Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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Online publication date: 20 September 1999

To cite this Article Ding, Lunhan , Li, Yue , Jiang, Yan , Cao, Zhe and Huang, Jiaxian(1999) 'PROPERTIES AND SYNTHESES OF COPOLYMERIC SUPPORT FOR ENZYMES IMMOBILIZATION FROM VINYLENECARBONATE AND POLY(VINYLENE ALCOHOL) N-METHYLOLACRYLAMIDE ETHER', Journal of Macromolecular Science, Part A, 36: 10, 1443 — 1457
To link to this Article: DOI: 10.1081/MA-100101607
URL: http://dx.doi.org/10.1081/MA-100101607

J.M.S.—PURE APPL. CHEM., A36(10), pp. 1443–1457 (1999)

PROPERTIES AND SYNTHESES OF COPOLYMERIC SUPPORT FOR ENZYMES IMMOBILIZATION FROM VINYLENE CARBONATE AND POLY(VINYLENE ALCOHOL) N-METHYLOLACRYLAMIDE ETHER

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Key Words: Vinylene Carbonate, Poly(vinylene alcohol), N-methyloacrylamide, Fixed Enzyme, Support, Trypsin

ABSTRACT

First, Poly(vinylene alcohol) (PVA) N-methylolacrylamide ether (E-PVA) was prepared, then dissolved it with vinylene carbonate(VCA) and water-soluble initiator in water. The solution was copolymerized in paraffin oil to yield hydrophilic beads containing cyclic carbonate groups. By attachment of trypsin, properties of the supports were determined and the result shows that amount of enzymes coupled to supports and specific activity of immobilized trypsin were related to content of VCA structure unit, reaction time, concentration of enzyme solution, etc.

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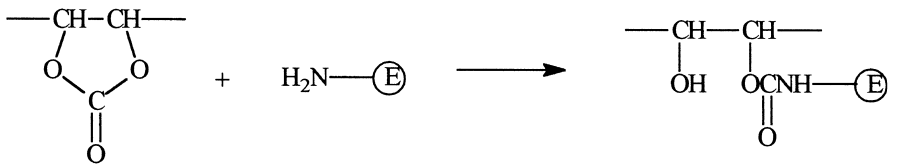


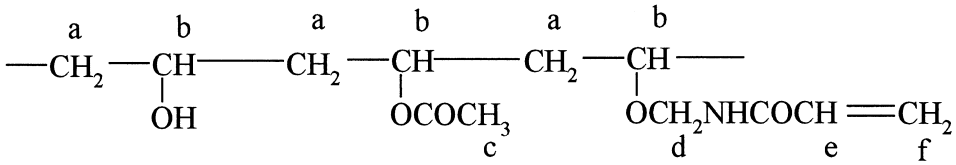
Scheme 1.

INTRODUCTION

Chains of poly(vinylene carbonate) (PVCA) contain cyclic carbonate groups as many as its structural units. It has been known that these cyclic carbonate groups can react with amino groups (Scheme 1) and thus be taken as effective functional groups for enzymes immobilization [1-9]. Generally cyclic carbonate groups on the polymer chain are stable under air atmosphere or in aqueous solution with neutral pH value at room temperature, thus the reactivity of supports prepared from vinylene carbonate can keep steady for much long time, and it will be convenient for practical use since activation of supports is not needed.

But due to its very hydrophobic property, PVCA itself cannot directly be used as the support of enzymes since hydrophobic materials may cause inactivation of enzymes as organic solvents may do [10-12]. Furthermore, supports swollen in aqueous solution will favor the diffusion of enzymes molecules into the supports and provide a comfortable microenvironment for enzymes. Therefore, the supports should not only contain cyclic carbonate groups, but also be hydrophilic.

Poly(vinylene alcohol) (PVA) is a usual water-soluble polymer and under weak acid condition it can combine with N-methylolacrylamide and yield PVA N-methylolacrylamide ether (E-PVA) as follows:



Scheme 2.

At a low degree of etherification, the reaction products are still water-soluble and, in particular, a part of side-groups of PVA bring acrylamide side groups which can lead to crosslinking reaction [13-14]. In this article, first PVA N-methylolacrylamide ether was prepared, then dissolved with VCA and water-soluble initiator in water. The solution was polymerized in paraffin oil to yield hydrophilic beads containing cyclic carbonate groups. By coupling with trypsin the properties of these supports were measured.

EXPERIMENTAL

Materials

PVA, 0588, was from Beijing Organic Chemical Factory. VCA was prepared as described in some references [15-16] . Trypsin, specific activity of 5.50 Unit/mg, and benzoyl-D,L-arginin-β-naphthylamide hydrochloride (BANA), both were products of Sino-American Biotechnology Company. All other reagents were of analytical grade.

Preparation of PVA N-methylolacrylamide Ether (E-PVA)

10 g of PVA was weighed and added into 100 ml of three-necked flask equipped with an electric stirrer and condenser, 18 ml of water was again added, and the flask was kept in a water-bath at 80°C and stirring was begun to dissolve PVA. After all PVA were dissolved, a solution was added which consisted of 10 g of N-methylolacrylamide, 0.7 g of NH_4Cl, 0.1 g hydroquinone, and 7 ml of water. Then, 0.8 ml of 85% phosphate acid was injected into the mixture. The reaction was kept in 80°C water bath for 2 hours. In order to wash out the remaining N-methylolacrylamide, the product was dissolved in water and precipitated repeatedly in acetone four times. Finally, the precipitation was dried in vacuum at room temperature to a fixed weight.

Synthesis of Copolymeric Beads of VCA and E-PVA

50 ml of paraffin oil and 2 drops of triethanol amine octadecenate were added to a 100 ml three-necked flask equipped with an electric stirrer, condenser, and inlet pipe of nitrogen. Furthermore, 2.5 g of PVA N-methyl-olacryamide ether was weighed and added to a 50 ml glass cup, then 10 ml of water was added to dissolve the polymer at room temperature. After the polymer was dissolved completely, 2.5 g of VCA was added and stirred continuously, till the

VCA was dissolved. To this mixture was added 2 ml of aqueous solution of 0.05 g $K_2S_2O_8$ and 2 ml of aqueous solution of 0.05 g $NaHSO_3$, the solution was mixed vigorously by stirring, then immediately transferred into the flask. The stirrer was turned on and the speed controlled to produce a suitable drop size. The flask first was kept in a 30°C water bath to react for 6 hours, and at 60°C for 4 hours. The beads were isolated by filtration, washed with petroleum ether, then washed with water and ethanol and dried in vacuum at 60°C for 24 hours. The yield was 77%.

Immobilization of Trypsin onto Support

The beads (accurately 30.0 mg) in a test tube were swollen in 3 ml of 0.2 M borate buffer at pH 8.0 for 12 hours. The solution was then taken out of the tube by a syringe and 3.00 ml of 3.00 mg/ml trypsin solution in 0.2M borate buffer at pH 8.0 was added to the tube. The suspension was incubated in an ice-water bath for 24 hours except where otherwise mentioned. The trypsin solution was taken out of the tube by a syringe and its absorbency at 280 nm was measured. Sometimes the trypsin solution was slightly turbid and its absorbency at 450 nm was read to eliminate the error caused by light scattering. The coupling yield and amount of proteins coupled to supports were determined according to Equations 1 and 2. The beads coupled by trypsin were washed with a cold 0.2M borate buffer at pH 8.0 for four times, then kept in a refrigerator.

$$\text{Coupling Yield (Y)} = \frac{A_o - A}{A_o} \tag{1}$$

$$\text{Amount of Protein coupled} = \frac{A_o - A}{A_o \times W} \times C \times V \tag{2}$$

where A_0 is the absorbency at 280 nm of initial typsin solutions, A is the absorbency at 280 nm of trypsin solutions after immobilization, C is concentration of trypsin solution (mg/ml), V is volume of trypsin solution (ml), W is weight of the dried support (mg).

Measurement of Activity of Trypsin

Measurement of Activity of Native Trypsin [17]

To a suitable test tube was added 0.10 ml of 0.25 percent benzoyl-D,L-arginin-β-naphthylamide hydrochloride (BANA) in absolute ethanol, 0.30 ml of

3.00 mg/ml trypsin solution in 0.2 M borate buffer at pH 8.0, and 1.10 ml of 0.2 M borate buffer at pH 8.0, the mixture was mixed and incubated for exactly 15 minutes at 37°C. The reaction was stopped by transferring 0.5 ml of 2 M HCl to the tube. 1.00 ml of 0.1 percent $NaNO_2$ solution was added and shaken vigorously to react for 2 minutes. After this treatment, 1.00 ml of 0.5 percent ammonium sulfamate, was added and coninuously shaken for 1 minute. Then, 2.00 ml of 0.05 percent N-(1-naphtyl) ethylene diamine dihydrochloride in absolute ethanol was added to the tube, mixed, and immersed in a 25°C water bath for 30 minutes. We made up a blank in a similar fashion but omitted the trypsin. The absorbency (A_{560}) at 560 nm was read using the blank as a reference. Then

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

$$\text{Specific Activity} = \frac{\text{total units}}{\text{mg protein}} = \frac{A_{560}}{t \times 0.01 \times W_1} \tag{4}$$

where t is reaction time (minute), W_1 is the weight of trypsin (mg), one BANA unit is defined as the amount of trypsin needed when the value of absorbency at 560 nm was raised 0.01 per minute.

Determination of Activity of Immobilized Trypsin

To a test tube containing immobilized trypsin from 30.0 mg dry supports, was added 1.40 ml of 0.2 M borate buffer at pH 8.0, 0.10 ml of 0.25 percent BANA in absolute ethanol. The solution was mixed and the tube was placed in a water bath at 37°C to react for exactly 15 minutes.The following steps were the same as those of native trypsin measurement (Equations 5-7).

$$\text{Specific Activity of immobilized trypsin} = \frac{A'_{560}}{0.01 \times t \times W} \tag{5}$$

$$\begin{aligned}\text{Retention of Activity} &= \frac{\text{total units of immobilized enzymes}}{\text{total units of native enzymes}} \times 100\% \\ &= \frac{A'_{560}}{t \times 0.01 \times V \times C \times G} \times 100\%\end{aligned} \tag{6}$$

$$\text{Relative Activity} = \frac{\text{total units of immobilized enzymes}}{\text{total units of native enzymes - total units of superate}} \times 100\% = \frac{A'_{560}}{t \times 0.01 \times V \times C \times G \times Y} \times 100\% \quad (7)$$

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

Measurement of the Water Absorbency of Beads

The dry beads were placed in deionized water (or other solutions) for 24 hours. The beads then were weighed wet after blotting to remove the surface water. The absorbency of beads was calculated as follows:

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

Estimation of Properties of Both Native and Immobilized Trypsin

Optimal pH value

The activity of native and immobilized trypsin in 0.2 M borate buffer with different pH were measured at 37°C.

Optimal Temperature

The activity of native and immobilized trypsin in 0.2 M borate buffer, pH 8.0 at different temperature were measured.

Determination of the Michaelis Constant

The Michaelis-Menten constant K_m of native and immobilized trypsin was evaluated using a BANA solution (ranging from 0.25% to 0.625%) in 0.2 M borate buffer, pH 8.0 at 37°C, on the basis of the Lineweaver-Burk plot (Equation 9):

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

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

RESULTS AND DISCUSSION

Preparation of copolymeric beads of VCA and E-PVA

Actually, solubility of VCA in water was very low, but we found that if water contains other water-soluable organic compounds, its solubility would be greatly improved. This property gives a great convenience for modifying ratios of monomers in the copolymerization system and makes it possible to carry out copolymerization in aqueous solution rather than in organic solvents.

PVA N-methylolacryamide ether (Scheme 2) was prepared first before the preparation of copolymeric beads. Its spectrum of ^{1}H-NMR is given in Figure 1. The etherification degree of PVA N-methylolacrylamide ether was determined to be about 5%. This macromolecule crosslinking agent and VCA were dissolved together in water and polymerized. The results of polymerization are listed in Table 1. From Table 1 it can be seen that all the products exhibited a good hydrophilicity. Although exact composition of copolymers could not be measured, it can be concluded that with increasing the VCA/E-PVA initial ratio, the content of the VCA unit contained in copolymer increased, since the corresponding water absorbency decreased.

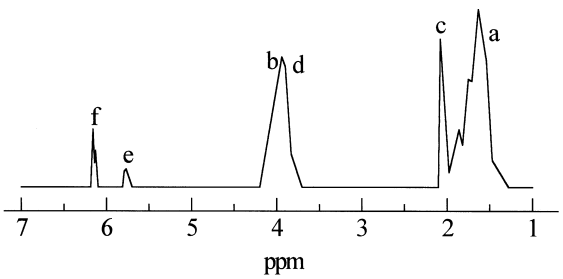


Figure 1. ^{1}H-NMR Spectrum of PVA N-methylolacrylamide ether.

TABLE 1. Water Absorbency of VCA and E-PVA Copolymer Beads

Initial ratio of VCA and E-PVA (g/g)	3/7	2/3	1/1	3/2
Water absorbency (g/g)	4.41	3.95	3.10	2.71

Influence of Initial Ratio of VCA and E-PVA on the Immobilization

Table 2 shows the effect of initial ratio of VCA and E-PVA on the immobilization of trypsin. It is evident that the coupling yield, as well as specific activity of immobilized trypsin, depended on the initial ratios of VCA and E-PVA, with an optimal at 2/3. This fact may be explained in the following way. With increasing the VCA content at the beginning, the content of cyclic carbonate groups increased and the support could combine more trypsin. However, when the ratio was much higher, the hydrophilicity of the support decreased and the support could not swell very well, thus some of the cyclic carbonate groups were wrapped up and could not react with trypsin [18]. Therefore, the amount of trypsin coupled and specific activity of trypsin immobilized decreased again, although the support contained much more cyclic carbonate groups.

TABLE 2. Influence of Initial Ratios of VCA and E-PVA on the Immobilization of Trypsin

VCA / E-PVA (g/g)	Enzyme Coupled (mg/g beads)	Coupling Yield (%)	Specific Activity of Enzyme Immobilized (U/g)	Retention of Activity (%)	Relative Activity (%)
3 / 7	42.6	14.2	58.0	3.51	24.8
2 / 3	88.2	29.4	154.7	9.38	31.9
1 / 1	92.0	30.7	139.3	8.45	27.5
3 / 2	61.0	20.3	97.3	5.31	26.1

Immobilization conditions: dry support: 300mg; enzyme solution: 3.00mL in 0.2M borate buffer at pH8.0, enzyme concentration 3.00 mg/mL, temperature 0 C; reaction time 24 hr; specific activity of native enzyme: 5.50 U/mg.

TABLE 3. Effect of Reaction Time on the Immobilization of Trypsin onto the Support

Time (hr)	Enzyme Coupled (mg/g beads)	Coupling Yield (%)	Specific Activity of Enzyme Immobilized (U/g)	Retention of Activity (%)	Relative Activity (%)
1	39.3	13.1	100	6.06	46.3
2	36.0	12.0	98.9	6.00	50.0
4	73.2	23.4	116.4	7.05	30.2
8	54.9	18.3	131.6	7.97	51.5
12	84.9	28.3	175.1	10.6	37.4
24	88.2	29.4	154.7	9.38	31.9

Immobilization conditions: dry support (VCA/E-PVA=2/3,) 300mg; enzyme solution: 3.00mL in 0.2M borate buffer at pH8.0, enzyme concentration 3.00 mg/mL, temperature 0C; specific activity of native enzyme: 5.50 U/mg.

Influence of Reaction Time on the Immobilization

Table 3 shows the effect of reaction time on the amount and specific activity of trypsin coupled to the beads. The immobilization nearly reached a maximum after 12 hours. It is known that cyclic carbonate groups of chain can react with amino groups very quickly in homogenous solution [19-20], but the reaction between cyclic carbonate groups of the support and trypsin molecules was limited by diffusion of trypsin into the support. Thus, combination of the support and trypsin needed much more time.

Moreover, with increasing the reaction time, relative activity of immobilized trypsin decreased although specific activity of the immobilized trypsin increased. This phenomena may be due to the fact that with increasing the amount of trypsin coupled, part of trypsin molecules may be covered by another part of trypsin molecules and cannot react with the substrate.

Influence of Trypsin Concentration on the Immobilization

As shown in Table 4, with increasing the trypsin concentration in the immobilization mixtures, both amount of enzymes coupled onto the matrix and specific activation of immobilized trypsin was raised, but simultaneously, coup-

TABLE 4. Effect of Trypsin Concentration on the Immobilization

Concentration of Trypsin (mg/mL)	Enzyme Coupled (mg/g beads)	Coupling Yield (%)	Specific Activity of Enzyme Immobilized (U/g)	Retention of Activity (%)	Relative Activity (%)
2	47.0	23.5	111.1	10.1	43.0
3	95.7	31.9	140.2	8.50	26.6
5	92.8	18.6	146.2	5.31	28.6
6	126.5	21.1	168.2	5.09	24.2

Immobilization conditions: dry support (VCA/E-PVA=1/1,) 300mg; enzyme solution: 3.00mL in 0.2M borate buffer at pH8.0, temperature 0°C; reaction time 24 hours; specific activity of native enzyme: 5.50 U/mg.

ling yield and relative activity decreased. Dramatic decreasing activity at high enzyme loaded indicates that enzymes becomes less efficient. This inefficiency may be due to a crowding of protein molecules on the support which reduces the accessibility of the substrates onto immobilized enzymes.

Influence of pH of the Reaction Medium on the Immobilization

According to the data shown in Table 5, optimal pH for trypsin immobilization was at pH 7.83. At lower pH, amino groups on the surface of protein were protonated and therefore, not effective as neucleophiles. At high pH value hydrolysis of cyclic carbonate groups would completely increase; furthermore, concentration of $-NH_3^+$and -COOH which have catalytic effect on the combination of the support and trypsin [19-20] decreased, consequently, the coupling yield and amount of trypsin coupled to the matrix decreased.

Comparison of Properties of the Immobilized and Native Trypsin

Optimal pH

Figure 2 shows the relationship between relative activity of trypsin (including both native and immobilized) and pH of the medium. Both native and immobilized trypsin are very sensitive to the pH of the reaction medium. The optimal pH for native trypsin is pH 8.5, and that for immobilized trypsin is pH 8.0. The difference may be due to the positive charges of enzymes in aqueous solution. It has been known that the isoeletric point (IEP) of trypsin is at pH 10.5,

TABLE 5. Effect of pH of the Medium on the Immobilization of Trypsin

pH	Enzyme Coupled (mg/g beads)	Coupling Yield (%)	Specific Activity of Enzyme Immobilized (U/g)	Retention of Activity (%)	Relative Activity (%)
6.12	35.2	11.7	22.4	1.36	11.6
7.32	66.2	22.1	110	6.67	30.2
7.83	92.4	30.8	141	8.54	27.7
8.00	92.0	30.7	139	8.45	27.5
8.54	33.6	11.2	54.0	3.27	29.2
9.00	5.86	1.95	26.7	1.61	83.0
10.09	11.9	3.98	30.4	1.84	46.3

Immobilization conditions: dry support (VCA/E-PVA=1/1,) 300mg; enzyme solution: 3.00mL in 0.2M borate buffer , enzyme concentration 3mg/mL, temperature 0°C; reaction time 24 hourr; specific activity of native enzyme: 5.50 U/mg.

so molecules of trypsin in pH neutral aqueous solution brings positive charges, which are the same as charges of substrates (BANA) in aqueous solution. Due to the repulsion between enzymes and substrates caused by the same electric charges, both of them cannot combine together at lower pH value of medium, but in aqueous solution with higher pH value positive charges will be weakened, enzymes and substrates can combine and result in the reaction. As far as the immobilized trypsin, part of the amino groups have reacted with cyclic carbonate groups, and naturally part of H_3N^+- change to be H_2N- according to electrolytical equilibrium, positive charges in the supports thus become less, and optimal pH for immobilized trypsin decrease slightly.

Optimal Temperature

Figure 3 shows the relationship between relative activity and reaction temperature for trypsin. The optimal temperature for both immobilized and native trypsin was at 37°C, but the curves did not overlap each other. Evidently, it indicates that the temperature effect on both immobilized and native trypsin were different. Generally, when the temperature was above 0°C, enzymes will become unstable and readily be denatured, whereas immobilized enzymes are more stable against the heat than a native one. Meanwhile, when the temperature

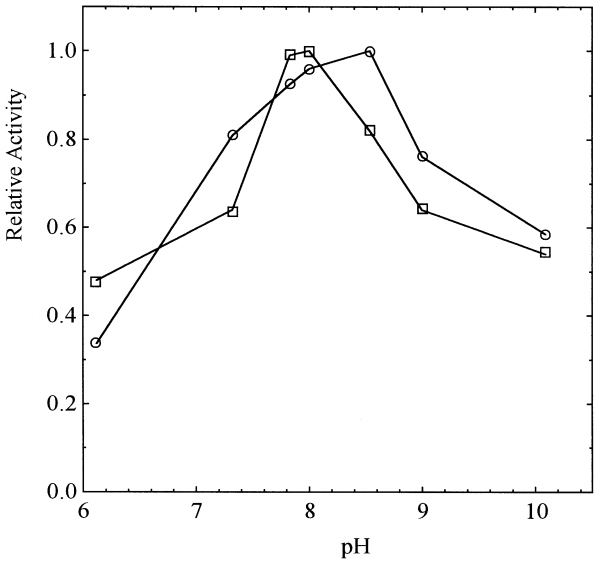


Figure 2. Effect of pH on relative activity of trypsin. □: immobilized trypsin, ○: native trypsin.

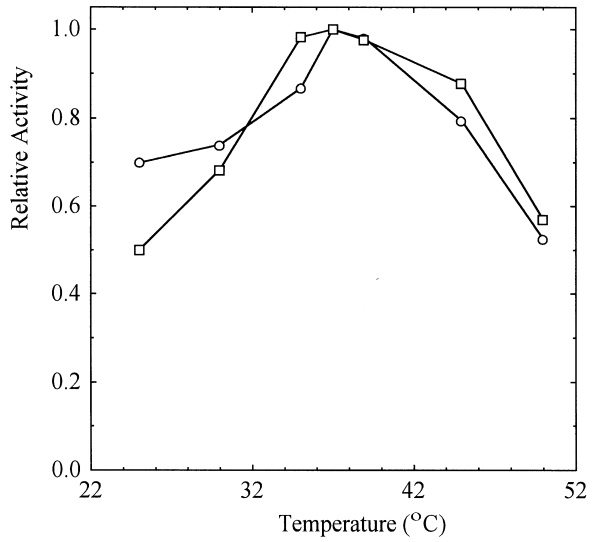


Figure 3. Effect of temperature on relative activity of trypsin. □: immobilized trypsin, ○: native trypsin.

increased, the remaining cyclic carbonate groups in the support might further react with trypsin and caused a part of immobilized trypsin to be inactivated. Therefore, the curve of immobilized trypsin did not act as native trypsin did.

K_m

K_m is the characteristic constant for enzymes catalytic reaction, thus determination of the apparent Michaelis-Menten constant (K_m) was performed for both native and immobilized trypsin. Figure 4 shows a Lineweaver-Burk plots obtained at various concentration of substrate. The native trypsin showed a K_m value of 15.2 mmol/L. In contrast to the native trypsin, immobilized trypsin exhibited higher K_m value (23.4 mmol/L). The discrimination may be due to limitation of substrate diffusion into the supports [21-23].

CONCLUSION

It was found in the experiment that VCA could easily dissolve in water together with E-PVA although it almost could not dissovle in water alone. Thus,

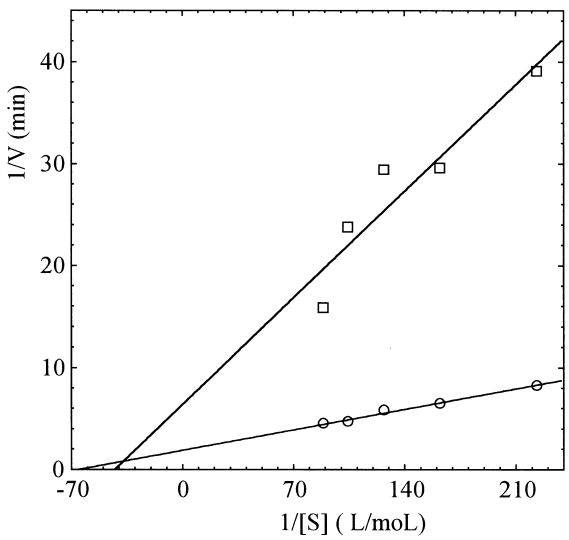


Figure 4. Linearweaver-Burk plot for trypsin. □: immobilized trypsin, ○: native trypsin

aqueous solution of both were copolymerized in paraffin oil by means of reverse-phase suspension polymerization and yielded a new hydrophilic support for enzyme immobilzation. By attachment of trypsin, properties of the supports were determined. The amount of enzymes coupled to supports and specific activity of immobilized trypsin were related to content of VCA structure unit, reaction time, concentration of enzyme solution, etc.

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Received November 30, 1998
Revision received February 28, 1999