controlling the activity of the immobilized enzyme and separating the immobilized enzyme from the batch by changing the pH of the medium. Compared with the free enzyme, the immobilized chymotrypsin had higher thermal stability, acid–base stability, and stability in use. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 2013–2018, 2001

Key words: immobilized chymotrypsin; interpolymer complex; cationic polymer, poly(allyltrimethyl ammonium chloride-*co*-acrylamide) [P(TM-*co*-AAm)]; poly(acrylic acid) (PAA)

INTRODUCTION

The immobilization of enzymes and cells is a basic bioengineering technique that locates the free enzymes or cells into a microenvironment with retention of their biological activities.[1,2] Immobilized enzymes or cells can be used repeatedly and continuously. Various methods of immobilization have been described in the literature, including physical absorption, covalent attachment, and gel entrapment. The interest in this field led to the development of intelligent immobilized enzyme systems, in which an enzymatic process can be controlled by externally applied stimuli, such as pH, temperature, light, electric fields, and mechanical force; pH-sensitive microcapsules, reversibly soluble polyelectrolyte complexes, and stimulus-sensitive polymer gels have been employed for this purpose.[3]

It is a new method to attempt to immobilize enzymes and cells with interpolymer complexes. Interpolymer complexes are formed by the secondary forces between various polymer chains, including hydrogen bonding, coulombic forces, van der Waals forces, electron-donating and electron-accepting interactions, and hydrophobic interactions in aqueous media.[4,5] These associations are sensitive to the surrounding environment and are generally accompanied by a change in structure. Thus, interpolymer complexes have potential applications for biosensors, bioseparations, and drug delivery.

Several studies on the immobilization of enzymes with polyelectrolyte complexes have been reported.[6–8] Hua et al. used two oppositely charged polyions, quaternized poly(vinyl dimethylaminobenzal) and potassium poly(acrolein bissulfate), to immobilize glucose oxidase.[8] The im-

Correspondence to: H. Wang (pxcheng@tjh.tjmu.edu.cn).

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mobilized enzymes exhibited the advantages of high specific activity, long storage life, and reproductivity of enzymatic reaction.

However, traditional polyelectrolyte complexes are hard and brittle in a dry state because of the limited molecular chain mobility, which results from their high ionic density. Their strengths decrease or even disappear when they are soaked in water because of the hydration of ionic bonds. Pure hydrogen-bonded complexes are unstable and easily affected by pH, they are readily disassociated into component polymers.

Incorporating relatively small amounts of ion–ion interactions can significantly enhance the miscibility of polymer pairs.[9,10] If such an ionic interaction was introduced into hydrogen-bonding complexes, the properties of the interpolymer complexes would be improved. We synthesized a new type of cationic polymer, poly(allyltrimethyl ammonium chloride-*co*-acrylamide) [P(TM-*co*-AAm)], and its interpolymer complex with poly(acrylic acid) (PAA).[11,12] The interaction forces between the two polymer chains were pH-dependent and reversible. In this study, we investigated the application of P(TM-*co*-AAm)/PAA complexes in the immobilization of chymotrypsin and the catalytic activity of the solid-phase enzyme.

EXPERIMENTAL

Materials

PAA, with a molecular weight of 6.4×10^4, was prepared by radical polymerization.[13] The cationic polymer P(TM-*co*-AAm), with a molecular weight of 6.8×10^4 and a cationic degree of 11.4%, was synthesized by radical polymerization, which has been described in detail.[10] Chymotrypsin (Biological Reagent (BR)), casein (Biological Reagent (BR)), and other reagents (Analytical Reagent (AR)) were used as available.

Immobilization of Chymotrypsin with the Complex of P(TM-*co*-AAm)/PAA

A given amount of the aqueous solution of chymotrypsin was added to an aqueous solution of P(TM-*co*-AAm) with stirring at room temperature. After the solution was stirred for 2 h, an equimolar amount of an aqueous solution of PAA was added dropwise with stirring. A white flocculent solid appeared. After the reaction had proceeded for 4 h, the products were separated by centrifugation. The precipitates were washed with deionized water. After immersion in a phosphate buffer (0.2*M*, pH = 6) for 24 h, the precipitates were dried *in vacuo* and then ground to a grain of 60 mesh. The supernatants were collected, and the enzyme activities were measured.

Assays of Chymotrypsin Activity

The measurements of enzyme activities were carried out as a reference.[14] One milliliter of a 10 mg/L chymotrypsin enzyme aqueous solution and l mL of a 20% casein-substrate aqueous solution were mixed after preincubation in a water bath with a constant temperature of 35°C for 15 min. The time was read on a stopwatch. After the mixture solution was kept at 35°C for 20 min, 3 mL of 5% trichloroacetic acid was added with shaking. The reaction solution was filtered after being kept at room temperature for 30 min. The absorbance at 280 nm of the filtrate was measured on an ultraviolet–visible spectrophotometer (UVIDEC-32.0, Shimadzu, Japan).

The activity of chymotrypsin was expressed in convenient units. One unit of chymotrypsin was defined as the amount of enzyme needed to form the hydrolysis products per minute, which brought about an absorbance increase of 1.00 at 280 nm.

The activity of the immobilized enzyme was calculated as follows:

$$\text{Activity yield (\%)} = \frac{\text{Activity of enzyme in solid phase}}{\text{Total activity of enzyme}}$$

$$\text{Relative activity (\%)} = \frac{\text{Total activity of enzyme in solid phase}}{\text{Total activity of enzyme} - \text{total activity of enzyme in supernatant}}$$

RESULTS AND DISCUSSION

Immobilization of Chymotrypsin with P(TM-*co*-AAm)/PAA Complexes

Chymotrypsin was immobilized with the interpolymer complexes of equimolar P(TM-*co*-AAm) and PAA. The activity yields of the enzymes, which were immobilized at pH 2 with different concentrations of the individual polymers P(TM-*co*-AAm) and PAA (0.025–0.4*N*), are shown in

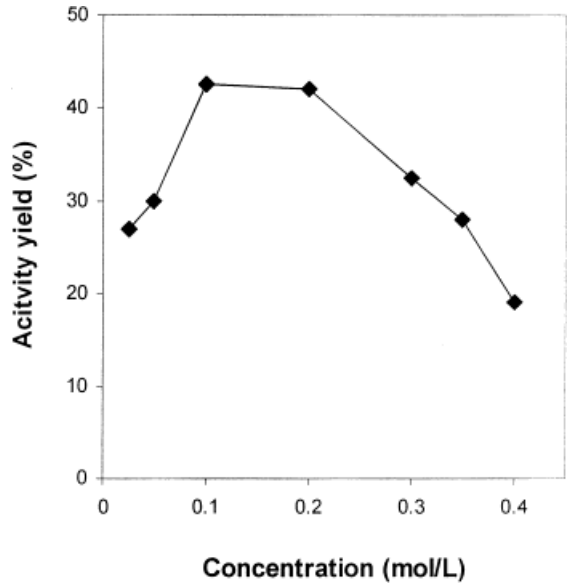


Figure 1 Plot of the activity yield of chymotrypsin versus the concentration of the individual polymer [P(TM-*co*-AAm) or PAA] during immobilization. pH = 2.

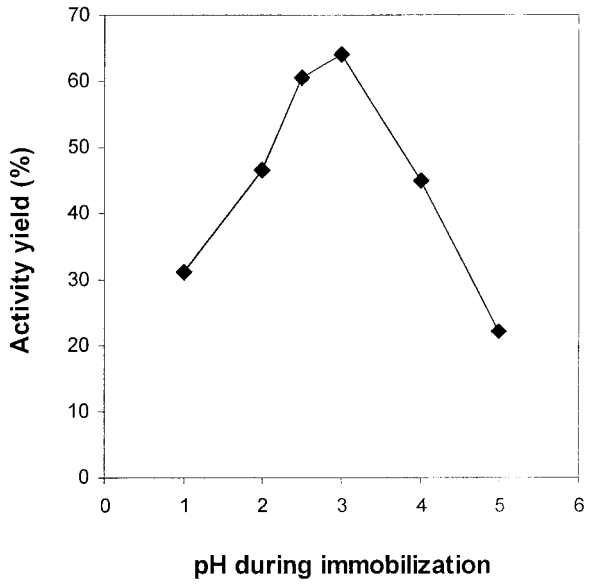


Figure 2 Plot of the activity yield of chymotrypsin versus pH in the immobilization process. [P(TM-*co*-AAm)] = [PAA] = 0.1*N*.

Figure 1. The enzyme activity yield increased with an increase in concentration and reached the maximum when the concentration of the polymer was 0.1–0.2*N*. At this concentration, a suitable network structure was formed for the entrapment of the enzyme. When the concentration was lower, the crosslinking density of the complex network was too low to immobilize the enzyme. The loose structures may have led to the leakage of the enzyme. At the higher concentration, the diffusion of the substrate and product were also limited by the lower pore capacity and pore diameter of the complex gels.

The chymotrypsin could be immobilized at different pH's, ranging from 1 to 5, which were controlled by the pH of a P(TM-*co*-AAm) and PAA solution. Figure 2 shows the activity yields of the enzymes immobilized at different pH's. It is clear that the pH in the immobilizing process had a strong effect on the activity yield of the immobilized enzyme. The optimum pH was around 3, and the activity yield decreased dramatically when the pH deviated from the optimum value. The free chymotrypsin was mostly stable at pH 3–3.5. At this pH, the ionization degree of PAA may have been suitable for the cationic degree of P(TM-*co*-AAm). The cooperative interaction between two polymers resulted in the effective intermolecular crosslinking network immobilizing the largest amount of enzyme.

When the other conditions were fixed at pH 3 and 0.1*N* PAA and P(TM-*co*-AAm), the concentration of chymotrypsin varied from 1 to 20 mg/L for the immobilization. The activity yield of immobilized enzyme increased with the concentration of chymotrypsin and showed a peak value at about 5 mg/L (Fig. 3). The activity yield began to decrease

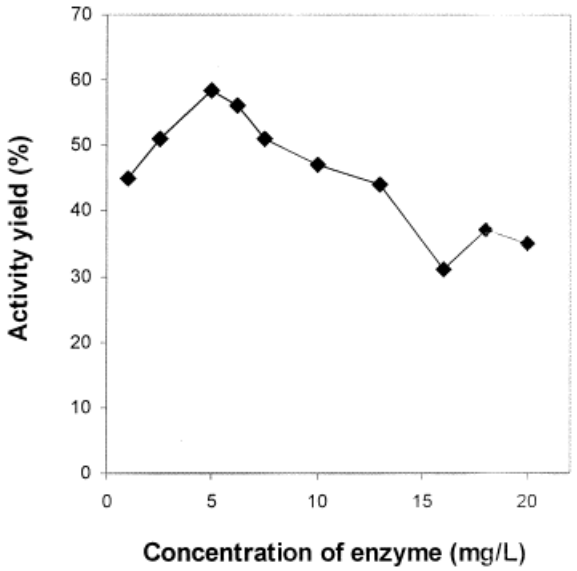


Figure 3 Plot of the activity yield of chymotrypsin versus the concentration of enzymes during immobilization. pH = 2; [P(TM-*co*-AAm)] = [PAA] = 0.1*N*.

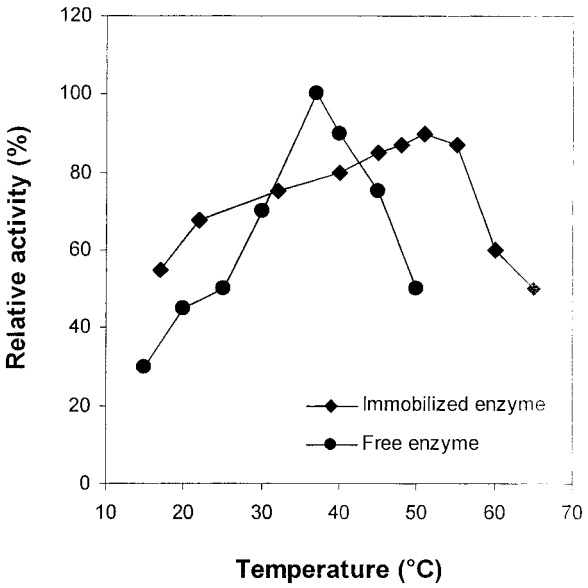


Figure 4 Temperature dependence of the relative activity of the enzyme (the highest activity of the free enzyme is taken to be 100%).

when the enzyme concentration was higher than this value. It suggests that there was a threshold for the quantity of enzyme immobilized under certain carrier conditions and that self-agglomeration between condensed enzyme molecules could happen over this threshold. Under the conditions of this experiment, the maximum amount of the immobilized chymotrypsin was 80–100 mg/g of the dry complex.

On the basis of the previously mentioned facts, the optimum conditions of chymotrypsin immobilization with the interpolymer complexes of P(TM-*co*-AAm)/PAA can be concluded to be pH 3 of the immobilizing medium, 5 mg/L of the free enzyme during mixing, and 0.1*N* PAA and P(TM-*co*-AAm). The average activity yield is 54–62%, which is much higher than the yields from the most common immobilizing methods.

Heat Resistance of the Immobilized Enzymes

The relative activities of the free and immobilized chymotrypsin at different temperatures (15–70°C) were measured in a glycine–NaOH buffer. The dependence of the relative activities of the enzymes on temperature is shown in Figure 4. The free chymotrypsin showed the highest activity when the temperature was at about 37°C. However, the activity of the free enzyme decreased when the temperature differed from this optimum. The immobilized enzyme showed the higher optimum temperature at about 51°C and the wider optimum temperature range of 45–56°C, indicating better temperature resistance than that of the free enzyme.

In a comparison with the chymotrypsin immobilized with carboxymethyl cellulose azide, which showed an optimum temperature 8°C higher than that of the free enzyme,[2] the complex carrier of P(TM-*co*-AAm)/PAA has advantages for enzyme immobilization. There is hydrogen bonding between —$CONH_2$ and —COOH, and there is a strong ionic interaction between —$N(CH_3)_3^+$ and —COO^-. When the enzyme is immobilized with complexes, the enzyme exists as the binding state because of the ionic interaction and hydrogen bonding between enzyme and carrier. The higher thermal stability of the enzyme resulting from the immobilization would contribute to the acceleration of the reaction at a higher temperature, which is a condition widely used in industry.

Acid–Alkali Resistance of Immobilized Enzymes

Free chymotrypsin has an optimum pH of 8, which is close to its isoelectric point, for catalyzing the hydrolysis of the substrate casein. When the pH is higher or lower than 8, the enzyme activity greatly decreases (Fig. 5). The relative activities of enzymes were determined at 51°C in a borax–NaOH buffer. After immobiliza-

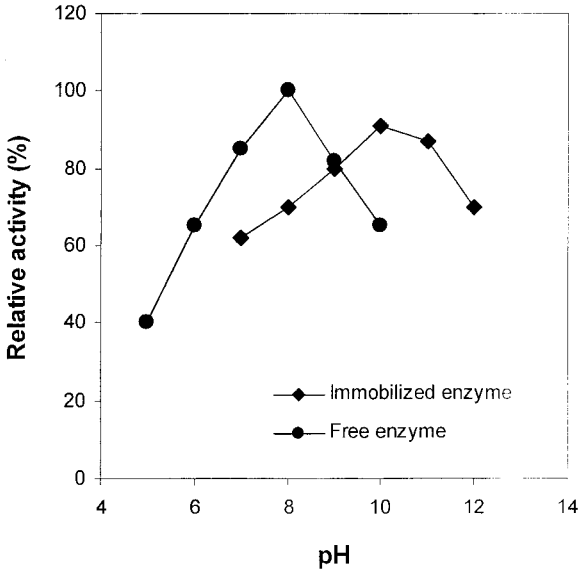
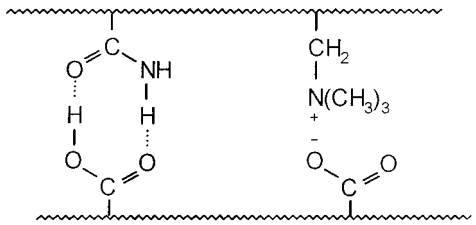


Figure 5 Medium pH dependence of the relative activity of the enzyme (the highest activity of the free enzyme is taken to be 100%).



Scheme 1

tion with the complex of P(TM-*co*-AAm)/PAA, the optimum pH of chymotrypsin shifts to pH 10, and the optimum pH range becomes wider (Fig. 5), suggesting an enhancement in the acid and alkali resistances of the enzyme. This is caused by the change of the enzyme microenvironment after the immobilization.

It is interesting to see the pH sensitivity peculiar to this kind of complex carrier. At the optimum pH of 10, the complex carrier appears as a swelling and dissolving equilibrium state, and the latter process is very slow. In this case, the immobilized enzyme has a higher relative activity (~90%) because of the higher permeability for the substrates and products. When the pH is adjusted to 6, the complex goes back to a contracted state, and the immobilized enzyme ceases to catalyze reactions and is very easily separated from the reaction mixture by filtration. This means that the enzyme reaction can be controlled conveniently by the pH of the reaction medium being changed. The influence of pH on the activity of the immobilized enzyme is explained by the ionization degree of PAA. For example, when the pH is 10, PAA is ionized, and most of the hydrogen bonds are dissociated. The interaction between the polymer chains comes from the relatively small amounts of ionic interaction. In this case, the complex carriers are in the swollen state (Scheme 1).

Stability of Storage and Repeated Use

The relative activity of the immobilized enzyme is slightly lower than that of the free enzyme, but it is useful for industrial applications because it has higher stability and can be used repeatedly. The immobilized chymotrypsin with the complex of P(TM-*co*-AAm)/PAA retains the approximate value of the initial relative activity after 30 days of storage at room temperature (~20°C), although the free chymotrypsin only keeps 50% of the initial relative activity (Fig. 6). Moreover, there is

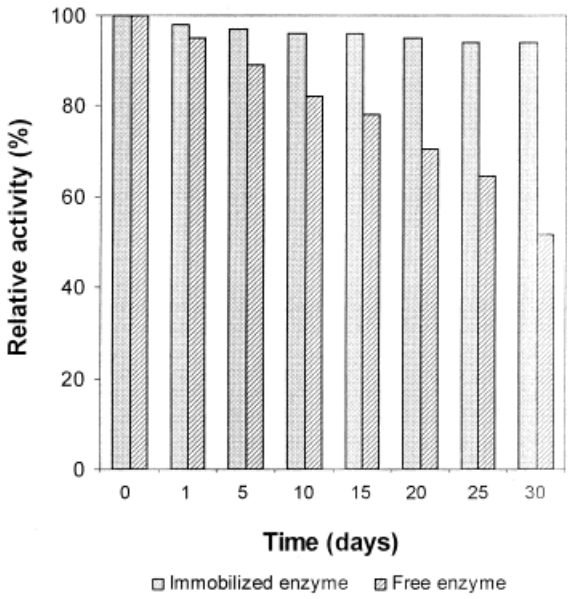


Figure 6 Dependence of the relative activity of the enzyme on the storage time at 20°C (the highest activity of the free enzyme is taken to be 100%).

little change in the shape and morphology of the immobilized enzyme.

After the catalytic reaction in the pH 10 borax–NaOH buffer, the pH was regulated to 6 to separate the immobilized enzyme for repeated use. After 10 such circulation uses, the immobilized chymotrypsin retained almost the initial relative activity of the first time (Fig. 7).

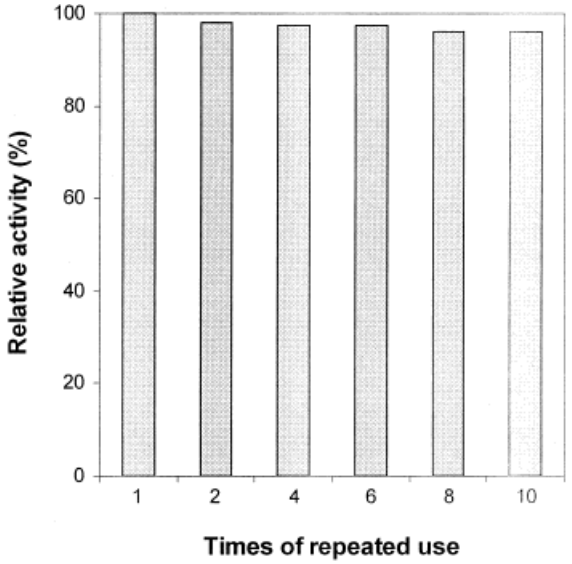


Figure 7 Dependence of the relative activity of the enzyme on the times of repeated use (the highest activity of the free enzyme is taken to be 100%).

CONCLUSIONS

Chymotrypsin was successfully immobilized with an interpolymer complex based on the cationic polymer P(TM-*co*-AAm) and PAA. The stability, pH, and storage of chymotrypsin were greatly improved after immobilization. The immobilized enzyme could be used repeatedly with the retention of activities. A small portion of the ionic interactions in the complexes led to a pH-sensitive network structure. When the pH was 10, the complex was in a swollen state, and the immobilized enzyme showed the highest activity. When the pH was changed to 6, the complex changed to a shrinkage state, and the activity of immobilized enzyme decreased. In this case, the immobilized enzyme was very easily separated from the batch for repeated use. These properties have potential applications in industry.

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