

PROPERTIES AND SYNTHESIS OF NEW SUPPORTS FOR IMMOBILIZATION OF ENZYMES BY COPOLYMERIZATION OF VINYLENE CARBONATE AND METHACRYLIC ACID

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Abstract Methacrylic acid first was neutralized with an aqueous solution of NaOH to pH = 6.0–7.0, vinylene carbonate (VCA) was added to the solution, then monomers were copolymerized in paraffin oil by means of reverse-phase suspension polymerization and hydrophilic copolymeric supports were prepared. The properties of the supports were determined using trypsin and results show that the amount of enzymes coupled to the supports and the specific activity of immobilized trypsin are related to the content of VCA structure units, reaction time and concentration of enzyme solution, etc.

Keywords Vinylene carbonate, Methacrylic acid, Fixed enzyme, Support, Trypsin

INTRODUCTION

Cyclic carbonate groups generally are stable, but can react with amino groups easily under very mild condition. It has been proved that the cyclic carbonate groups of poly(vinylene carbonate) are effective and convenient functional groups for enzyme immobilization^[1, 2]. The hydrophobicity of poly(vinylene carbonate) prevents it from being employed directly as a solid support for the fixation of enzymes, since enzymes immobilized onto highly hydrophobic supports tend to be inactivated or denatured. It is therefore necessary to increase the hydrophilicity of supports by some method. Poly(methacrylic acid) gel shows highly hydrophilic and water swellable properties^[3, 4], so in this paper methacrylic acid was employed as the comonomer for VCA. As a result, the hydrophilicity of the supports was greatly improved and their matrix exhibited very good properties on coupling with trypsin.

EXPERIMENTAL

Materials

Methacrylic acid (MA) obtained from Beijing Chemical Factory was distilled under reduced pressure. To a 20.0 g portion of redistilled methacrylic acid diluted with 10 mL of water, was added dropwise 22.3 g of 33.3 wt% NaOH aqueous solution in ice-water bath to neutralize methacrylic acid solution till pH = 6.0. VCA was prepared as described in some references^[5, 6]. Trypsin was a product from Sino-American Biotechnology Company. All other reagents were of analytical grade.

Preparation of Microspheric Beads from VCA and MA

The microspheric beads were prepared by reverse-phase suspension copolymerization using paraffin oil as the

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Revised December 7, 1999

continuous phase and water as the solvent for monomers. For a typical experiment, 50 mL of paraffin oil and a drop of triethanolamine monooleate were added to a 100 mL three-necked flask equipped with an electric stirrer and condenser. The reaction flask was kept under nitrogen atmosphere. An aqueous solution composed of 0.6 g VCA, 2.4 g neutralized MA solution, 0.18 g *N,N'*-methylene bisacrylamide, 0.03 g K₂SO₄, 0.03 g NaHSO₃, and 3 mL water was added to the paraffin oil. The stirrer was turned on and its speed regulated to produce liquid drops with suitable size. The flask was kept in a water bath at 30°C to react for 6 h and then at 60°C for 4 h. The beads were isolated by filtration, washed successively with petroleum ether, water and ethanol, and dried in vacuum at 60°C for 24 h. The yield was over 90%.

Immobilization of Trypsin onto Support

The beads (30.0 mg, precisely) were placed a test tube and swollen in 3 mL of 0.2 mol/L borate buffer at pH 8.0 for 12 h. Then the aqueous solution was removed from the tube by a syringe, and 3.00 mL of 3.00 mg/mL trypsin solution in 0.2 mol/L borate buffer at pH 8.0 was added to the tube. The suspension was incubated in an ice-water bath for 24 h except where otherwise mentioned. The trypsin solution was removed from the tube by a syringe and its absorbance was measured at 280 nm. Sometimes the trypsin solution was slightly turbid and its absorbance at 450 nm was recorded to eliminate any error caused by light scattering. Coupling yield and amount of proteins coupled to the supports were determined according to Eqs. (1) and (2). The beads coupled to trypsin were washed with cold 0.2 mol/L borate buffer at pH 8.0 for four times, and then kept in refrigerator.

$$\text{Coupled yield (Y)} = \frac{A_0 - A}{A_0} \times 100\% \quad (1)$$

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

where A_0 is the absorbance of initial trypsin solution at 280 nm, A is the absorbance of trypsin solution at 280 nm after immobilization, C is the concentration of trypsin solution (mg/mL), V is the volume of trypsin solution (mL), W is the weight of the dried support (mg). The calibration curve with trypsin concentration ranging between 0.5 and 5 mg/mL is presented in Fig. 1.

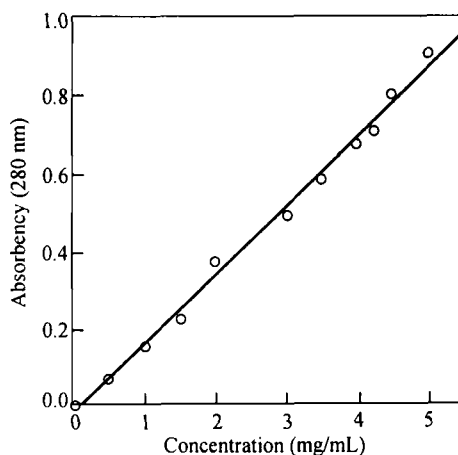


Fig. 1 The standard curve of absorbency at 280 nm plotted as a function of concentration of trypsin solution

Measurement of Activity of Trypsin

In a suitable test tube, a mixture of 0.10 mL of 0.25% benzoyl-*D,L*-arginin- β -naphthylamide hydrochloride (BANA) in absolute ethanol, 0.30 mL of 3.00 mg/mL trypsin solution in 0.2 mol/L borate buffer at pH 8.0, and 1.10 mL of 0.2 mol/L borate buffer at pH 8.0 was incubated for exactly 15 min at 37°C. The reaction was stopped by adding 0.50 mL of 2 mol/L HCl to the tube. 1.00 mL of 0.1% NaNO₂ solution was added and the tube

was shaken vigorously for 2 min. After that 1.00 mL of 0.5% ammonium sulfate was added and shaken for 1 min. 2.00 mL of 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in absolute ethanol was then added to the tube. The tube was immersed in a 25°C water bath and stirred for 30 min. A blank was made in a similar fashion but omitting the trypsin. Absorbance (A_{560}) at 560 nm was determined using the blank as a reference. Then

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

$$\text{Specific activity} = \frac{\text{total units}}{\text{mg protein}} = \frac{A_{560}}{t \times 0.01 \times W_1} \quad (4)$$

where t is the reaction time (min), W_1 is the weight of trypsin (mg), one BANA unit is defined as the amount of trypsin needed when the value of absorbance at 560 nm rises by 0.01 per min.

Determination of the Activity of Immobilized Trypsin

To a test tube containing immobilized trypsin from 30.0 mg dried supports, 1.40 mL of 0.2 mol/L borate buffer at pH 8.0 and 0.10 mL of 0.25% BANA in absolute ethanol were added. The mixture was stirred and the tube was placed in a water bath at 37°C to react for exactly 15 min. The following steps were the same as those of the native trypsin measurement.

$$\text{Specific activity of immobilized trypsin} = \frac{A_{560}}{0.01 \times t \times W} \quad (5)$$

$$\begin{aligned} \text{Retention of activity} &= \frac{\text{total units of immobilized enzymes}}{\text{total units of native enzymes}} \times 100\% \\ &= \frac{A_{60}}{t \times 0.01 \times V \times C \times G} \times 100\% \end{aligned} \quad (6)$$

$$\begin{aligned} \text{Relative activity} &= \frac{\text{total units of immobilized enzymes}}{\text{total units of native enzymes} - \text{total units of remained in solution}} \times 100\% \\ &= \frac{A_{560}}{t \times 0.01 \times V \times C \times G \times Y} \times 100\% \end{aligned} \quad (7)$$

where A'_{560} is the absorbance of the reaction solution at 560 nm catalyzed by immobilized trypsin, t is the reaction time (min), W is the weight of dried beads (mg), G is specific activity of native trypsin (units/mg), V is the volume of initial trypsin solution (mL), C is the concentration of initial trypsin solution (mg/mL), Y is the coupling yield.

Measurement of the Absorbance of Beads in Water

The dried beads were placed in deionized water (or other solutions) for 24 h, then they were weighed after blotting with filter paper to remove the surface water. The absorbance of beads was calculated as follows:

$$\text{Water absorbance (g/g)} = \frac{\text{weight of wet beads} - \text{weight of dried beads}}{\text{weight of dry beads}} \quad (8)$$

Estimation of the Properties of Both Native and Immobilized Trypsin

Optimal pH value

The activities of native and immobilized trypsin in 0.2 mol/L borate buffer at different pH were measured at 37°C.

Optimal temperature

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

Determination of the michaelis constant

The Michaelis-Menten constant K_m of native and immobilized trypsins was evaluated using BANA solution (ranging from 0.25% to 0.625%) in 0.2 mol/L borate buffer (pH 8.0) at 37°C, on the basis of the Lineweaver-

Burk plot:

$$\frac{1}{V} = \frac{K_m}{V_m} \cdot \frac{1}{[S]} + \frac{1}{V_m} \quad (9)$$

where V is the initial reaction velocity calculated from the absorbance of solution and reaction time (5 min), $[S]$ is the concentration of the substrate, V_m is the maximum reaction velocity and K_m is the Michaelis-Menten constant.

RESULTS AND DISCUSSION

Preparation of Copolymeric Beads of VCA and Methacrylic Acid

Methacrylic acid was neutralized by an aqueous solution of sodium hydroxide to prevent the decomposition of VCA during copolymerization. It was found that VCA hardly dissolves alone in water. However, if VCA and methacrylic acid or other water-soluble organic monomers were put in water together, its solubility would be improved greatly. This property provides a highly convenient means of modifying the ratios of monomers in the copolymerization system and makes it possible to perform copolymerization in aqueous solution rather than in organic solvents^[1]. The water absorption of VCA-MA copolymeric beads at different ratios of monomers is illustrated in Table 1. It shows that copolymeric beads have a good swelling performance in water due to the presence of a large amount of dissociable groups, such as $-\text{COONa}$ and $-\text{COOH}$, present in the chains of copolymer.

Table 1. Water absorbence of VCA-MA copolymer beads

VCA/MA (g/g)	1/4	1/3	1/2
Water absorbence of copolymer (g/g)	10.04	8.874	6.701
Water absorbency of copolymer in pH7.32 0.2 mol/L borate buffer solution (g/g)			6.656

Influence of Amount of VCA on the Immobilization

The amounts of trypsin coupling onto the supports at different ratios of VCA/MA are listed in Table 2. The network of the supports contained not only many cyclic carbonate groups, but also a large number of dissociating groups of $-\text{COONa}$ and $-\text{COOH}$. All these functional groups could combine with enzyme molecules, thus the coupling yields of these supports, which were calculated from the concentration change of trypsin solutions before and after immobilizing reaction, were very high. But they are only apparent coupling yields, since enzymes coupled by $-\text{COONa}$ or $-\text{COOH}$ groups could be washed away easily by buffer solution. It was found that the support without VCA structural units having the same degree of crosslinking of also could fix a large amount of trypsin in the solution. However, after washing with buffer solution for 5 times, the enzyme activity of the support almost dropped to zero. Therefore, it is reasonable that the specific activity of fixed trypsin was not very high compared with its coupling yield and depended on the content of VCA units.

Table 2. Influence of VCA content on the immobilization of trypsin^a

VCA/MA (g/g)	Enzyme coupled (mg/g beads)	Coupling yield (%)	Specific activity of enzyme immobilized (U/g)	Retention of activity (%)	Relative activity (%)
1/2	178	59.4	188	11.4	19.2
1/3	154	51.2	121	7.32	14.3
1/4	139	46.3	84.9	5.14	11.1

^a Immobilization conditions: dried support (crosslinking degree 6%) 300 mg; Enzyme solution: 3.00 mL in 0.2 mol/L borate buffer at pH 7.32; Enzyme concentration: 3.00 mg/mL; $T = 0^\circ\text{C}$; Reaction time: 24 h; Specific activity of native trypsin: 5.50 U/mg

Influence of Reaction Time on the Immobilization

Due to the high swelling power of the support trypsin molecules diffused easily into the network of the support and the immobilization nearly reached a maximum after 2 h as illustrated in Table 3, but its specific activity of

fixed trypsin was not yet high. Probably this was due to the fact that most of the trypsin was coupled with carboxyl groups at the beginning of the reaction. This combination was reversible and enzymes would be washed away before the activity of the fixed trypsin was measured. However, with the advance of reaction time trypsin originally combined with carboxyl groups was released and reacted with cyclic carbonate groups. Therefore the specific activity of immobilized trypsin increased gradually with increasing reaction time.

Table 3. Effect of reaction time on the immobilization of trypsin onto the support^a

Time (h)	Enzyme coupled (mg/g beads)	Coupling yield (%)	Specific activity of enzyme immobilized (U/g)	Retention of activity (%)	Relative activity (%)
1	147	46.0	85.6	5.18	11.3
2	159	52.9	106	6.41	12.1
4	174	56.9	128	7.77	13.7
8	175	58.3	132	8.03	13.8
12	177	59.1	191	11.6	19.6
24	172	57.2	196	11.9	20.8

^a Immobilization conditions: dried support (VCA/MA = 1/2, crosslinking degree 6%) 300 mg; Enzyme solution: 3.00 mL in 0.2 mol/L borate buffer at pH 7.32; Enzyme concentration: 3.00 mg/mL; $T = 0^{\circ}\text{C}$; Specific activity of native trypsin: 5.50 U/mg

Influence of Trypsin Concentration on the Immobilization

As shown by the data in Table 4, with increasing trypsin concentration in the immobilization mixture, the amount of trypsin coupled rose and simultaneously the specific activity of fixed trypsin also increased. Increasing trypsin concentration, however, significantly influenced the relative activity of the fixed trypsin on the matrix. A dramatic decrease of relative activity indicates that enzymes become less efficient. This efficiency drop may be due to the crowding of trypsin on the support, which reduces the accessibility of substrates with immobilized enzyme^[7].

Table 4. Effect of trypsin concentration on the immobilization onto the supports^a

Concentration of trypsin (mg/mL)	Enzyme coupled (mg/g beads)	Coupling yield (%)	Activity of enzyme immobilized (U/g)	Retention of activity (%)	Relative activity (%)
1.00	32	32.0	99.1	27.0	84.4
2.00	106	53.0	152	13.8	26.0
3.00	172	57.2	196	11.9	20.8
5.00	318	63.7	185	6.73	10.6
6.00	391	65.2	210	6.32	9.71

^a Immobilization conditions: dried support (VCA/MA = 1/2, crosslinking degree 6%) 300 mg; Enzyme solution: 3.00 mL in 0.2 mol/L, borate buffer at pH 7.32; $T = 0^{\circ}\text{C}$; Reaction time: 24 h; Specific activity of native trypsin: 5.50 U/mg

Influence of pH of Reaction Mixture on the Immobilization

The effects of pH of the reaction mixture on the immobilization of trypsin are listed in Table 5. It is evident that the coupling yield depends on the pH, with an optimum efficiency at pH 7.83, whereas the maximum specific activity of coupled trypsin is at pH 7.32. This difference may be due to the fact that the coupling reaction involves two kinds of mechanisms, one is the combination of cyclic carbonate groups with trypsin, and the other is the adsorption of trypsin by carboxyl groups. Generally, a high pH value of the reaction medium favors the reverse adsorption reaction between the trypsin molecules and the support, and conversely, a low pH value of the reaction medium benefits the combination between trypsin and cyclic carbonate groups due to the existence of $-\text{NH}_3^+$ and $-\text{COOH}$ which could catalyze the combination of both^[8,9].

Comparison of the Properties of Native and Immobilized Trypsin

Optimal pH

It can be observed from Fig. 2 that the optimal pH values for native and immobilized tryptins are 8.5 and 7.8, respectively. It has been known that the isoelectric point of trypsin is pH 10.5, thus a trypsin molecule in neutral

aqueous solution has positive charges the same as the substrates (BANA) in aqueous solution. Obviously raising the pH of the medium would weaken the positive charges of trypsin and substrates, and favor their combination. However as far as immobilized trypsin is concerned, the network of the support contains many dissociable groups, such as --COONa and --COOH , which could dissociate according to the electrolytic equilibrium and cause the support to carry charges. In this case, the substrates can diffuse into the support and therefore the optimal pH for immobilization of trypsin decreased markedly.

Table 5. Effect of pH of the medium on the immobilization of trypsin^a

pH	Enzyme coupled (mg/g beads)	Coupling yield (%)	Specific activity of enzyme immobilized (U/g)	Retention of activity (%)	Relative activity (%)
6.12	22.4	7.46	34.0	2.06	27.6
7.32	150	49.9	174	10.5	21.1
7.83	188	62.6	159	9.64	15.4
8.00	176	58.6	147	8.92	15.3
9.00	130	43.3	133	8.04	18.6
10.09	56.8	18.9	113	6.88	36.4

^a Immobilization conditions: dried support (VCA/MA=1/2, crosslinking degree 6 %) 300mg; Enzyme solution 3.00 mL in 0.2 mol/L borate buffer; Enzyme concentration 3.00 mg/mL, $T = 0^{\circ}\text{C}$; Reaction time 24 h; Specific activity of native trypsin: 5.50 U/mg

Optimal temperature

The relationship between relative activity and reaction temperature for trypsin is shown in Fig. 3. The optimal temperature for both the immobilized and the native trypsin is at 37°C , but the curves do not overlap with each other. Evidently, this indicates the effect of temperature on immobilized and on native trypsin was different. On the one hand, when the temperature is above 0°C , enzymes would become unstable and readily denature, whereas the immobilized enzymes are more stable against heat than native ones. On the other hand, as the temperature increases, the remaining cyclic carbonate groups on the support may further react with trypsin and part of the immobilized trypsin becomes inactivated. Therefore, the curve of immobilized trypsin does not agree with that of native trypsin.

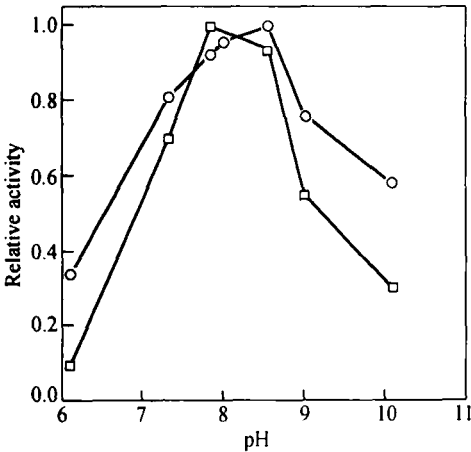


Fig. 2 Effect of pH on relative activity of trypsin
□ Immobilized trypsin
○ Native trypsin

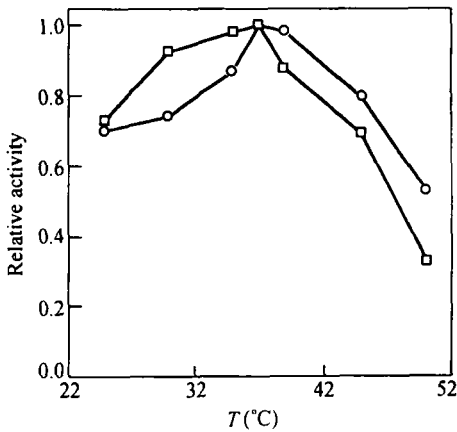


Fig. 3 Effect of temperature on relative activity of trypsin
□ Immobilized trypsin
○ Native trypsin

K_m
 K_m is the characteristic constant for the enzyme's catalytic reaction, thus a determination of the apparent Michaelis-Menten constant (K_m) was performed for native and immobilized trypsin. Lineweaver -Burk plots

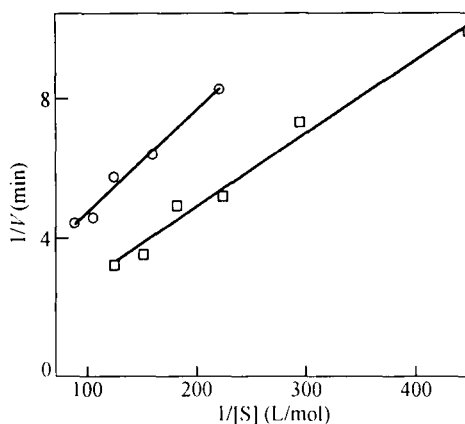


Fig. 4 Linearweaver-Burk plot for trypsin
 □ Immobilized trypsin
 ○ Native trypsin

obtained at various concentration of substrate are shown in Fig. 4. The native trypsin shows a K_m value of 15.2 mmol/L. In contrast to the native trypsin, immobilized trypsin exhibits a higher K_m value (30.7 mmol/L). The discrimination may be due to the limitation of substrate that diffuses into the supports^[10-12].

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