New Supports for Enzyme Immobilization Based on Copolymers of Vinylene Carbonate and β-Hydroxyethylene Acrylate

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ABSTRACT: Vinylene carbonate (VCA), β-hydroxyethylene acrylate, and *N,N′*-methylene bisacrylamide were dissolved in water, and the aqueous solutions were copolymerized via reverse-phase suspension copolymerization in paraffin oil. A series of hydrophilic and beaded supports containing reactive cyclic carbonate groups for enzyme immobilization were obtained. The supports were examined by coupling with trypsin, and the results showed that the amount of enzymes coupled to the supports and the specific activity of the immobilized trypsin were related to the content of VCA structure units and the reaction time. Meanwhile, the optimal pH and temperature, as well as the Michaelis–Menten constant K_m, for both native and immobilized trypsin were measured. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 83: 94–102, 2002

Key words: vinylene carbonate; β-hydroxyethylene acrylate; enzymes; immobilization; trypsin; gels; supports

INTRODUCTION

Enzymes are widely applied as biocatalysts in various fields, such as the chemical and pharmaceutical industries and clinical analysis.[1,2] Because immobilized enzymes have the advantages of being handled in batch and continuous systems and being removed easily from reaction media, enzymes bound to matrices have attracted a great deal of interest in recent decades. Many carriers and techniques for the activation of the carriers have been developed. Two major techniques for binding enzymes to a matrix can be distinguished: (1) the entrapment of a protein with the lattice of a polymer, such as crosslinked polyacrylamide[3] or silicone resin,[4] and (2) the establishment of covalent linkages between proteins and supports.[5,6] Because of the stable linkages between polymer matrices and proteins, the covalent fixation of biomolecules has received wide attention. The supports activated by CNBr[5] and glutaraldehyde[7] are two of those used most intensively. However, both have problems, such as procedures for activation that are too complicated and the removal of toxic residuals, especially cyanide ions.[8]

It is well known that cyclic carbonate groups can readily combine with amino groups of biomolecules under very mild conditions.[9–12] Moreover, these highly reactive groups show good stability under the usual conditions (e.g., storage in an aqueous solution with a neutral pH).[9,10] Cyclic carbonate groups can be introduced onto the sup-

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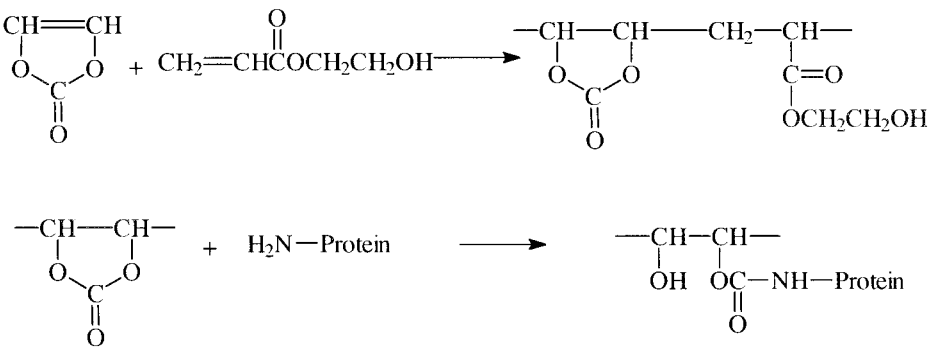

Scheme 1

ports by the treatment of polymers containing hydroxyl groups with ethyl chloroformate in anhydride organic solvents[8–12] or even directly by the copolymerization of vinylene carbonate (VCA) with some hydrophilic vinyl monomers.[13–15] It is evident that the copolymerization of VCA should be a feasible and direct way of preparing the reactive supports (Scheme 1). Therefore, in this study, with water as the solvent of the comonomers, new hydrophilic supports containing reactive cyclic carbonate groups were prepared via reverse-phase suspension copolymerizations of aqueous solutions of VCA and β-hydroxyethylene acrylate (HEA) in paraffin oil, and the utility of the reactive supports was examined by the immobilization of trypsin.

EXPERIMENTAL

Materials

VCA was prepared as described in the literature.[16,17] HEA was distilled before use (bp = 630–65°C/2 mmHg). Trypsin, with a specific activity of 5.50 U/mg, was a product of Sino-American Biotechnology Co. (Beijing, China). All other reagents were analytical-grade.

Preparation of Microspheric Beads from VCA and HEA

The microspheric beads were prepared by reverse-phase suspension copolymerization with paraffin oil as the continuous phase and water as the solvent for monomers. For a typical experiment, to a 100-mL, three-necked flask equipped with a mechanical stirrer, N_2 inlet, and condenser, 50 mL of paraffin oil and 0.05 g of triethanolamine monooleate (TM) were placed. An aqueous solution composed of VCA (1.5 g, 17.4 mmol), HEA (1.5 g, 12.9 mmol), *N,N′*-methylene bisacrylamide (0.15 g, 0.97 mmol; its percentage was calculated and regarded as the crosslinking degree of the beads), $K_2S_2O_8$ (0.03 g, 0.13 mmol), $NaHSO_3$ (0.03 g, 0.29 mmol), and 3 mL of water was added to the flask. The mixture was stirred at a fixed speed to produce a suitable droplet size. The copolymerization first was maintained in a water bath at 30°C to react for 6 h and then in a water bath at 60°C to react for 4 h. Subsequently, the beads were isolated by filtration, rinsed with petroleum ether, further washed with water and ethanol, and dried in a vacuum at 70°C to a constant weight. The yield was 2.7 g (90%).

Immobilization of Trypsin onto the Support

The beads (precisely 30.0 mg) in a test tube were swollen in 3 mL of a 0.2*M* borate buffer (pH 8.0) for 12 h. Then, the solution was carefully removed from the tube with a syringe, and 3.00 mL of a 3.00 mg/mL trypsin solution in a 0.2*M* borate buffer (pH 8.0) was added to the tube. The suspension was incubated in an ice–water bath for 24 h, unless otherwise mentioned. The trypsin solution was removed with a syringe, and its absorbency at 280 nm was measured. Occasionally, the trypsin solution was slightly turbid, and its absorbency at 450 nm was used to eliminate the error caused by light scattering. The coupling yield and amount of proteins coupled to supports were determined according to eqs. (1) and (2). The trypsin-coupled supports were washed four times with a cold 0.2*M* borate buffer (pH 8.0) and stored in a refrigerator:

$$\text{Coupling Yield} = \frac{A_0 - A}{A_0} \times 100\% \qquad (1)$$

$$\text{Amount of Enzymes Coupled} = \frac{A_0 - A}{A_0 \times W} \times C \times V \quad (2)$$

where A_0 is the absorbency at 280 nm of initial trypsin solutions, A is the absorbency at 280 nm of trypsin solutions after immobilization, C is the concentration of trypsin solutions (mg/mL), V is the volume of trypsin solutions (mL), and W is the weight of the dried supports (g).

Measurement of the Activity of Trypsin

Measurement of the Activity of Native Trypsin

An assay mixture consisting of 0.10 mL of a 0.25% absolute ethanol solution of benzoyl-D,L-arginin-β-naphthylamide hydrochloride (BANA; 0.30 mL) of a 3.00 mg/mL trypsin solution in a 0.2*M* borate buffer (pH 8.0) and 1.10 mL of a 0.2*M* borate buffer (pH 8.0) was prepared. The mixture was incubated for precisely 15 min at 37°C. The reaction was stopped by the transference of 0.50 mL of a 2*M* HCl aqueous solution to the tube. To the reaction mixture, 1.00 mL of a 0.1% $NaNO_2$ aqueous solution was added, and the mixture was shaken vigorously for 2 min. After this treatment, 1.00 mL of a 0.5% ammonium sulfamate aqueous solution was added during continued shaking for 1 min. Finally, 2.00 mL of a 0.05% absolute ethanol solution of *N*-(1-naphtyl) ethylene diamine dihydrochloride was added to the tube. The solution was mixed and then immersed in a 25°C water bath for 30 min. A blank was prepared in a similar fashion, but the trypsin was omitted. The absorbency at 560 nm (A_{560}) was read with the blank as a reference. The activity was calculated as follows:

$$\text{Total Activity (BANA Unit)} = \frac{A_{560}}{t \times 0.01} \quad (3)$$

$$\text{Specific Activity} = \frac{\text{Total Units}}{\text{Protein (g)}} = \frac{A_{560}}{t \times 0.01 \times W_1} \quad (4)$$

where t is the reaction time (min), W_1 is the weight of trypsin (g), and one BANA unit is defined as the amount of trypsin needed when A_{560} rises 0.01 per minute.

Determination of the Activity of Immobilized Trypsin

To a test tube containing immobilized trypsin prepared from 30.0-mg dry supports, 1.40 mL of a 0.2*M* borate buffer (pH 8.0) and 0.10 mL of a 0.25% BANA absolute ethanol solution were added. The solution was mixed and placed in a water bath at 37°C to react for exactly 15 min. Subsequent steps were identical to those used for native trypsin measurements:

$$\text{Specific Activity of Immobilized Trypsin} = \frac{A'_{560}}{0.01 \times t \times W} \quad (5)$$

$$\text{Retention of Activity} = \frac{\text{Total Units of Immobilized Enzymes}}{\text{Total Units of Native Enzymes}} \times 100\% = \frac{A'_{560}}{t \times 0.01 \times V \times C \times G} \times 100\% \quad (6)$$

$$\text{Relative Activity} = \frac{\text{Total Units of Immobilized Enzymes} \times 100\%}{\text{Total Units of Native Enzymes} - \text{Total Units of Filtrate}} = \frac{A'_{560}}{t \times 0.01 \times V \times C \times G \times Y} \times 100\% \quad (7)$$

where A'_{560} is the absorbency at 560 nm of a reaction solution catalyzed by immobilized trypsin, W is the weight of dry beads (g), G is the specific activity of native trypsin (unit/g), V is the volume of the initial trypsin solution (mL), C is the concentration of the initial trypsin solution (g/mL), and Y is the coupling yield.

Estimation of the Properties of Both Native and Immobilized Trypsin

Optimal pH Value

The activity of native and immobilized trypsin in a 0.2 mol/L borate buffer with different pHs was measured at 37°C.

Optimal Temperature

The activity of native and immobilized trypsin in a 0.2 mol/L borate buffer (pH 8.0) at different temperatures was measured.

Determination of the Michaelis Constant

The Michaelis–Menten constant K_m of native and immobilized trypsin was evaluated with BANA

solutions (ranging from 0.25% to 0.625%) in a 0.2 mol/L borate buffer (pH 8.0) at 37°C on the basis of a Lineweaver–Burk plot:

$$\frac{1}{V} = \frac{K_m}{V_m} \cdot \frac{1}{[S]} + \frac{1}{V_m} \tag{8}$$

where V is the initial reaction velocity calculated from the absorbency of the reaction solution at 540 nm after incubation in a 37°C water bath for 5 min, [S] is the concentration of the substrate, and V_m is the maximum reaction velocity.

Storage Stability of Immobilized Trypsin Beads

Immobilized trypsin beads were stored in a 0.2*M* borate buffer (pH 8.0) at 4°C for certain times, and the activities were determined.

Reusability of Immobilized Trypsin Beads

The activity of immobilized trypsin prepared from 30.0-mg dry beads was determined first. The suspensions of the beads in the substrate solution were filtered, and the beads were rinsed with a cold 0.2*M* borate buffer (pH 8.0) and used for the next activity determination. The process was repeated 10 times, and the activity was determined in each case.

Characterization of Poly(vinylene carbonate/ β-hydroxyethylene acrylate) [P(VCA/HEA)] Beads

IR Spectra

Fourier transform infrared (FTIR) spectra of the beads were obtained with an FTIR spectrophotometer (Nicolet Magna-IR 750 spectrometer, Madison, WI). For FTIR spectra, the beads and KBr were thoroughly mixed, this mixture was pressed to form a tablet, and the spectrum was recorded.

Bead Size and Distribution

Micrographs of the beads were taken with a scanning electron microscope (Hitachi X-650, Japan). We determined the size distribution of the polymeric beads by measuring at least 100 particles for each sample from the scanning electron micrographs.

Swellabilities of Beads

The dry beads were placed in deionized water (or other solutions) for 24 h. The beads then were weighed after being blotted for removal of the surface water. The swelling degree of the beads was calculated as follows:

$$\text{Swelling Degree (g/g)} = \frac{\text{Weight of Wet Beads} - \text{Weight of Dry Beads}}{\text{Weight of Dry Beads}} \tag{9}$$

RESULTS AND DISCUSSION

P(VCA/HEA) Beads

P(VCA/HEA) beads were produced by the reverse-phase suspension copolymerization of aqueous solutions of VCA and HEA. Paraffin oil was chosen as a dispersion medium in this study because of its insolubility with the comonomers and its high viscosity, which favored the stabilization of monomer droplets. TM, an oil-soluble emulsifier, was used as the dispersion agent of suspension polymerization.

Generally, VCA hardly dissolves alone in water.[13–15] Thus, Mauz and coworkers[13,15] carried out copolymerizations of VCA with *N*-vinyl pyrrolidone in dimethylformamide or other organic solvents to prepare the reactive supports. However, we found that VCA can readily dissolve in aqueous solutions preliminarily containing HEA. This behavior enables us to directly use water as the solvent of comonomers and perform the reverse-

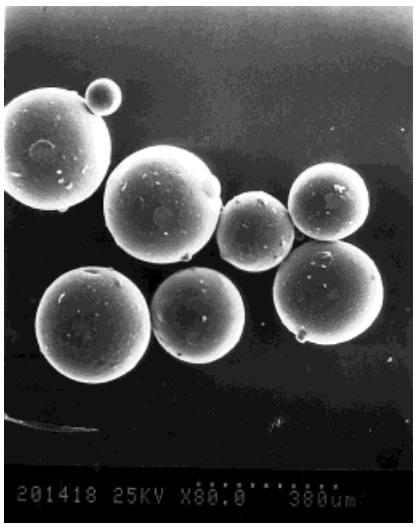

Figure 1 Representative scanning electron micrograph of P(VCA/HEA) beads.

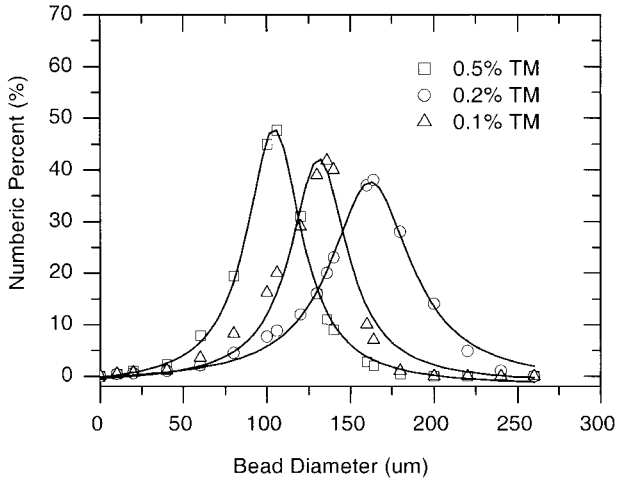

Figure 2 Size distribution of P(VCA/HEA) beads prepared by reverse-phase suspension polymerization with different amounts of emulsifier (TM).

phase suspension copolymerization in the oil phase. The merits of using water as the solvent of the monomers are obvious, including no pollution derived from organic solvent residuals, low cost for manufacturing, and the possibility of employing a simple redox initiating system (i.e., $K_2S_2O_8$ and $NaHSO_3$) that provides the convenience of carrying out the copolymerization at a low temperature. Because the decomposition of VCA and other side reactions may occur in aqueous solutions at a high temperature and the viscosity of paraffin oil may decline steeply as the temperature is raised, a low polymerization temperature surely will offer great benefits to reverse-phase suspension copolymerizations. With $K_2S_2O_8$ and $NaHSO_3$ as the initiator and co-initiator, the copolymerizations first were carried out at 30°C for several hours until most of the monomers were consumed and then at 60°C to complete the polymerization. The yields of the copolymers were pretty high (>90%), and the suspension polymerization proceeded perfectly even though polymeric stabilizers were absent.

As a result, P(VCA/HEA) beads with various contents of VCA and crosslinker were prepared successfully. A representative scanning electron micrograph of the dry P(VCA/HEA) beads prepared in this study is shown in Figure 1. Note that the beads possessed a spherical shape. The smoothness of the bead surface may be an indication that the beads contained no porosity.

The size distributions of the beads obtained with different amounts of the dispersion agent (TM) are given in Figure 2. The results show that the number of large beads decreased with an increasing amount of the stabilizer (i.e., TM) while the rate of the stirring, phase ratio, and comonomer concentrations were kept constant.

The swelling behaviors of the beads were measured, and the data are given in Table I. All the beads exhibited suitable swellability in aqueous solutions; this makes it possible for protein molecules to penetrate the supports and react with the reactive groups in the supports. Moreover, the swelling degree of the beads was dependent on the amount of hydrophobic VCA structure units and the crosslinking agent. With an increasing amount of VCA structure units and crosslinking agent, swelling degrees of the supports decreased.

Immobilization of Trypsin onto P(VCA/HEA) Supports

Figure 3 shows IR spectra for a P(VCA/HEA) support and a trypsin-coupled support. We can see that the two spectra are almost identical, except that the absorbency at 1821 cm^{-1}, which corresponds to carbonyl groups of cyclic carbonate, becomes much weaker for the trypsin-coupled sup-

Table I Swelling Degree of P(VCA/HEA) Copolymer Beads

Feed Ratio of VCA/HEA (g/g)	Crosslinking Degree (%)	Swelling Degree (g/g)	Swelling Degree in pH 8.0 0.2 mol/L Borate Buffer
1/2	20	1.117	1.078
1/2	15	1.555	1.543
1/2	10	3.220	3.198
1/2	5	3.590	3.642
1/3	5	3.789	3.800
1/4	5	3.956	4.071
1/1	5	2.292	2.153
1/5	5	3.989	3.923

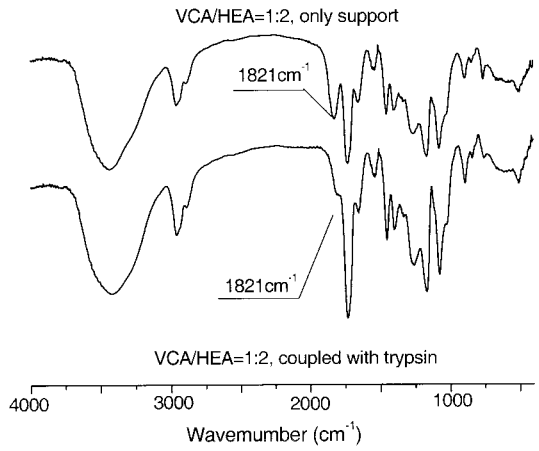

Figure 3 IR spectra of pure P(VCA/HEA) beads and the same beads coupled with trypsin.

port. However, the absorbent peak at 1821 cm^{-1} still can be observed in the trypsin-coupled supports. Obviously, this is an indication that some of the cyclic carbonates still remained in the supports.

The results of binding trypsin onto P(VCA/HEA) beads with different VCA contents are shown in Table II. Because of the high yield of copolymerization, the feed ratios of VCA and HEA can be considered the approximate compositions of the copolymers. The coupling yield and amount of trypsin coupled to the matrix rose gradually with increasing VCA content; simultaneously, the specific activity and relative activity of immobilized trypsin were reduced, and the specific activity reached its maximal value at HEA/VCA = 4/1.

From the data of Table I, we can see that the supports at a crosslinking degree of 5% possessed a good swelling performance in water (swelling degree $>$ 2 g/g). Thus, the amount of trypsin coupled to the matrix mainly was dependent on the content of VCA structure units. However, it is interesting to note from the data in Table II that the specific activity of immobilized trypsin did not increase with the increasing amount of trypsin coupled to the supports. Moreover, it also can be seen that the less enzyme the supports combined, the higher the relative activity the immobilized enzymes would show. These results evidently were due to the crowding of protein molecules on the supports, which reduced the accessibility of the substrate to immobilized enzymes.[19] Nevertheless, a high content of cyclic carbonates on the supports also could produce multiple attachments of enzymes with the matrix, which might further lead to the denaturing of trypsin. Hence, the content of VCA in the copolymers should be maintained within a suitable range through control of the feed ratios of comonomers in aqueous solutions, so that an ideal result of enzyme immobilization can be obtained.

Table III depicts the effect of reaction time on the amount and specific activity of trypsin coupled to the beads. With the reaction time prolonged, the immobilization reaction proceeded slowly and reached its maximum after nearly 8 h. The rate of immobilization was determined by the reaction rate between H_2N— and cyclic carbonate groups, as well as the diffusion rate of trypsin into the supports. Because of the large scale of trypsin molecules, the diffusion rate of trypsin was very slow, so it took more time to finish the immobilization reaction.

Properties of the Immobilized and Native Trypsin

Optimal pH

Figure 4 shows the correlation between the relative activity of trypsin (both native and immobi-

Table II Influence of VCA Content on the Immobilization of Trypsin

Initial Ratio of HEA/VCA (g/g)	Enzyme Coupled (mg/g of beads)	Coupling Yield (%)	Specific Activity of Enzyme Immobilized (U/g)	Retention of Activity (%)	Relative Activity (%)
5/1	43.2	14.4	173	10.5	72.8
4/1	72.8	24.3	229	13.9	57.1
3/1	98.9	33.0	218	13.2	40.0
2/1	102	33.9	214	13.0	38.2
1/1	113	37.9	118	7.14	18.7

Immobilization conditions: dry support (crosslinking degree = 5%) 30 mg; enzyme solution 3.00 mL in 0.2 mol/L borate buffer at pH 8.0; enzyme concentration = 3.00 mg/mL; temperature = 0°C; reaction time = 24 h; specific activity of native trypsin = 5.50 U/mg.

Table III Effect of Reaction Time on the Immobilization of Trypsin onto the Support

Time (h)	Enzyme Coupled (mg/g of beads)	Coupling Yield (%)	Specific Activity of Enzyme Immobilized (U/g)	Retention of Activity (%)	Relative Activity (%)
1	42.9	14.3	183	11.0	77.3
2	57.6	19.2	136	8.28	43.0
4	55.4	18.5	158	9.55	51.7
8	71.6	23.9	214	13.0	54.6
12	72.8	24.3	212	12.8	52.9
24	73.9	24.6	227	13.8	56.1

Immobilization conditions: dry support (VCA/HEA = 1/4, crosslinking degree = 5%) 30 mg; enzyme solution = 3.00 mL in 0.2 mol/L borate buffer at pH 8.0; enzyme concentration = 3.00 mg/mL; temperature = 0°C; specific activity of native trypsin = 5.50 U/mg.

lized) and the pH of the medium. Both native and immobilized trypsin was very sensitive to the pH of the reaction medium. The native trypsin showed an optimal pH of 8.5, and the optimal pH for the immobilized one moved to pH 8.0. Because the immobilized trypsin was prepared in an aqueous solution of pH 8.0, we could envision that new NH_2— groups might be generated from NH_3^+— groups according to the dissociation equilibrium when the pH of the medium was raised above 8.0. These new amino groups might further react with remaining cyclic carbonate groups on the supports and thus lead to the inactivation of the immobilized trypsin due to excess covalent linkages between the supports and trypsin. Therefore, the optimal pH for the immobilized trypsin was the same as the pH at which the immobilized trypsin was prepared.

Optimal Temperature

Figure 5 shows the relationship between the relative activity of trypsin and the reaction temperature. The optimal temperature for both immobilized and native trypsin was 37°C, but the curves do not overlap each other. This indicates that the effects of temperature on both immobilized and native trypsin were different. On the one hand, the enzymes might have become unstable and been readily denatured when the reaction temperature was above 0°C, whereas the immobilized enzymes were more stable against the heat. On the other hand, the remaining cyclic carbonate groups on the supports further reacted with trypsin when the reaction temperature was raised, and so some of the immobilized trypsin might be

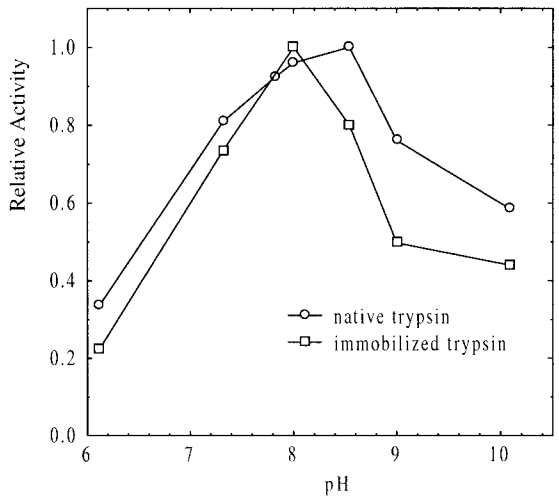

Figure 4 Effect of pH on the relative activity of trypsin.

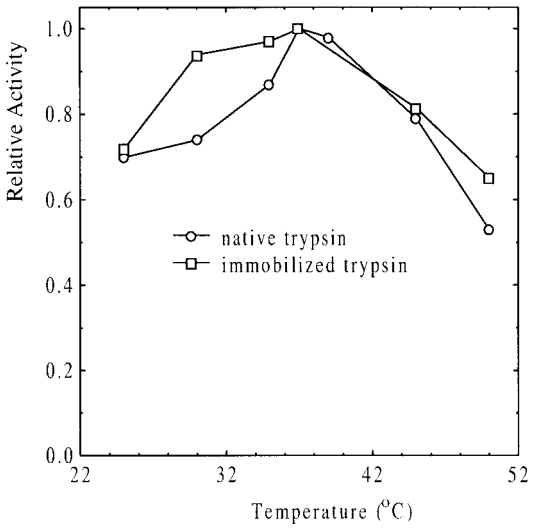

Figure 5 Effect of temperature on the relative activity of trypsin.

inactivated. Consequently, the curve for the immobilized trypsin was inconsistent with that for the native one.

K_m

K_m is a characteristic constant for an enzyme catalytic reaction; therefore, its determination was performed for the native and immobilized trypsin. Figure 6 shows Lineweaver–Burk plots obtained at various concentrations of the substrate. The native enzyme showed a K_m value of 15.2 mmol/L. In contrast to the native trypsin, the immobilized trypsin exhibited a higher K_m value (21.7 mmol/L). The discrimination was obviously due to the limitation of substrate diffusion onto the supports.[18,19]

Storage Stability

Suspensions of the immobilized trypsin in 0.2*M* borate at pH8.0 were stored at 4°C, and the activity was measured at certain intervals. The residual activity was 82% after 1 month and 71% after 6 months.

Reusability

As immobilized enzymes can be used repeatedly, the reusability of the immobilized trypsin was evaluated. Figure 7 shows the effect of repeated use on the activity of the immobilized trypsin. A residual activity of about 86% was obtained after the immobilized trypsin was reacted 10 times with BANA.

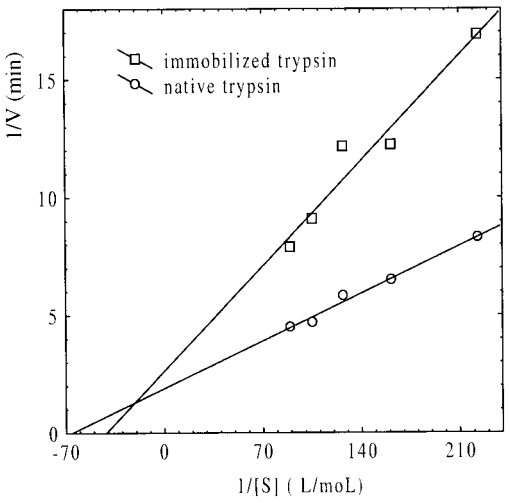

Figure 6 Lineweaver–Burk plot for trypsin.

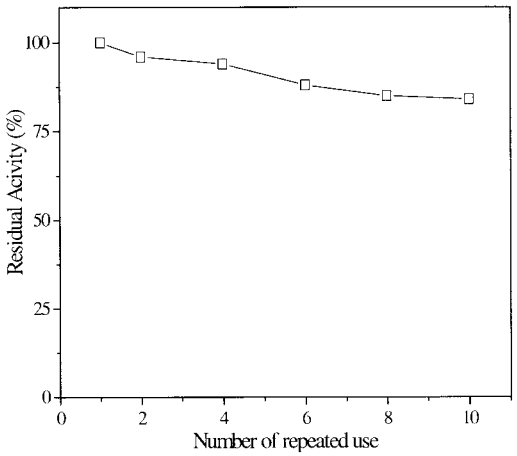

Figure 7 Effect of repeated use on the activity of immobilized trypsin.

CONCLUSIONS

Because VCA can easily dissolve in an aqueous solution of HEA, hydrophilic microspheric copolymers containing cyclic carbonate groups were prepared successfully from an aqueous solution of VCA and HEA via reverse-phase suspension polymerization in paraffin oil. With trypsin as a model enzyme, P(VCA/HEA) supports were evaluated, and the results show that the coupling yield and the specific activity of the immobilized trypsin mainly were dependent on the content of VCA structure units and the reaction time. The optimal pH for the immobilized trypsin was pH 8.0, lower than that for the native trypsin. Both the native and immobilized trypsin showed an optimal temperature of 37°C. K_m values also were determined: 15.2 mmol/L for the native trypsin and 21.7 mmol/L for the immobilized trypsin.

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