0273-2289/01/95/0011/$12.75

New Supports for Enzyme Immobilization Based on Copolymers of Vinylene Carbonate and Acrylamide

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Received December 1, 1999; Revised April 20, 2001; Accepted April 23, 2001

Abstract

In this study, a series of beadlike and hydrophilic supports containing reactive cyclic carbonate groups for enzyme immobilization were prepared via reverse-phase suspension copolymerization of the aqueous solutions of vinylene carbonate (VCA), acrylamide (AA), and *N*,*N*'-methylene bisacrylamide in paraffin oil. The supports were used as a matrix for immobilization of trypsin and showed a considerable capacity to couple with trypsin and reasonable retention of activity for the immobilized trypsin, depending on the immobilization conditions, such as the content of VCA structural units, reaction time, and pH of the medium.

Index Entries: Vinylene carbonate; trypsin; immobilization enzyme; support; acrylamide.

Introduction

Because the recovery and reuse of enzyme in aqueous solutions are limited, a great deal of interest has been focused on enzyme immobilization. Additionally, the immobilized enzymes have the advantages of being used in both batch and continuous modes and of being easily removed from the reactor. Hence, many techniques for enzyme immobilization have been developed in recent decades. Owing to the stable linkages between

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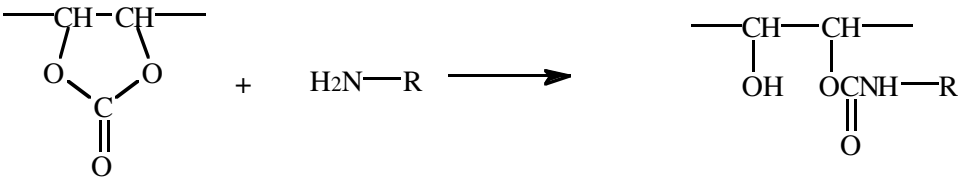

Scheme 1. Reaction of cyclic carbonate and amino group.

proteins and the supports, covalent binding of enzymes onto the supports has gained wide and intensive attraction. Supports activated by CNBr *(1)* and glutaraldehyde *(2)* are two of the most frequently used. However, both methods have problems, such as very complicated procedures for activation and removal of the toxic residuals, especially cyanide ions *(3)*.

It is already known that cyclic carbonate groups can readily combine with amino groups of biomolecules under very mild conditions (Scheme 1) *(4)*. Thus, several kinds of polymer supports containing carbonate groups have been prepared and evaluated previously *(4–10)*. Cyclic carbonate groups can be introduced onto the polymer supports by treatment of the polymers containing hydroxyl groups with ethyl chloroformate in anhydrous organic solvents *(4)* or even directly by copolymerization using vinylene carbonate as a comonomer *(5–9)*. In these regards, copolymerization of vinylene carbonate with other hydrophilic monomers should be a feasible way to prepare the reactive supports, since the reactive supports can be obtained directly after the polymerization and no further activation procedure is needed. Therefore, in the present study, using water as the solvent of comonomers, a series of beadlike hydrophilic supports containing cyclic carbonate groups for enzyme immobilization were synthesized via reverse-phase suspension polymerization of the aqueous solutions of VCA and acrylamide in paraffin oil. In addition, the performance of the supports coupled with trypsin in a variety of conditions was determined.

Materials and Methods

Reagents

VCA was prepared following our previous procedures *(11)*. Trypsin had a specific activity of 5.50 U/mg and was a product of Sino-American Biotechnology. All other reagents were of analytical grade.

Preparation of Microspheric Beads from VCA and AA

The microspheric beads were prepared via reverse-phase suspension copolymerization using paraffin oil as the continuous phase and water as the solvent for monomers. In a typical experiment, in a 100-mL three-necked flask equipped with a mechanical stirrer, N_2 inlet, and condenser, 50 mL of paraffin oil and a drop of triethanolamine monooleate were placed. An aqueous solution composed of 1.0 g of VCA, 1.5 g AA, 0.125 g of

N,*N*'-methylene bisacrylamide, 0.025 g of $K_2S_2O_8$, 0.025 g of $NaHSO_3$, and 10 mL of water was then added to the flask. The mixture was stirred at a fixed speed to produce suitable sized droplets. The suspension polymerization first was maintained in a water bath at 30°C to react for 6 h, then in a water bath at 60°C to react for another 4 h. Finally, the beads were filtered; rinsed with petroleum ether, water, and ethanol; and dried in a vacuum at 60°C to constant weight. Yield was >95%.

Immobilization of Trypsin onto Supports

The beads (30.0 mg) in a test tube were swollen in 3 mL of 0.2 *M* borate buffer, pH 8.0, for 12 h. Then the solution was removed from the tube using a syringe, and 3.00 mL of 3.00 mg/mL of trypsin solution in 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 except where otherwise mentioned. The trypsin solution was removed from the tube carefully using a syringe, and its absorbancy at 280 nm was measured. Occasionally, the trypsin solution was slightly turbid, and its absorbancy at 450 nm was used to eliminate the error caused by light scattering. Coupling yield and amount of proteins coupled to supports were determined according to Eqs. 1 and 2. The trypsin-coupled beads were washed four times with cold 0.2 *M* borate buffer, pH 8.0, and stored at 0°C.

$$\text{Coupling yield } (Y) = [(A_0 - A)/A_0] \times 100\% \quad (1)$$

$$\text{Amount of trypsin coupled} = [(A_0 - A)/(A_0 \times W)] \times C \times V \quad (2)$$

in which A_0 is the absorbancy at 280 nm of initial trypsin solutions, A is the absorbancy at 280 nm of trypsin solutions after immobilization, C is the concentration of initial trypsin solution (mg/mL), V is the initial volume of trypsin solution (mL), and W is the weight of the dried support (mg).

Measurement of Trypsin Activity

Native Trypsin Activity

An assay mixture consisting of 0.10 mL of 0.25% absolute ethanol solution of benzoyl-D,L-arginin-β-naphthylamide hydrochloride (BANA), 0.30 mL of 3.00 mg/mL of trypsin solution in 0.2 *M* borate buffer (pH 8.0), and 1.10 mL of 0.2 *M* borate buffer (pH 8.0) was prepared. The mixture was incubated for 15 min at 37°C. The reaction was stopped by transferring 0.5 mL of a 2 *M* HCl aqueous solution to the tube. To the reaction mixture, 1.00 mL of 0.1% $NaNO_2$ solution was added, and then the mixture was shaken vigorously for 2 min. After this treatment, 1.00 mL of 0.5% aqueous solution of ammonium sulfate was added during continued shaking for 1 min. Finally, 2.00 mL of 0.05% absolute ethanol solution of *N*-(1-naphtyl) ethylene diamine dihydrochloride was added to the tube. The solution was mixed, and then the tube was immersed in a 25°C water bath for 30 min. A blank was prepared in a similar fashion without the trypsin. The absor-

bancy (A_{560}) at 560 nm was read using the blank as a reference. Then activity was calculated as follows:

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

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

in which t is the reaction time (min), W_1 is the weight of trypsin (mg), and one BANA unit for trypsin activity is defined as the amount of trypsin needed when the value of absorbancy at 560 nm is raised 0.01/min.

Immobilized Trypsin Activity

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

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

$$\begin{aligned}\text{Retention of activity} &= \frac{\text{total units of immobilized enzymes}}{\text{total units of native enzymes}} \times 100\% \\ &= \frac{A_{560}}{t \times 0.01 \times V \times C \times G} \times 100\%\end{aligned} \quad (6)$$

$$\begin{aligned}\text{Relative activity} &= \frac{\text{total units of immobilized enzymes} \times 100\%}{\text{total units of native enzymes} - \text{total units remained in solution}} \\ &= \frac{A'_{560}}{t \times 0.01 \times V \times C \times G \times Y} \times 100\%\end{aligned} \quad (7)$$

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

Characterizations of Poly(VCA/AA) Beads

Degrees of swelling of the beads in aqueous solutions were determined according to Ding et al. *(8)*. Micrographs of the beads were taken with a scanning electron microscope (Hitachi X-650).

Estimation of Properties of Both Native and Immobilized Trypsin

Optimal pH Value

The activity of both native and immobilized trypsin in 0.2 *M* borate buffer at various pH values was measured at 37°C.

Optimal Temperature

The activity of native and immobilized trypsin in 0.2 *M* borate buffer at pH 8.0 at different temperatures was measured.

Determination of Michaelis Constant

The Michaelis-Menten constant K_m of native and immobilized trypsin was evaluated using different concentrations of BANA solution in 0.2 *M* borate buffer, pH 8.0, at 37°C, on the basis of the Lineweaver-Burk plot:

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

in which V is the initial reaction rate, V_m is the maximum reaction rate, $[S]$ is the concentration of the substrate, and K_m is the Michaelis-Menten constant.

Results and Discussion

Reverse-Phase Suspension Copolymerization of VCA and AA

Generally, VCA is sparingly soluble in water *(5–9)*. Thus, to prepare the supports containing cyclic carbonate groups, Mauz et al. *(5)* carried out copolymerization of VCA and *N*-vinyl pyrrolidone in dimethylformamide or other organic solvents. In the present study, we found that VCA could readily dissolve in aqueous solutions preliminarily containing AA, which makes it possible to use water as the solvent of comonomers and to perform reverse-phase suspension copolymerization in an oil phase. The merits of using water as the solvent for copolymerization are obvious: no pollution derived from organic solvent residuals, low cost for manufacture, and the possibility to employ a simple redox initiating system (i.e., $K_2S_2O_8$ and $NaHSO_3$) that provides the convenience of carrying out the copolymerization at low temperature. Because the decomposition of VCA and the other side reactions may occur in aqueous solutions at high temperature, and the viscosity of paraffin oil may decline steeply as the copolymerization temperature is raised, a low polymerization temperature is favorable for obtaining a high yield of beaded copolymer products. In the present study, the copolymerizations were first carried out at 30°C for several hours until most of the monomers had been consumed, and then at 60°C to complete all the monomers. As a result, the yields of copolymerizations were >90%, and the suspension polymerizations proceeded steadily even in the absence of polymeric stabilizers.

Accordingly, poly(VCA/AA) beads with various amounts of VCA content and crosslinker were prepared successfully. A representative scanning electron micrograph of the dry poly(VCA/AA) beads prepared is shown in Fig. 1. Note that most of the beads possessed a perfect globule shape. Smoothness of the surface may be an indication that no porosity existed in the beads. For attachment of trypsin, the dried beads above 60 meshes were collected and used as the supports.

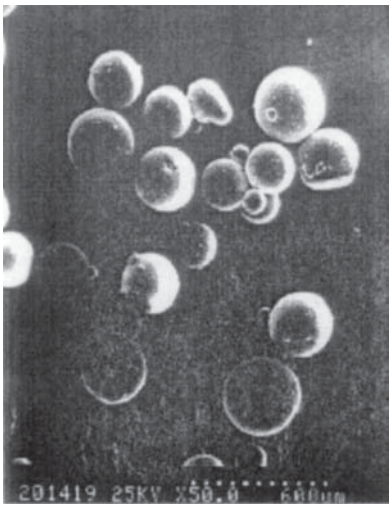

Fig. 1. Representative scanning electron micrograph of poly(VCA/AA) beads.

Table 1
Degree of Swelling of VCA/AA Copolymer Beads

Initial ratio of VCA:AA (g/g)	Degree of crosslinking (%)	Yield (%)	Degree of swelling (g/g)	Degree of swelling in pH 8.0 0.2 mol/L borate solution (g/g)
1:1	5	94	4.82	
1:2	5	96	6.14	
1:3	5	95	7.01	6.04
1:4	5	98	6.01	
1:3	10	96	5.01	
1:3	15	96	4.72	
1:3	20	95	2.94	

The degree of swelling of the beads was measured, and the results are given in Table 1. It can be seen that all the beads exhibited a suitable swellability in aqueous solutions, which enabled protein molecules to penetrate into the supports and react with cyclic carbonate groups in the supports. The degree of swelling of the beads was dependent on the amount of VCA structural units and the crosslinking agent. Increasing of the amount of VCA structural units and *N*,*N*'-methylene bisacrylamide (crosslinker) led to a decrease in the degree of swelling of the supports.

Influence of VCA Content on Immobilization

The results of binding trypsin onto the supports with different VCA contents are shown in Table 2. Because of the high yield of copolymerizations, the feed ratios of VCA and AA could be considered as the approxi-

Table 2
Influence of VCA Content on Immobilization of Trypsin[a]

VCA:AA (g/g)	Enzyme coupled (mg/g beads)	Coupling yield (%)	Specific activity of enzyme immobilized (U/g)	Retention of activity (%)	Relative activity (%)
1:1	232	77.2	203	12.3	15.9
1:2	210	70.1	235	14.2	20.3
1:3	171	57.1	244	14.8	25.9
1:4	162	54.0	223	13.5	25.0

[a]Immobilization conditions: 300 mg of dried support (5% crosslinking degree), 300 mL of enzyme solution in 0.2 *M* borate buffer (pH 8.0) with an enzyme concentration of 3.00 mg/mL, a temperature of 0°C, a reaction time of 24 h, and a specific activity of 5.50 U/mg of native enzyme.

mate composition of copolymers. The coupling yield and the amount of trypsin coupled to the matrix gradually increased with an increase in the VCA content, and simultaneously the specific activity and relative activity of the immobilized trypsin reached their maximal value at VCA:AA = 1:3. From the data in Table 1, one can see that all the supports at 5% of crosslinking degree possessed a good swelling performance in aqueous solutions. Consequently, the amount of trypsin coupled to the matrix was mainly dependent on the content of the VCA structural unit. However, the specific activity of immobilized trypsin did not increase with an increase in the amount of trypsin coupled to the supports (Table 2). The results may be owing to excess covalent linkages, which lead to the inactivation of trypsin, between trypsin and the matrix that contained a high amount of cyclic carbonate groups *(8)*. The reduction in the relative activity of the immobilized trypsin with an increase in coupling yield can be considered the evidence for this conclusion. Hence, to obtain promising results for enzyme immobilization, the VCA content in copolymer supports should be maintained in a suitable range by controlling feed ratios of comonomers in aqueous solutions.

Influence of Reaction Time on Immobilization

Table 3 shows the effect of reaction time on the amount of trypsin coupled to the beads and the specific activity of the immobilized trypsin. The immobilization proceeded slowly and approached a maximum after nearly 12 h. It is known that the reaction between cyclic carbonate groups and organic amine occurs very quickly in homogeneous solutions *(12,13)*. However, the same reaction that occurs on the supports may proceed much more slowly because the diffusions of large enzymes into the supports become the rate-limiting step in this case. In addition, the relative activity of the immobilized trypsin decreased with an increase in reaction time, although the specific activity of the immobilized trypsin increased. The

Table 3
Influence of Reaction Time on Immobilization of Trypsin onto Support[a]

Time (h)	Enzyme coupled (mg/g beads)	Coupling yield (%)	Specific activity of enzyme immobilized (U/g)	Retention of activity (%)	Relative activity (%)
1	51.2	17.0	119	7.21	42.4
2	101	33.8	185	11.2	33.1
4	116	38.8	196	11.9	30.6
8	138	45.9	216	13.1	28.5
12	169	56.3	235	14.2	25.3
24	171	57.1	244	14.8	25.9

[a]Immobilization conditions: 300 mg of dry support (5% crosslinking degree, VCA:AA = 1:3), 3.00 mL of enzyme solution in 0.2 *M* borate buffer (pH 8.0) with an enzyme concentration of 3.00 mg/mL, a temperature of 0°C, and a specific activity of 5.50 U/mg of a native enzyme.

Table 4
Effect of pH of Medium on Immobilization of Trypsin[a]

pH value	Enzyme coupled (mg/g beads)	Coupling yield (%)	Specific activity of enzyme immobilized (U/g)	Retention of activity (%)	Relative activity (%)
6.12	12.8	4.28	33.1	2.01	46.9
7.32	190	63.5	214	13.0	20.4
7.83	175	58.2	182	11.0	19.0
8.54	107	35.6	187	11.3	31.8
9.00	54.4	18.1	122	7.42	41.0

[a]Immobilization conditions: 300 mg of dry support (5% crosslinking degree, VCA:AA = 1:3), 3.00 mL of enzyme solution in 0.2 *M* borate buffer with an enzyme concentration of 3.00 mg/mL, a temperature of 0°C, a reaction time of 24 h, and a specific activity of 5.50 U/mg of native enzyme.

results can be ascribed to a crowding of protein molecules, which reduced the accessibility of substrates to the immobilized enzymes *(14)*.

Influence of pH of Medium on Immobilization

From the data in Table 4, it can be determined that the optimal pH of the medium for trypsin immobilization was 7.32. At lower pH, amino groups of proteins were protonated to a considerable extent and, therefore, were not effective as nucleophiles. At a higher pH, hydrolysis of cyclic carbonates increased greatly. In addition, the concentrations of $-NH_3^+$ and –COOH that were favorable to the combinations between cyclic carbonate groups and amino groups decreased *(12,13)*. Consequently, the coupling yield and the amount of trypsin coupled to the matrix decreased.

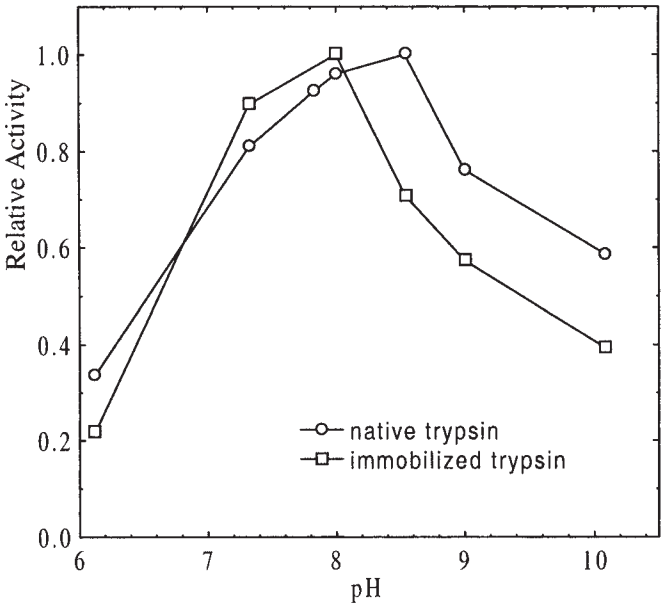

Fig. 2. Effect of pH on relative activity of trypsin at 37°C.

Comparison of Properties of Immobilized and Native Trypsin

Optimal pH

The relationships between the relative activity of trypsin (both native and immobilized) and pH of the medium are shown in Fig. 2. Both the native and immobilized trypsin were very sensitive to the pH of the reaction medium. The optimal pH for native trypsin was 8.5, and that for immobilized trypsin switched to 8.0. Since the immobilized trypsin in this study generally was prepared from the buffer solution at pH 8.0, it is reasonable to believe that the additional NH_2– groups would be produced from NH_3^+– groups according to ionization equilibrium when pH of the medium was raised. The new amino groups may further combine with the remaining cyclic carbonates on the supports and thus lead to the inactivation of the immobilized trypsin owing to excess covalent linkages with the supports. Therefore, the optimal pH for the immobilized trypsin was the same as the pH of the solution in which the immobilized trypsin was prepared.

Optimal Temperature

Figure 3 shows the relationships between the relative activity and reaction temperature for trypsin. The optimal temperature for both immobilized and native trypsin was at 37°C; however, the curves did not overlap each other. This indicates that the effects of temperature on both immobilized and native trypsin were different. Normally, enzymes may become unstable and readily be denatured when the temperature goes above 0°C,

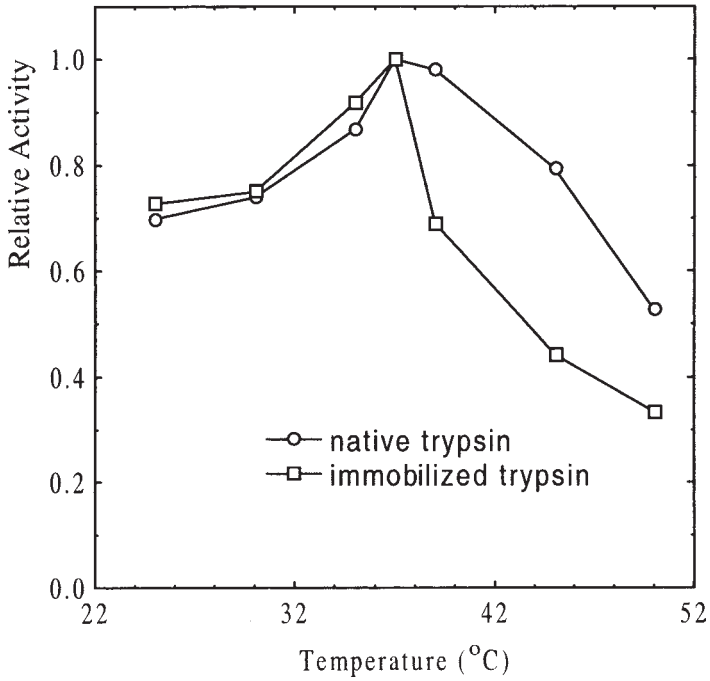

Fig. 3. Effect of temperature on relative activity of trypsin at pH 8.0.

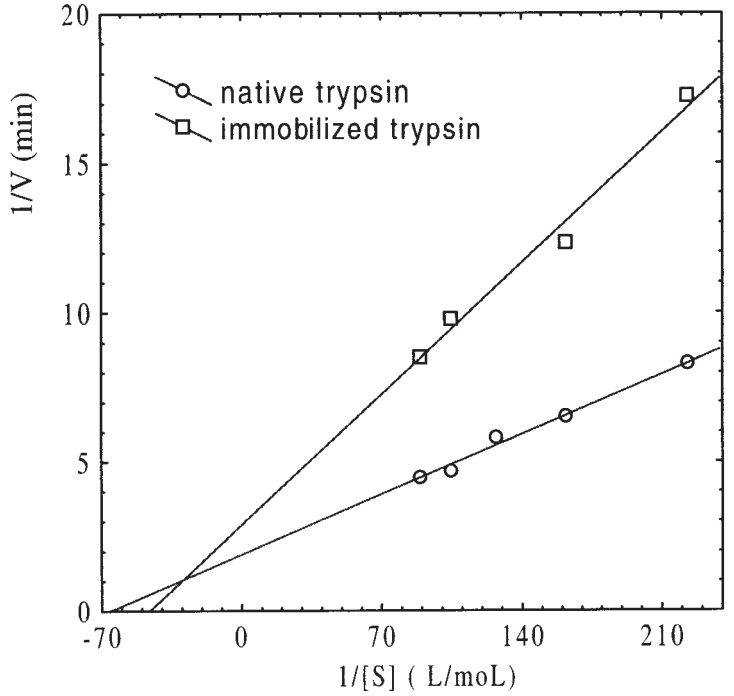

Fig. 4. Lineweaver-Burk plot for trypsin (37°C, pH 8.0).

whereas the immobilized enzymes are more stable against the heat. On the other hand, the remaining cyclic carbonate groups in the supports might further react with trypsin when the reaction temperature was raised, and thus the part of immobilized trypsin would be inactivated. Consequently, the curve of the immobilized trypsin was inconsistent with that of the native trypsin.

K_m Value

K_m is the characteristic constant for enzymes' catalytic reaction. Therefore, determination of the apparent Michaelis-Menten constant (K_m) was performed for the native and immobilized trypsin. Figure 4 shows the Lineweaver-Burk plots obtained at various concentrations of substrate. The K_m for the native trypsin was 15.2 mmol/L. By contrast, the immobilized trypsin showed a higher K_m (21.7 mmol/L). This discrimination may be owing to limitation of substrate diffusion onto the supports *(15,16)*.

Acknowledgment

This work was supported by the National Nature Sciences Foundation of China (grant no. 59973009).

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