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Preparation, Characterization, and Application of a Novel Immobilized Carboxypeptidase B

P. SÜDI, ERZSÉBET DALA, AND B. SZAJÁNI*

Reanal Factory of Laboratory Chemicals, Budapest 70, PO Box 54, Hungary

ABSTRACT

Pig pancreas carboxypeptidase B has been immobilized by covalent attachment to a polyacrylamide-type bead support possessing carboxylic functional groups activated by water-soluble carbodiimide. The optimum conditions of immobilization were determined. The activation of the support and the coupling reaction were performed in 0.1 *M* sodium citrate/sodium phosphate buffer (pH 4.5) using a support-carbodiimide-enzyme weight ratio 4:8:1 at 0–4°C. Under such conditions, the highest activity achieved was 6700 U/g solid. The catalytic properties and stability of immobilized carboxypeptidase B were studied and compared with the corresponding properties of the soluble enzyme. The specific activity of the immobilized enzyme calculated on bound protein basis was about 70% of that of soluble enzyme. The optimum pH for the catalytic activity of the immobilized carboxypeptidase B was practically identical with that of soluble enzyme (pH 7.6–7.7). The apparent optimum temperature of the immobilized carboxypeptidase B was about 7°C higher than that of the soluble enzyme. With hippuryl-L-arginine as substrate, K_{mapp} value of the immobilized enzyme was tenfold higher than the K_m value of the soluble enzyme. The conformational stability of the enzyme was markedly enhanced by the strongly hydrophylic microenvironment in a wide temperature and pH range. The immobilized carboxypeptidase B was used for stepwise digestion of cytochrome C.

Index Entries: Carboxypeptidase B, immobilized; support, polyacrylamide type; properties, immobilized carboxypeptidase B; cytochrome C digestion.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Carboxypeptidase B (peptidyl-L-lysine [L-arginine] hydrolase, EC 3.4.17.2) selectively splits the COOH-terminal arginine and lysine residues from the peptide chains. Therefore the enzyme is an important tool for the protein structure studies. The soluble enzyme is rather unstable and applicable only for a single test. The immobilization makes possible the repeated application in batch and continuous process as well. In spite of the practical importance, only a few attempts have been made to immobilize carboxypeptidase B (*1–4*).

We have used polyacrylamide type bead supports possessing various functional groups for the immobilization of carboxypeptidase B by covalent bonding. The best result was achieved when carboxylic functional groups were activated by water-soluble carbodiimide. The immobilized enzyme was characterized and applied for stepwise digestion of cytochrome C.

MATERIALS AND METHODS

Carboxypeptidase B was isolated from pig pancreas (5) as a solution containing 0.1 *M* sodium chloride and 1 μM PMSF. Before use the enzyme solution was dialyzed against buffer or distilled water.

Polyacrylamide-type bead supports were commercial products of Reanal Factory of Laboratory Chemicals (Budapest, Hungary). L-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate was purchased from Fluka AG, Buchs, Switzerland. Hippuryl-L-arginine was a preparation of Sigma Chemical Company, St. Louis, MO. All other chemicals were reagent grade commercial preparations of Reanal.

Methods of Immobilization

Immobilization on Akrilex AH

Akrilex AH dry beads (0.5 g) were suspended in 32 mL of 0.1 *M* potassium phosphate (pH 7.0). Then, 4 mL of 2 *M* HCl and 16 mL of $NaNO_2$ solution (4%) was added dropwise to the suspension at 0–4°C. After 15 min stirring, the activated support was washed with 0.1 *M* potassium phosphate buffer (pH 7.0). Then, 0.37 g of carboxypeptidase B dissolved in 60 mL of buffer was added. The suspension was stirred for 6 h, and after 16 h, it stood for an additional 2 h. The gel was filtered by suction and successively washed 3 times with 100 mL of 0.1 *M* potassium phosphate (pH 7.0), 3 times with 100 mL of the same buffer containing 1.0 *M* sodium chloride, three times again with 100 mL of buffer to remove the unbound proteins, and, finally, with distilled water to remove the phosphate ions. The product was then lyophilized.

Immobilization on Akrilex A

One g of Akrilex A dry beads was suspended in 25 mL of 0.1 *M* potassium phosphate (pH 7.0) and 0.66 g of carboxypeptidase B dissolved in 54 mL of buffer was added. The suspension was stirred for 10 min at 0–4°C then 1 g of 1-cyclohexyl-3-(2-morpholino-methyl) carbodiimide metho-*p*-toluene sulfonate in a buffer solution was added and the volume was filled up to 100 mL with buffer. The reaction mixture was held at 0–4°C for 2 d with 2 times the 6 h period of agitation.

Then washing was performed as described above and the product was lyophilized.

Immobilization on Bromoacetyl-Akrilex

One g of Bromoacetyl-Akrilex dry beads were suspended in 25 mL of 0.1 *M* potassium phosphate (pH 8.5) and 0.18 mg of carboxypeptidase B dissolved in water was added. The volume was filled up to 50 mL and the reaction mixture was held at 0–4°C for 24 h with an 8-h period of agitation.

Washing was performed as described above and the product was lyophilized.

Immobilization on Akrilex C

Carboxypeptidase B was covalently attached to the support using a method successfully applied for the immobilization of other enzymes (*6–16*). The carboxylic functional groups of support were activated by water-soluble carbodiimide.

The general method of the immobilization was the following.

One g of Akrilex C xerogel was suspended and swollen in 50 mL of 0.1 *M* buffer and 1–2 g of 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-*p*-toluene sulfonate dissolved in 20 mL cold (0°C) buffer, was added under continuous stirring and cooling in an ice bath. After 10 min, 15.5–62.0 mL of carboxypeptidase B solution (16.5 mg/mL in buffer) was added, and the pH was adjusted to the starting pH value. The mixture was incubated at 0–4°C during 48 h using 2 6-h periods of agitation. The gel was filtered by suction and successively washed 3 times with 100 mL of 0.1 *M* potassium phosphate buffer (pH 7.0), 3 times with 100 mL of the same buffer containing 1.0 *M* sodium chloride, again 3 times with 100 mL of buffer to remove the unbound proteins, and, finally, with a large volume of distilled water to remove the phosphate ions. The product was lyophilized.

Measurement of Protein

Protein determinations were performed according to the method of Lowry et al. (*17*), as modified by Schacterle and Pollack (*18*). The amount of immobilized protein was calculated as the difference between the amount

of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of Carboxypeptidase B Activity

The activity determination of the soluble carboxypeptidase B was performed with the method of Folk et al. (*19*) using hippuryl-L-arginine as substrate. For the measurements a modified Spektromom 203 spectrophotometer (MOM, Hungarian Optical Works, Budapest, Hungary) was applied.

At the activity tests of the immobilized carboxypeptidase B, 1 mg of immobilized enzyme (loaded polymer beads) was suspended and swollen in 1 mL of 0.025 *M* Tris/HCl buffer containing 0.1 *M* NaCl for 5 min and then 5 mL of 0.012 *M* hippuryl-L-arginine dissolved in the same buffer was added. The reaction mixture was continuously stirred for appropriate periods of time (3–15 min) at 25°C. The immobilized enzyme was then filtered of quickly (a few seconds) and the filtrate was assayed according to the ninhydrin method (*20,21*). For the calculation an L-arginine calibration curve was used. One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol hippuryl-L-arginine/min at pH 7.65 and at 25°C.

Heat Stability Tests on Soluble and Immobilized Carboxypeptidase B

The experiments were performed on 0.025 *M* potassium phosphate (pH 5.5–7.0) and Tris/HCl buffer (pH 7.0–8.0) containing 0.1 *M* sodium chloride, with reaction mixtures of 1.0 mL vol at 60°C. After appropriate times of incubation, the samples were rapidly cooled in an ice bath and the residual activities were assayed by the standard method at 25°C.

Thin-Layer Chromatography

In order to determine the amino acids and peptides Fixion 50×8 chromatoplates with a strongly acidic cation exchanger layer (Reanal) were used.

RESULTS

Immobilization of Carboxypeptidase B

The results of immobilization experiments using different type of supports are summarized in Table 1. As shown by the data the highest activity both on dry weight and wet gel basis was achieved when Akrilex C, a support possessing carboxylic functional groups activated by water-soluble carbodiimid was used.

Table 1
Immobilization of Carboxypeptidase B on Various Supports by Covalent Bonding[a]

Support	Functional groups	Functional group density, mmol/g solid	Immobilized protein, %	Immobilized activity, %	Activity loss, %	Activity of immobilized enzyme	
						U/g solid	U/mL wet gel
Akrilex AH	acid hydrazid	4	17	0.11	71.9	11	1.3
Akrilex A	primer amino	2	2.5	0.2	16.8	26	1.4
Akrilex bromoacetyl	bromoacetyl	2	19	0	53	0	0
Akrilex C-100	carboxyl	6.2	55	40	60	6700	67

[a]For experimental details see the text.

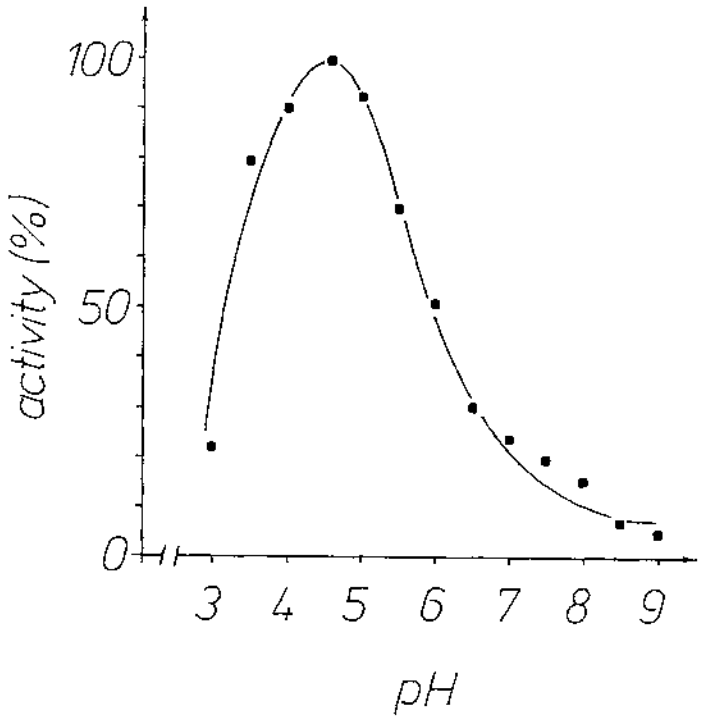

Fig. 1. Effect of pH of coupling reaction mixture on the activity of immobilized carboxypeptidase B. Experiments were performed in 0.1 *M* sodium citrate/sodium phosphate (pH 3.0–6.5) or 0.1 *M* Tris-maleate (pH 6.5–9.0) at 0–4°C. The Akrilex C-100 support was activated by 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate.

The most favorable conditions for the immobilization of carboxypeptidase B were determined. The optimal pH for the coupling was found to be pH 4.5 (Fig. 1). The effects of relative amount of reaction partners are presented in Table 2. A tight correlation was experienced between the specific activity of the soluble enzyme introduced into the coupling reaction mixture and that of the immobilized enzyme produced (Fig. 2).

Catalytic Activity

In optimal conditions, i.e., pH 4.5 and support-carbodiimide-enzyme weight ratio 4:8:1, the highest activity achieved was 6,700 U/g dry support; the specific activity of the bound enzyme calculated on protein basis was 72.7% of that of the soluble enzyme introduced into the reaction mixture. The loading was about 100 mg protein/g dry support.

pH Dependence of Catalytic Activity

The experiments were performed in 25 m*M* sodium acetate (pH 4.0–6.0), 25 m*M* potassium phosphate (pH 6.0–7.5), and 25 m*M* Tris-HCl (pH

Table 2
Effect of the Composition of the Coupling Reaction Mixture on the Immobilization of Carboxypeptidase B[a]

Composition of reaction mixture			
AC-100,[b] g	CMC,[c] g	CPB,[d] g	Activity of immobilized enzyme (U/g solid)
1	2	0.5	4,800
1	1	0.5	5,250
1	2	0.25	6,700
1	2	1.0	5,630

[a]For experimental details see the text.
[b]Akrilex C-100.
[c]L-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate.
[d]Carboxypeptidase B.

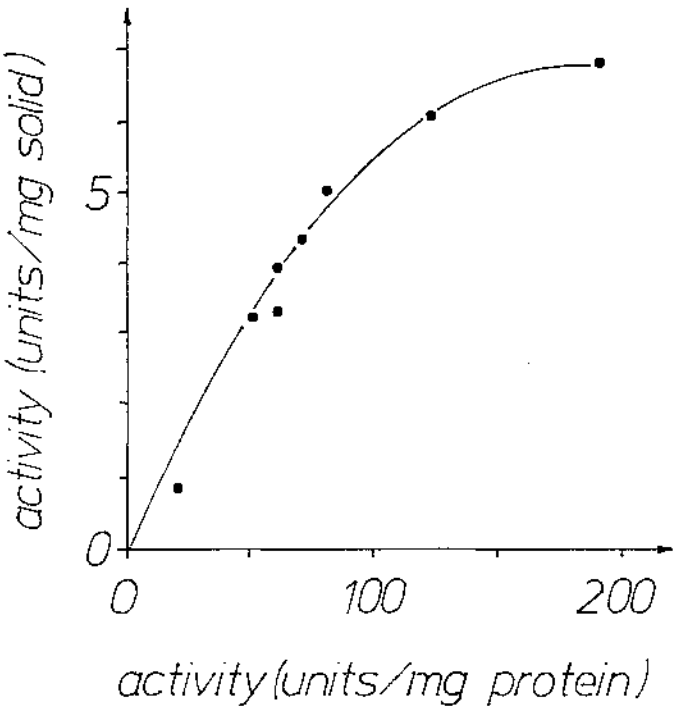

Fig. 2. Effect of specific activity of the soluble enzyme used for the coupling reaction on the activity of immobilized carboxypeptidase B. Experiments were performed in 0.1 *M* sodium citrate/sodium phosphate (pH 4.5) at 0–4°C. The reaction mixture (85.5 mL) contained 1 g of Akrilex C-100, 2 g of l-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate.

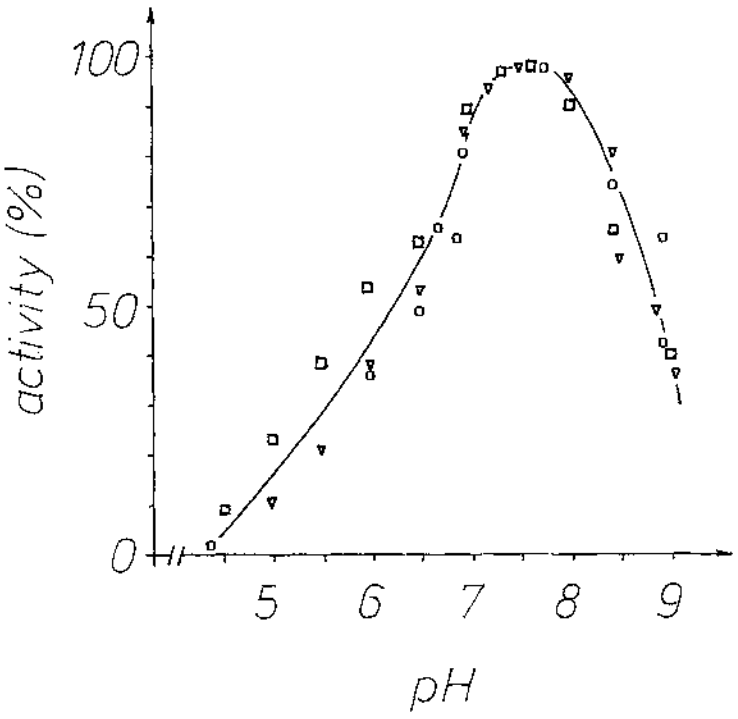

Fig. 3. Effect of pH on the catalytic activity of immobilized carboxypeptidase B at different ionic strength. Experiments were performed in 25 m*M* sodium acetate (pH 4.0–6.0), 25 m*M* potassium phosphate (pH 6.0–7.5), and 25 m*M* Tris-HCl (pH 7.0–9.5), respectively, at 25°C. The ionic strength was adjusted using sodium chloride. o, $\Gamma = 0.3$; △, $\Gamma = 1.0$; □, $\Gamma = 2.0$. The maximum activity was taken as 100%.

7.0–9.5), respectively, at 25°C. The ionic strength was adjusted by addition of sodium chloride to 0.3, 1.0, and 2.0. The optimum pH for the catalytic activity of the immobilized carboxypeptidase B was pH 7.6–7.7 that is practically identical with that of soluble enzyme. The optimum value remained unaltered in the ionic strength range of 0.3–2.0 (Fig. 3).

Dependence of Catalytic Activity on Temperature

The temperature dependence of the activity of soluble and immobilized carboxypeptidase B was studied in 25 m*M* Tris-HCl buffer containing 0.1 *M* sodium chloride at the optimum pH for catalytic activity in the temperature range 20–70°C, with hippuryl-L-arginine as substrate. Initial velocities were derived by measuring the activity after 15 min incubation at selected temperatures (Fig. 4). The apparent temperature optimum of the immobilized carboxypeptidase B (55°C) is somewhat higher than that of the soluble enzyme (48°C).

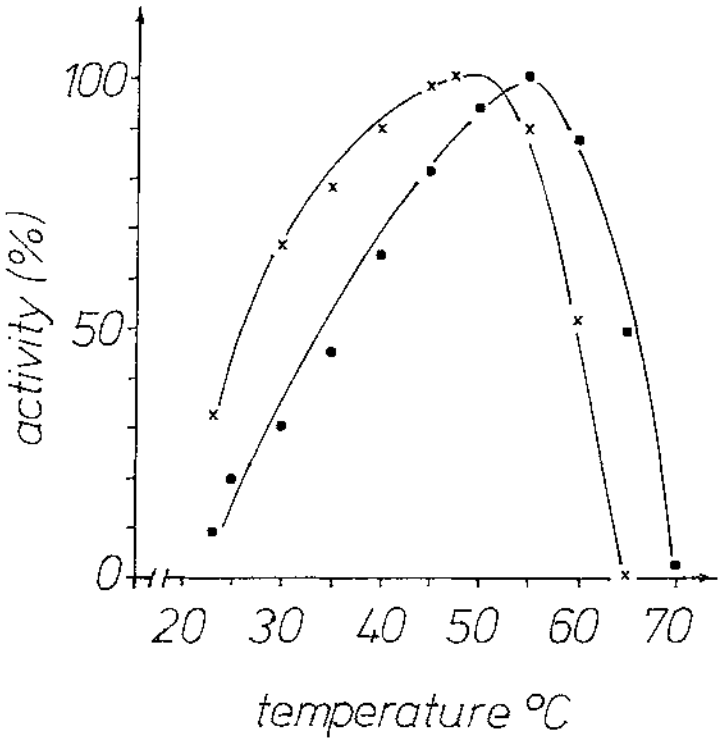

Fig. 4. Effect of temperature on the activity of soluble (x) and immobilized (●) carboxypeptidase B. Experiments were performed in 25 m*M* Tris-HCl buffer containing 0.1 *M* sodium chloride at pH 7.65. With both soluble and immobilized enzyme the maximum activity was taken as 100%.

Effect of Substrate Concentration on the Catalytic Reaction

The effect of hippuryl-L-arginine concentration on the initial rates of catalyzed reaction was investigated in the concentration range of 0–30 m*M* at the optimum pH for catalytic activity. Experiments were carried out in 25 m*M* Tris-HCl buffer containing 0.1 *M* sodium chloride. For calculation of K_m values, kinetic plots according to Hanes (22) were used. From the plots, K_m of soluble carboxypeptidase B was calculated to be 2.8 m*M* and K_{mapp} of immobilized enzyme to be 28 m*M*.

Heat Stability

The rates of heat inactivation of soluble and immobilized carboxypeptidase B were investigated in the temperature range between 50–65°C at the optimum pH for catalytic activity in 25 m*M* Tris-HCl buffer containing

Table 3
Heat Stability of Soluble and Immobilized Carboxypeptidase B[a]

Temperature, °C	+ 1/2 app (min)	
	Soluble enzyme	Immobilized enzyme
50	245	460
55	56	150
60	27	60
65	1.5	30

[a]Experiments were carried out in 25 m*M* Tris-HCl buffer containing 0.1 *M* sodium chloride (pH 7.65). Enzyme concentrations used were soluble enzyme, 0.01 mg protein/mL; immobilized enzyme, 1.0 mg solid/mL.

0.1 *M* sodium chloride. It was found that the conformational stability markedly increased as an effect of the immobilization (Table 3).

The pH Dependence of Heat Inactivation ("pH Stability")

The pH stability of soluble and immobilized carboxypeptidase B was compared at selected pH values in 25 m*M* potassium phosphate (pH 6.0–7.5) and 25 m*M* Tris-HCl buffer (pH 7.3–9.0) containing 0.1 *M* sodium chloride at 60°C. The half life values derived from the time curves at different pH values are presented in Fig. 5. As it can be seen from the figure, the heat stability of immobilized carboxypeptidase B exceeded that of the soluble enzyme at every pH. The optimum pH for the heat stability of the immobilized enzyme was shifted to a more acidic value (pH 6.7–6.8) than that of the soluble carboxypeptidase B (pH 7.5).

Storage Stability

The immobilized carboxypeptidase B preserved its original catalytic activity at least for a year at room temperature (20–25°C).

Practical Use of the Immobilized Carboxypeptidase B

The practical use of the immobilized carboxypeptidase B was tested in a preliminary experiment. Horse heart (0.25 g) cytochrome C heme-octapeptide fragment was dissolved in 25 mL of distilled water and pH was adjusted to 7.65 by sodium carbonate. Then 10 mg of immobilized carboxypeptidase B (5000 U/g solid) was added and the mixture was stirred at 25°C. In every 10 min sample (10 μL) was withdrawn and tested using TLC method. After 10 min incubation lysine was detectable and after 50 min the digestion was complete (Fig. 6).

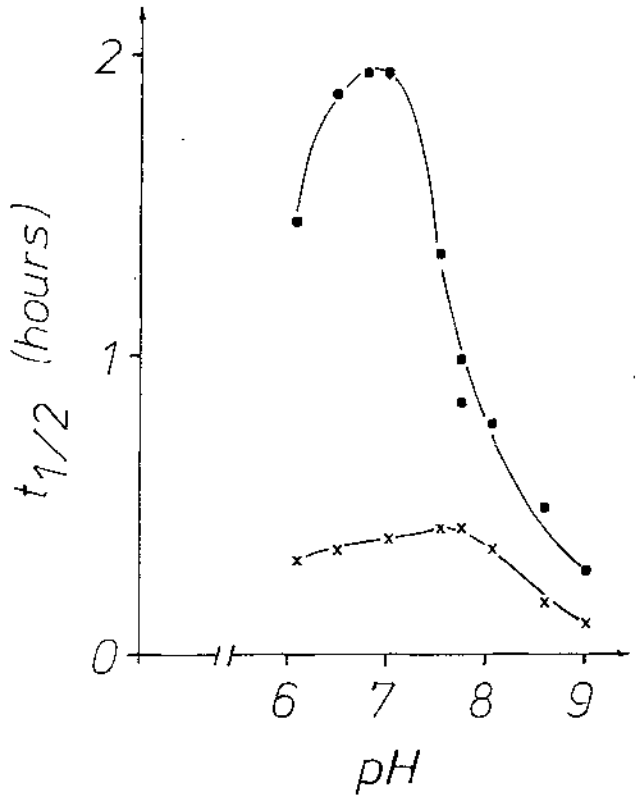

Fig. 5. The pH dependence of heat stability ("pH stability") of soluble (x) and immobilized (●) carboxypeptidase B. Experiments were carried out in 25 m*M* potassium phosphate (pH 6.0–7.5) and 25 m*M* Tris-HCl buffer (pH 7.3–9.0) containing 0.1 *M* sodium chloride at 60°C. Enzyme concentrations used were soluble enzyme, 0.01 mg protein/mL; immobilized enzyme, 1.0 mg solid/mL.

DISCUSSION

For protein analytical purposes carboxypeptidase B isolated from pig pancreas was immobilized on polyacrylamide type bead supports possessing various functional groups. Polyacrylamide beads were chosen because of their advantageous chemical and mechanical properties. The highest activity both on dry weight and swollen gel volume basis was achieved when the support had carboxylic functional groups activated by water-soluble carbodiimide. It is supposed that the relatively bulky side-chains of carbodiimide made possible to avoid the damages in the active centre as shown by the high specific activity on protein basis. The polyanionic microenvironment did not influence the optimum pH for catalytic activity. The enzyme-substrate interaction was significantly altered by the immobilization. In the strongly hydrophylic microenvironment the enzyme showed an enhanced conformational stability.

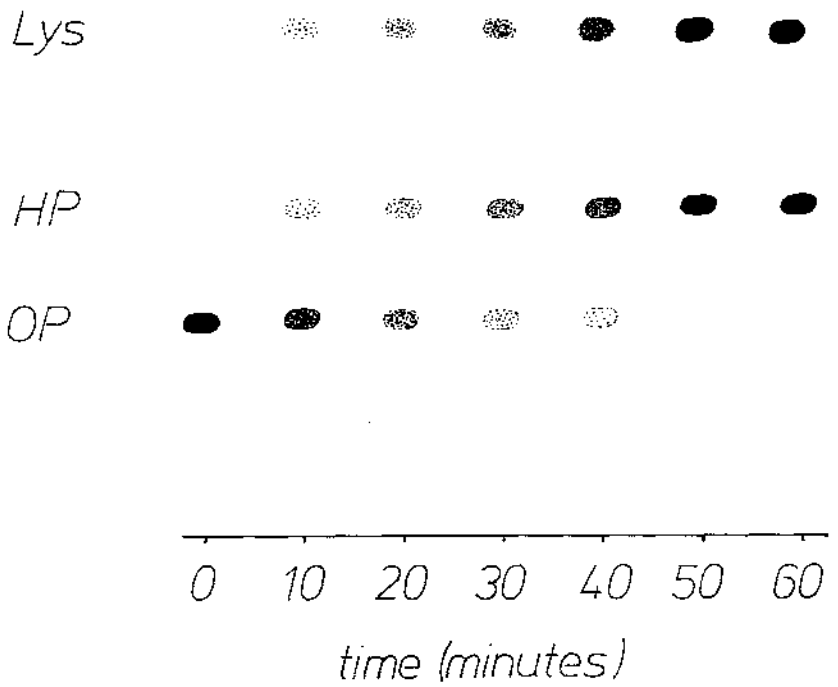

Fig. 6. Digestion of cytochrome C heme-octapeptide with immobilized carboxypeptidase B. OP, heme-octapeptide; HP, heme-heptapeptide. For experimental details see the text.

The Akrilex C-100-carboxypeptidase B is suitable for protein chemistry as was supported by the cytochrome C heme-octapeptide digestion. Additional experiments are in progress.

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