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Activity of Carboxypeptidase A Bound to a Modified Cellulosic Matrix

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ABSTRACT

Carboxypeptidase A immobilized on acid chloride of oxidized cellulose showed the following features: (a) as indicated by the linearity of reaction kinetics, the immobilized enzyme action is not diffusion controlled; (b) greater flow rates are achievable with less clogging during continual usage since the enzyme is attached to a porous screen; (c) ease of handling; and (d) no apparent electrostatic interaction with the support material that is uncharged. The immobilized enzyme retained 60% of the original activity. The half-life of free enzyme was only 20 min, whereas for immobilized enzyme it was enhanced up to 2 h 48 min. It could be recovered and repeatedly used.

Index Entries: Carboxypeptidase A, activity of; carboxypeptidase A, binding of to a modified cellulose matrix; solid supported enzymes, preparation and properties of; immobilized enzyme action; enzyme derivatives, chemical nature, stability, and kinetic behavior of; oxidized cellulose.

INTRODUCTION

During the past few years there has been considerable interest in the preparation and properties of the solid supported enzymes (*1–5*). Immo-

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bilization of enzymes and other biochemicals is of interest because of research and industrial applications of the solid-phase products (*6*). Covalent coupling has been the most popular approach to immobilization, presumably because of the accessibility and stability of the bound-enzyme product. Usually, the carboxyl, phenolic, or amino groups on the enzyme surface are reacted with a solid support. Studies on water-insoluble derivatives of several enzymes, in which the biologically active protein is covalently bound to a high-molecular-weight support material, have shown that the chemical nature, stability, and kinetic behavior of the enzyme derivative are generally markedly affected upon immobilization (*7–9*).

Enzymes have been insolubilized by covalent attachment to various organic polymers (*10–15*) and cellulose derivatives (*16–18*). The work in our laboratory on oxidized cellulose (*19*) has indicated that it can form a convenient support whose biodegradability (*20*) in solutions can be controlled by such factors as degree of oxidation and pH of solution. In the present investigations, preparation of acid chloride of oxidized cellulose and its coupling to carboxypeptidase A (CPA) has been described. On oxidizing cellulose with periodate, the glucose moieties in the cellobiose units (A) are converted into dialdehydes (B) (*21*). These dialdehydes on treatment with sodium chlorite give the acid of oxidized cellulose (C), which on further treatment with thionyl chloride is converted into its chloride (D) (Scheme 1).

The aim of the present work was to prepare and characterize water-insoluble derivatives of CPA that would retain its native enzymatic activity for longer periods of time. The conditions for optimal recovery of enzymatic activity and of immobilized protein were determined.

MATERIALS AND METHODS

Cellulose powder was purchased from E. Merck, Germany, and NCbz-glycyl-L-phenylalanine was obtained from Sigma, USA. All other chemicals were of BDH analytical reagent grade.

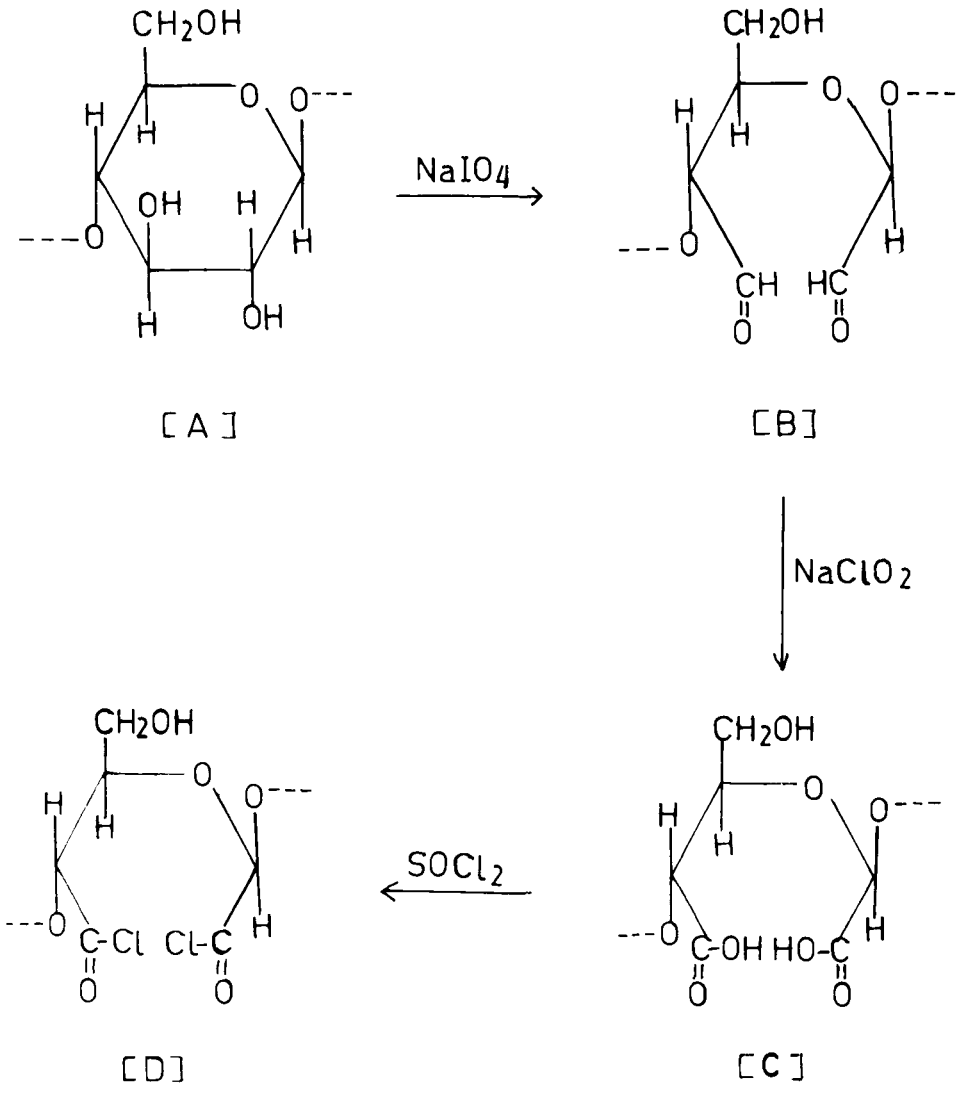
Preparation of Acid Chloride of Oxidized Cellulose

Preparation of Oxidized Cellulose

Oxidized cellulose was prepared according to the method of Nevell (*21*) and has already been used for CPA immobilization in our laboratory (*19*).

Acid of Oxidized Cellulose

Acid of oxidized cellulose was obtained by treating oxidized cellulose with sodium chlorite. To prepare 100 mL of 0.2*M* sodium chlorite solution, 1.81 gm of sodium chlorite was dissolved in about 75 mL of water in a volumetric flask. Exactly 20 mL of 5*M* acetic acid was added,



Scheme I. Preparation of acid chloride of oxidized cellulose from cellulose

and the mixture was made up to the mark with water. The solution was used at once because of its instability.

The oxidized cellulose (1 g) was treated for 72 h with 0.2*M* sodium chlorite solution (50 mL) in a thermostat at 20°C. It was then filtered out, washed with 0°C water, steeped for 30 min in 0.1*N* HCl at 0°C, and freed from acid by steeping it in numerous portions of 0°C water for 24 h. Finally, it was dried either in a vacuum desiccator over calcium chloride or directly by exposure to air.

Acid Chloride of Oxidized Cellulose

Acid chloride of oxidized cellulose was formed by the action of thionyl chloride upon the acid. It may be noted that both the by-products are

gaseous. In practice, thionyl chloride in excess (20–75%) of the theoretical quantity was used. Some of this was volatilized with the gaseous by-products, and the remainder was easily recovered by fractional distillation.

The acid of oxidized cellulose (10 g) was taken with 12 g (8 mL) of redistilled thionyl chloride in a 250-mL round-bottomed flask. The flask was fitted with a reflux condenser carrying a $CaCl_2$ guard tube. The flask was heated on a waterbath with occasional stirring for 1 h or until the evolution of HCl gas and SO_2 almost ceased. The reaction mixture was cooled and transferred cautiously to a Claisen flask condenser and a receiver. The excess $SOCl_2$ (bp 77°C) was distilled off slowly, and the distillation continued until the temperature rose rapidly to about 120°C; this ensured that all the $SOCl_2$ was removed. The acid chloride of oxidized cellulose formed was immediately used for CPA immobilization.

Immobilization of Carboxypeptidase A

About 200 mg of the carrier was equilibrated with 5 mL of CPA solution (2.1 mg/mL) in 0.05*M* acetate buffer at 2°C at various pHs, (4–8) for periods varying from 2 to 8 h. The mixture was stirred occasionally. Protein uptake by the carrier was followed by determining the protein concentration spectrophotometrically in the supernatant. The immobilization was found to be maximum at pH 6.5 (Fig. 1) for a fixed period of time (Table 1). The product was washed with 0.05*M* acetate buffer, pH 6.5, and distilled water, in succession. The amount of enzyme coupled was estimated by spectrophotometrically determining the protein in filtrate plus washings and subtracting it from total protein taken. The enzyme activity was determined by colorimetric method (*22*). Twenty milligrams of immobilized sample was taken for assaying.

Isolation and Purification of Carboxypeptidase A

Carboxypeptidase A was purified from goat pancreas, as described by Dua and Srivastava (*23*).

Assay for Carboxypeptidase A Activity

Colorimetric Method

The CPA activity was determined by the method of Folk and Gladner (*22*), except that the assay mixture was incubated at 50°C for 10 min. The absorbance of the ninhydrin colored product of the released amino acid was read at 565 nm against a blank. The specific activity was expressed in enzyme units/mg proteins, one enzyme unit being equal to one μmol of amino acid liberated/min under assay conditions. A standard curve was prepared using L-phenylalanine.

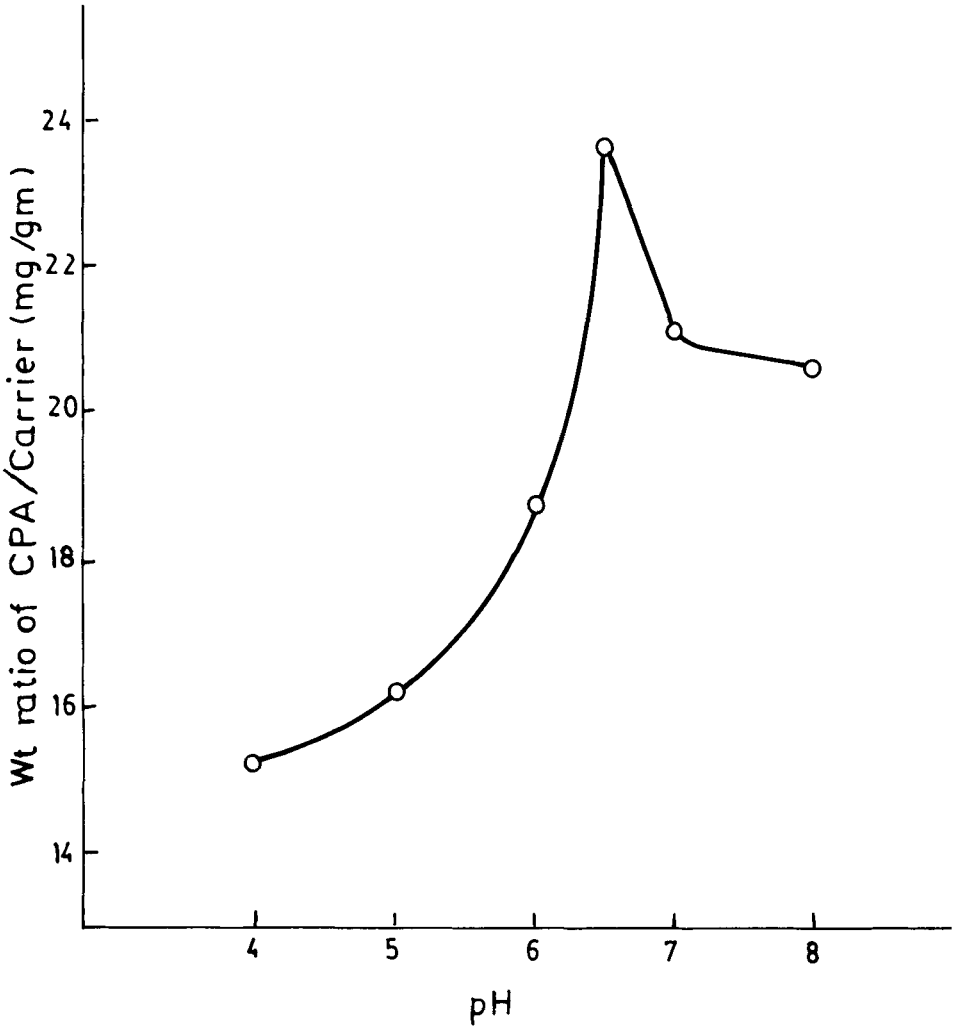


Fig. 1. Optimal pH for immobilization of CPA; 5 mL of CPA (protein: 2.1 mg/mL) in 0.05*M* acetate buffer was stirred with 200 mg of carrier for 6 h at 2°C at various pHs and the fixed CPA determined.

TABLE 1
Effect of Time on Degree of Protein Coupling and Specificity of Immobilized Carboxypeptidase A

	Acid chloride of oxidized cellulose	
Coupling time, h	Protein, mg/g	Specific activity, %
2	16.9	63.3
4	20.2	61.2
6	23.7	60.5
8	23.9	60.4

Determination of Optimum pH and Optimum Temperature

For optimum pH, aliquots of free (0.1 mL) and immobilized enzyme (20 mg) were incubated for 10 min at 50°C with 0.025*M* substrate at different pHs, and CPA activity was determined from released amino acid.

For optimum temperature, aliquots of free (0.1 mL) and immobilized enzyme (20 mg) were incubated for 10 min with 0.025*M* substrate in Tris-HCl buffer, pH 7.5, at different temperatures; released amino acid was determined with ninhydrin reagent.

Reusability of the Immobilized Enzyme

Twenty milligrams of immobilized sample was taken and its enzyme activity determined. The carrier was recovered by filtration and reused after 24 h. The process was repeated several times to check reusability of the immobilized enzyme.

Determination of the Michaelis–Menten Constant (K_m) at Different Ionic Strengths and Half Life

The K_m values of immobilized and free enzymes at different ionic strengths were determined by the method of Lineweaver and Burk (*24*) by plotting $1/v$ against $1/s$.

For half-life, the immobilized enzyme (20 mg) was suspended in acetate buffer, pH 6.5, and incubated at 50°C. Every 30 min a sample was withdrawn and its activity determined. Similar experiments were repeated with the native enzyme, samples of which were withdrawn every 5 min.

RESULTS AND DISCUSSION

Screening of different supports for immobilizing CPA was carried out in order to choose suitable supports for immobilization. The acid chloride of oxidized cellulose is very reactive and is hydrolyzed in the presence of water, but the hydrolysis is retarded at lower temperatures. In the present study, the maximum immobilization for CPA was achieved with 6 h contact time at pH 6.5 in 0.05*M* acetate buffer (Fig. 1), whereas the maximum immobilization for other supports, i.e., oxidized cellulose (*19*), copolymer styrene–maleic anhydride (*25*), and copolymer ethylene–maleic anhydride (*26*), tried in our laboratory, was at pH 6.0 in 0.05*M* acetate buffer. As pH increases, the amino groups of the enzyme become more reactive toward the support, and the immobilized enzyme retains 60% of the original activity (Table 1). The lowering of specific activity might be a result of rigidity and hindered accessibility of the enzyme to the substrate.

The native and immobilized enzymes showed optimal activity at pH 7.5, as seen in Fig. 2. The pH for optimal activity of the immobilized en-

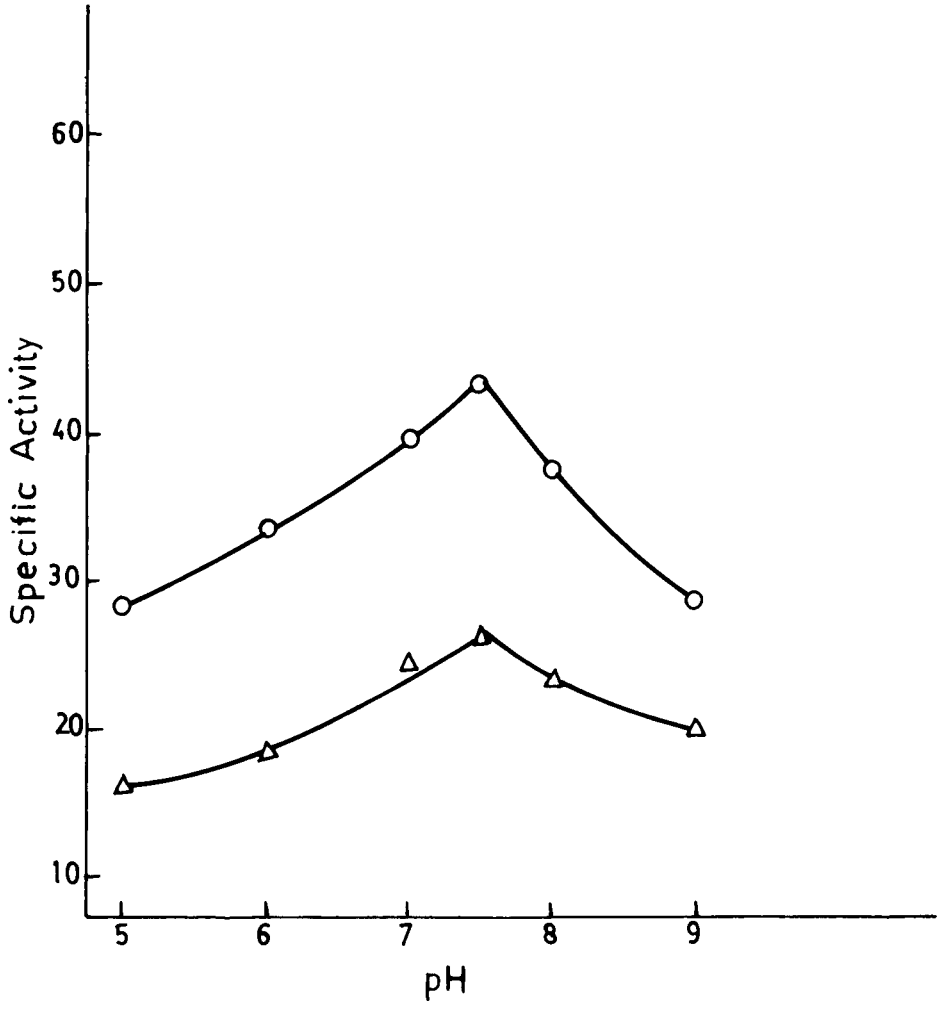


Fig. 2. Determination of optimum pH for CPA activity. Aliquots of free (0.1 mL) and immobilized enzyme (20 mg) were incubated with 0.025*M* substrate of different pHs for 10 min at 50°C in a waterbath and CPA activity was determined from released amino acid; ○, native enzyme; △, immobilized enzyme.

zyme could be influenced by microenvironments, such as chemical modification of enzyme, diffusional effects, and steric factors, which generally shift the pH activity profile to either side of the native enzyme. However, in our case, the immobilized and solubilized enzymes have optimal activity at the same pH.

The optimal temperature for native and immobilized enzymes was 50°C (Fig. 3). The activity increase between 30 and 50°C for the bound enzyme is not as large compared to soluble enzyme. Exposure of soluble and bound enzyme to increasing temperatures above 50°C caused a more rapid inactivation of the soluble enzyme.

The half-life of native enzyme was 20 min, whereas that of immobilized enzyme was enhanced to 2 h 48 min (Fig. 4). The enzyme is thus

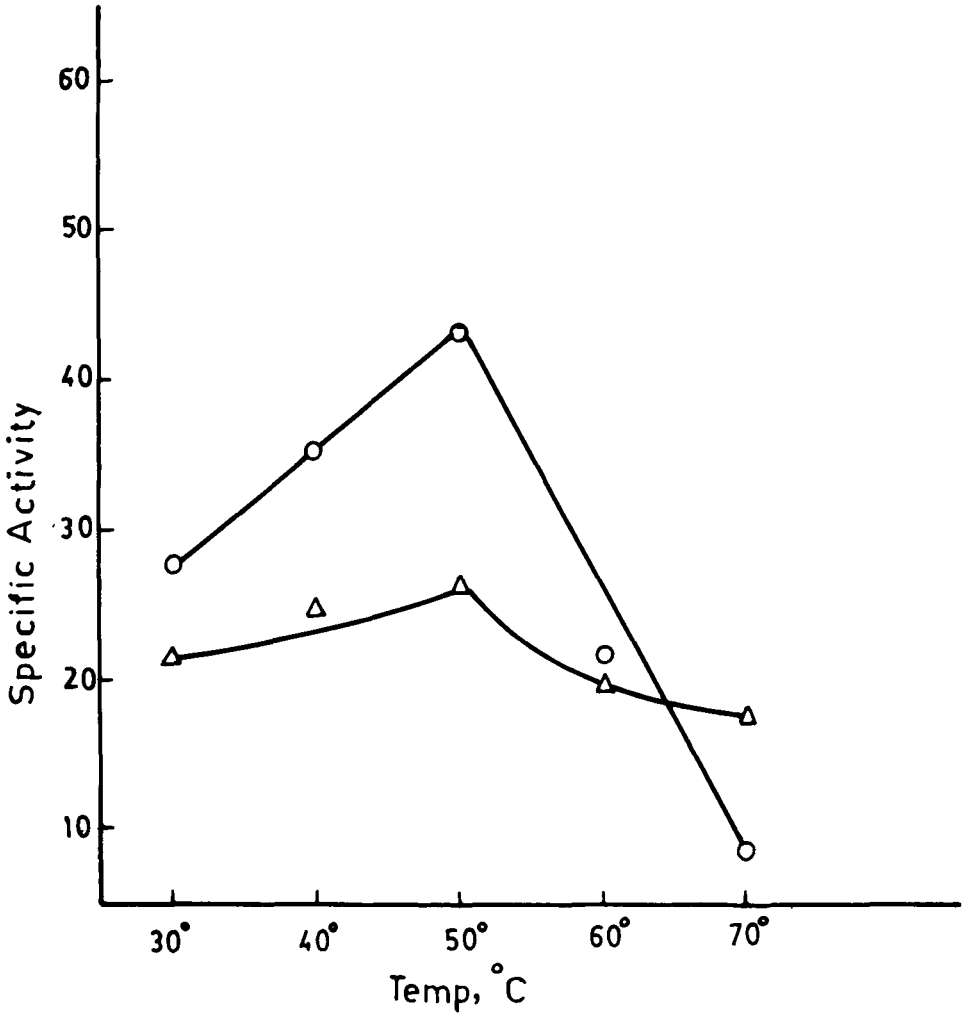


Fig. 3. Determination of optimum temperature for CPA activity. Aliquots of free (0.1 mL) and immobilized enzyme (20 mg) were incubated for 10 min with 0.025*M* substrate at different temperatures and amino acid released determined with ninhydrin reagent; ○, native enzyme; △, immobilized enzyme.

stabilized on immobilization. Possibly, the bound enzyme is in a conformation with less freedom of movement than that of the free enzyme, and such a restriction reduces denaturation.

In the case of proteolytic enzymes, autodigestion often leads to inactivation. In such a case, insolubilization should increase stability, since the covalent binding of the enzyme molecules to the carrier matrix prevent their interaction with each other. The immobilized CPA showed no more than 8% activity loss after 6 mo of storage at 4°C. Reusability data of immobilized CPA is depicted in Fig. 5. It is evident from the figure that enzyme activity is reduced to half its value after 30 runs.

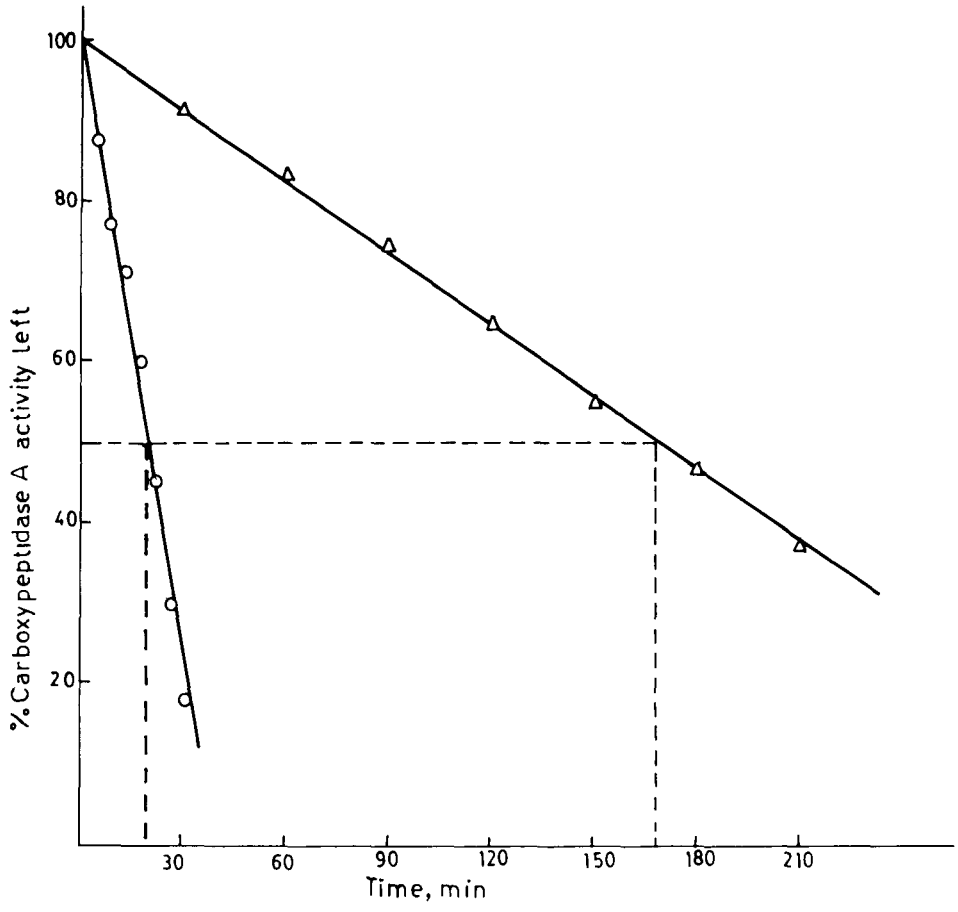


Fig. 4. Determination of half life of CPA. Aliquots of free and immobilized enzyme were heated at 50°C in a waterbath and samples withdrawn at regular time intervals and assayed for CPA activity; ○, native enzyme; △, immobilized enzyme.

The data from Fig. 6 show that the K_m values of immobilized CPA were lower than those of free enzymes at low ionic strengths ($\tau/2$ = 0.05–0.1). Upon increase of the ionic strength from 0.1 to 5.0, the native enzyme did not show much variation, but the K_m of immobilized enzyme continued to increase up to the ionic strength ($\tau/2$) of 2.0 and then became constant. In the region of higher ionic strengths, the K_m of immobilized CPA was higher than that of native enzyme. The microenvironmental effects, diffusional effects, and chemical modification of the enzyme may be invoked to explain the differences in K_m. The attachment of the enzyme to the support may be creating an environment surrounding the bound enzyme that does not favor equal partition of the substrate between the bulk of the substrate solution and the surface of the polymer matrix. If the partition were such that at equilibrium the concentration of the substrate was more in the environment of the bound enzyme than

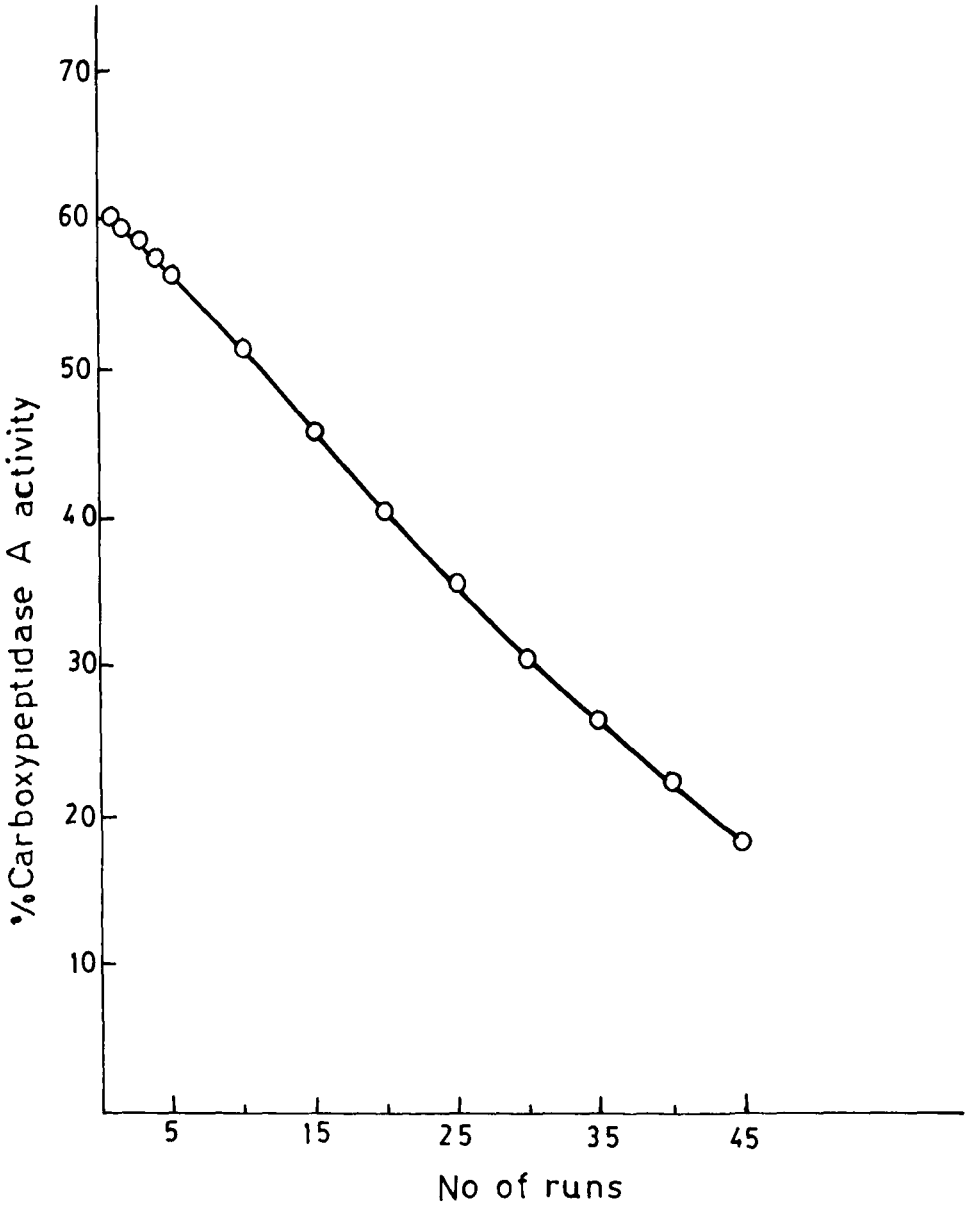


Fig. 5. Reusability of the immobilized CPA. Immobilized enzyme (20 mg) was incubated with 0.025*M* substrate for 10 min at 50°C and released amino acid was determined for CPA activity. Immobilized CPA was collected by centrifugation and assayed for CPA activity.

that measured in the bulk of the substrate, then a decrease in the observed K_m would occur.

The differences in K_m values of the soluble and insoluble enzyme forms could be explained by diffusional limitations on the support. It is well known that particles in suspension are surrounded by films of essentially stagnant liquid through which molecules can pass only by diffu-

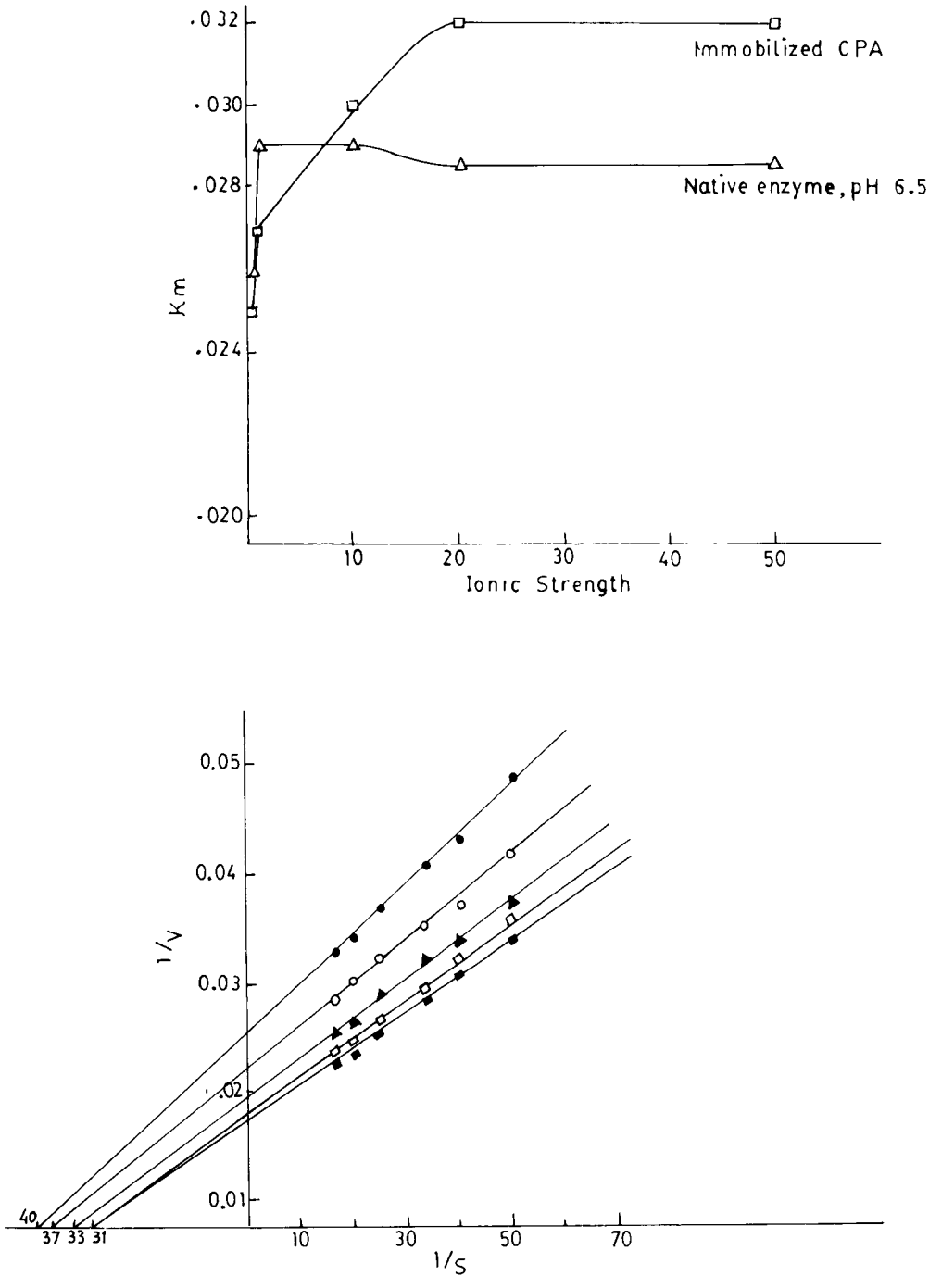


Fig. 6. Effect of ionic strength ($\tau/2$) on K_m of native and immobilized CPA. The K_m was determined by plotting $1/v$ vs $1/s$ at different ionic strengths: ●, $\tau/2$ = 0.05; ○, $\tau/2$ = 0.1; ▲, $\tau/2$ = 1.0; □, $\tau/2$ = 2.0; ■, $\tau/2$ = 5.0.

sion (*9,27*). Diffusional properties of this layer may influence the kinetic parameters. Goat CPA has fairly large turnover and diffusion of the sub-

strate, and products released from the active site can be rate limiting in such a case. Hence, rate of the reaction was determined at different shaking speed, but even at speeds four times those normally used, no change in activity was observed. This indicates that in this case diffusional effect is not significant.

The steric hindrance by the supporting matrix also influences the kinetic parameters of the immobilized enzyme. Because of steric hindrance of some active sites, it is likely that some individual molecules may be attached in such a way as to alter the tertiary structure slightly without destroying the enzymatic activity. This could result in true changes in K_m for the molecule with respect to its substrate. Though a particular value for a kinetic parameter may be measured, it is only a net value averaged over a large number of enzyme molecules bound to the support in large numbers in different ways.

CONCLUSIONS

Thus, although there is a loss in enzymatic activity on immobilization of CPA, there are many practical advantages in terms of their increased stability, greater resistance to denaturation, and reduced susceptibility to autolysis, making them suitable for repeated use over longer periods of time. By virtue of their insolubility, they can be recovered from the reaction media without contamination of the substrate and products. With material packed in columns, it has been shown that it is possible to exercise greater control over the rate and extent of conversion of substrate into products (*28*).

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