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R. D. Dua[a]; Padma Vasudevan[ab]; Sanjay Kumar[a]
[a] Biochemistry Laboratory Department of Chemistry, Indian Institute of Technology, New Delhi, India
[b] Centre for Rural Development and Appropriate Technology, Indian Institute of Technology, New Delhi, India

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Immobilized Carboxypeptidase A for Sequential Analysis

R. D. DUA, PADMA VASUDEVAN,* and SANJAY KUMAR

Biochemistry Laboratory
Department of Chemistry
Indian Institute of Technology
New Delhi 110016, India

ABSTRACT

The use of carboxypeptidase for sequence determination is attractive because of its technical simplicity. In chemical methods all molecules of a peptide are made to go through a degradation cycle before a new cycle is started. In the enzymatic degradation of a protein, the order of the amino acid residues is not necessarily determined in a stepwise fashion but rather from the rate at which the amino acids appear in the digest, i.e., an amino acid appearing faster than another presumably precedes it in the sequence. Under favorable circumstances the rate of appearance of the amino acids released during digestion give sufficient evidence for the C-terminal sequence to be deduced. The present work pertains to a study on the use of immobilized carboxypeptidase A columns for sequential analyses.

*Present address: Centre for Rural Development and Appropriate Technology, Indian Institute of Technology, New Delhi 110016, India.

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INTRODUCTION

Sequential analyses of peptides and proteins have generally been made through degradation by chemical and enzymatic methods. Among the various enzymes used so far, carboxypeptidases have been most useful because of their capability to remove amino acids one residue at a time from the C-terminus of polypeptide chains. When a carboxypeptidase acts on a protein, amino acids are removed until a residue exposed in the C-terminal position is released at such a slow rate that hydrolysis is effectively ended [1, 2]. Carboxypeptidase A rapidly releases amino acids with an aromatic or large aliphatic side chain [3], while carboxypeptidase B releases the basic amino acids lysine and arginine much faster than any of the other common protein amino acids.

The preparation and properties of carboxypeptidase A and carboxypeptidase B have been reviewed by Neurath [4]. An extensive review of the use of carboxypepdidases was made by Harris [5]. In an endgroup determination, a sample of protein is incubated with carboxypeptidase A or B or a mixture of both if arginine or lysine residue is present at the C-terminus. In order to slow down the rate of release of amino acids, either the enzyme/substrate ratio is kept low or digestion is done at room temperature instead of at 37°C. The amino acids released were determined. A plot of the different amino acids released as a function of time could give a clue of the sequencing of amino acids in a protein. The method has limitations when applied to a protein which has more than one residue of a particular amino acid and when the protein under investigation has more than one type of peptide chain and the enzyme releases amino acids from both. In such cases, kinetic experiments have to be supported by experiments on the isolation and determination of the C-terminal peptide by "diagonal" methods, substrate peptide maps, and cyanogen bromide cleavage [3].

Recent literature has discussed such applications of analytical techniques as field desorption mass spectrometry [6], fast bombardment mass spectrometry [7] and gas-liquid chromatography [8]. However, in all these methods the enzymes used cannot be recovered. Repeated use of an enzyme would be possible by using the immobilized enzyme technique [9]. Moreover, the flow rate of an immobilized enzyme can be so adjusted that in a certain time only one amino acid is released. This eliminates the necessity of keeping the enzyme/substrate ratio low and thus lead to more rapid analysis.

A search of the literature has shown that very little work has been done in this direction. Two different approaches have been tried. In one, C-terminal amino acids of peptides and protein were determined by adding the amino acids to a solid phase column through the N-terminus and eluting with a specific carboxypeptidase [10]. In the other, immobilization of carboxypeptidase A on CNBr activated sepharose was used for amino acid sequencing of the insulin B chain [11].

The present study shows that columns of immobilized carboxypeptidase A on a cellulosic carrier [9] can be used repeatedly for sequen-

tial analysis of amino acids in a tetrapeptide. The unknown peptide can be treated with the immobilized enzyme and the solutions, drawn after different times, can be analyzed by paper chromatography to determine the required residence in the column. The subsequent procedure is discussed in this paper.

EXPERIMENTAL

Cellulose powder (from E. Merck, Germany), NCbz-glycyl-L-phenylalanine, L-Leu-L-Leu-L-Val-L-Tyr, Dowex 50W (from Sigma, U.S.A.). All other chemicals were of BDH analytical reagent grade.

Isolation and Purification of Carboxypeptidase A

Dua and Srivastava purified carboxypeptidase A from goat (the main animal of slaughter in India) pancreas [12]. It was characterized in this laboratory for its substrate specificity, the effect of ion and ionic strength, buffer effect, and the requirements and role of metal ions [13-15].

Assay for Carboxypeptidase A Activity

Colorimetric Method. Carboxypeptidase A activity was determined by the method of Folk and Gladner [16], except that the assay mixture was incubated at 50°C for 10 min. The absorbance of the ninhydrin color 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 1 μmol of amino acid liberated per minute under assay conditions.

Oxidized cellulose was prepared according to the method of Nevell [17]. The degree of oxidation of the samples used for the present experiments was 50%, unless otherwise stated. Protein was determined by the method of Lowry et al. [18].

Immobilization of Carboxypeptidase A

Carboxypeptidase A was immobilized on oxidized cellulose (50%) [9]. The immobilization was found to be maximum at pH 6 for a fixed period of time [9], the amount of carboxypeptidase A attached to 2 g of oxidized cellulose being 12.78 mg. The enzyme activity in the immobilized sample was 78.75 μmol of phenylalanine liberated/mg/min.

Sequential Analysis of Tetrapeptide

A 0.025-M solution of tetrapeptide L-Leu-L-Leu-L-Val-L-Tyr (MW 506.0) along with a certain amount of immobilized enzyme (250.0 mg) was taken and kept in a waterbath at 50°C with constant stirring. After every 30 s, a 0.1-mL solution was pipetted out into different tubes. It was seen by paper chromatography that the enzyme took about 80 s to liberate tyrosine, whereas valine was freed in 2 min, 50 s. Leucine was broken from the peptide chain in 6 min. These results were used for sequential analysis.

Immobilized carboxypeptidase A (1.8 g) was taken in columns of known dimensions (1 × 30 cm). The tetrapeptide (0.025 M) was loaded into column I, kept at 50°C. The flow rate was so adjusted that a 1 mL fraction came in in about 80 s. Five fractions of 1 mL each were collected (5 mL contains 0.6325×10^5 μg of tetrapeptide). The different fractions were analyzed by paper chromatography and also by TLC. The amino acid tyrosine liberated, and the tripeptide and unbroken tetrapeptide left, were separated in a Dowex 50W column by varying the pH of the buffer. The tyrosine liberated was estimated by the ninhydrin method. Residual tripeptide and tetrapeptide were estimated by the Biuret method. The tripeptide eluted in different fractions was pooled and concentrated by lyphilization to 5 mL (0.3047×10^5 μg) of tripeptide. This was again loaded into the freshly immobilized enzyme column II of the same dimensions and the same protein content at 50°C. The whole procedure was repeated with a flow rate of 1 mL/3 min. Valine was liberated from the tripeptide. The residual dipeptide (0.1157×10^5 μg) was again loaded onto the freshly immobilized enzyme column III of the same dimensions at 50°C, and the process was repeated with a flow rate of 1 mL/2 min.

The sequencing of amino acids was also studied at different temperatures (30, 40, 50, and 60°C) to see where the maximum release of C-terminal amino acid occurred. In all cases one column was used for each of the following hydrolytic stages: Column I, tetrapeptide to tripeptide; Column II, tripeptide to dipeptide; and Column III, dipeptide to amino acid. The same column was used repeatedly for that particular temperature and stage.

Repeated Use of a Column for Enzyme Activity

About 1.8 g of immobilized enzyme (12.78 mg carboxypeptidase A attached to 2 g of oxidized cellulose) was placed in a column of known dimensions (1 × 30 cm). The substrate NCbz-glycyl-L-phenylalanine (0.025 M) was loaded into the column and the enzyme activity [16] was determined every 24 h.

Michaelis-Menten Constant (K_m) through Columns

The substrate NCbz-glycyl-L-phenylalanine at different molarities was loaded into different columns of the same dimensions and with the same protein content. The fractions at 0 min and after 10 min were collected from each column, and the activity was determined [16].

RESULTS AND DISCUSSION

As shown above, the flow rates of columns were so adjusted that at a given time only one amino acid (C-terminal) was released. The penultimate amino acid was released in the second run in the next column. If carboxypeptidase A releases two amino acids together, it would obviously be difficult to decide which was released first.

The amount of tetrapeptide, tripeptide, and dipeptide loaded into the different columns (I, II, and III) and the C-terminal amino acid released from the respective peptides at 50°C are shown in Table 1.

The rate of hydrolysis of a peptide bond by carboxypeptidase A is affected by the nature of both the residues forming it, but the effect of the C-terminal amino acid (that is, the one released) predominates. As seen from Table 1, tyrosine is liberated from the tetrapeptide in less time than leucine and valine, since the flow rate for column I was only 1 mL/80 s while those for Columns II and III were 1 mL/3 min and 1 mL/2 min, respectively. The relative rates of hydrolysis of C-terminal amino acids are in agreement with the literature [3].

The effect of temperature on the breakage of tetrapeptide, tripeptide, and dipeptide by the immobilized enzyme is shown in Table 2. As is evident from Table 2, the liberation of C-terminal amino acid from all three peptides is a maximum at 50°C, and this has also been reported to be the optimum temperature for the free enzyme. The decrease in activity above 50°C may be due to denaturation of the enzyme.

Tables 3 and 4 show the use of the columns (I, II, and III), for the second and third times, respectively, at different temperatures. The results indicate that the immobilized enzyme columns can be used repeatedly although the activity decreases with every peptide run. This may be due to elution of the enzyme. A temperature of 50°C continues to be the optimum in all cases.

Enzyme activity was also determined by using an immobilized enzyme column repeatedly with NCbz-glycyl-L-phenylalanine as substrate. It is clear from Fig. 1 that enzyme activity is reduced to half its value after 24 runs.

Michaelis-Menten Constant (K_m)

A plot of 1/V versus 1/S for NCbz-glycyl-L-phenylalanine gives a straight line. From Fig. 2 it is seen that K_m of the immobilized en-

TABLE 1

Column I,[a] μg		Column II,[a] μg		Column III,[a] μg	
Tetrapeptide loaded	0.6325×10^5	Tripeptide loaded	0.3047×10^5	Dipeptide loaded	0.1157×10^5
Tetrapeptide used	0.4807×10^5	Tripeptide used	0.1782×10^5	Dipeptide used	0.0724×10^5
Tyrosine released	0.1760×10^5	Valine released	0.0625×10^5	Leucine released	0.0433×10^5
Tripeptide left	0.3047×10^5	Dipeptide left	0.1157×10^5		

[a] Flow rates as indicated in the Experimental section.

TABLE 2

Temperature, °C	Column I, % tetrapeptide broken (L-Leu-L-Leu-L-Val-L-Tyr)	Column II, % tripeptide broken (L-Leu-L-Leu-L-Val)	Column III, % dipeptide broken (L-Leu-L-Leu)
30	54.33	42.82	49.58
40	62.64	50.77	57.23
50	77.81	60.12	69.67
60	36.56	31.53	34.85

TABLE 3. Immobilized Enzyme Columns Used Second Time

Temperature, °C	Column I, % tetrapeptide broken (L-Leu-L-Leu-L-Val-L-Tyr)	Column II, % tripeptide broken (L-Leu-L-Leu-L-Val)	Column III, % dipeptide broken (L-Leu-L-Leu)
30	49.84	40.12	47.11
40	58.13	45.95	54.91
50	73.56	56.64	66.76
60	31.94	27.45	29.68

TABLE 4. Immobilized Enzyme Columns Used Third Time

Temperature, °C	Column I, % tetrapeptide broken (L-Leu-L-Leu-L-Val-L-Tyr)	Column II, % tripeptide broken (L-Leu-L-Leu-L-Val)	Column III, % dipeptide broken (L-Leu-L-Leu)
30	47.18	37.84	43.56
40	54.83	42.48	51.66
50	69.96	53.17	63.18
60	28.13	24.77	25.49

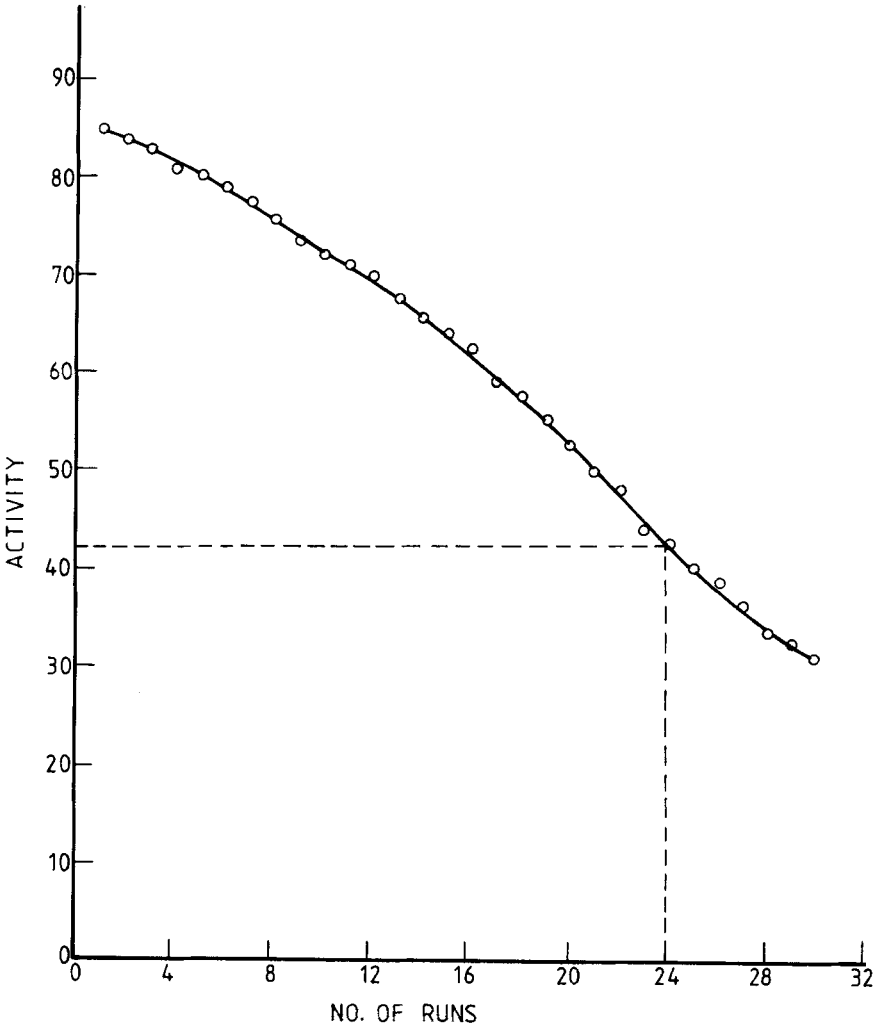

FIG. 1. Enzyme activity is reduced to half its value after 24 runs.

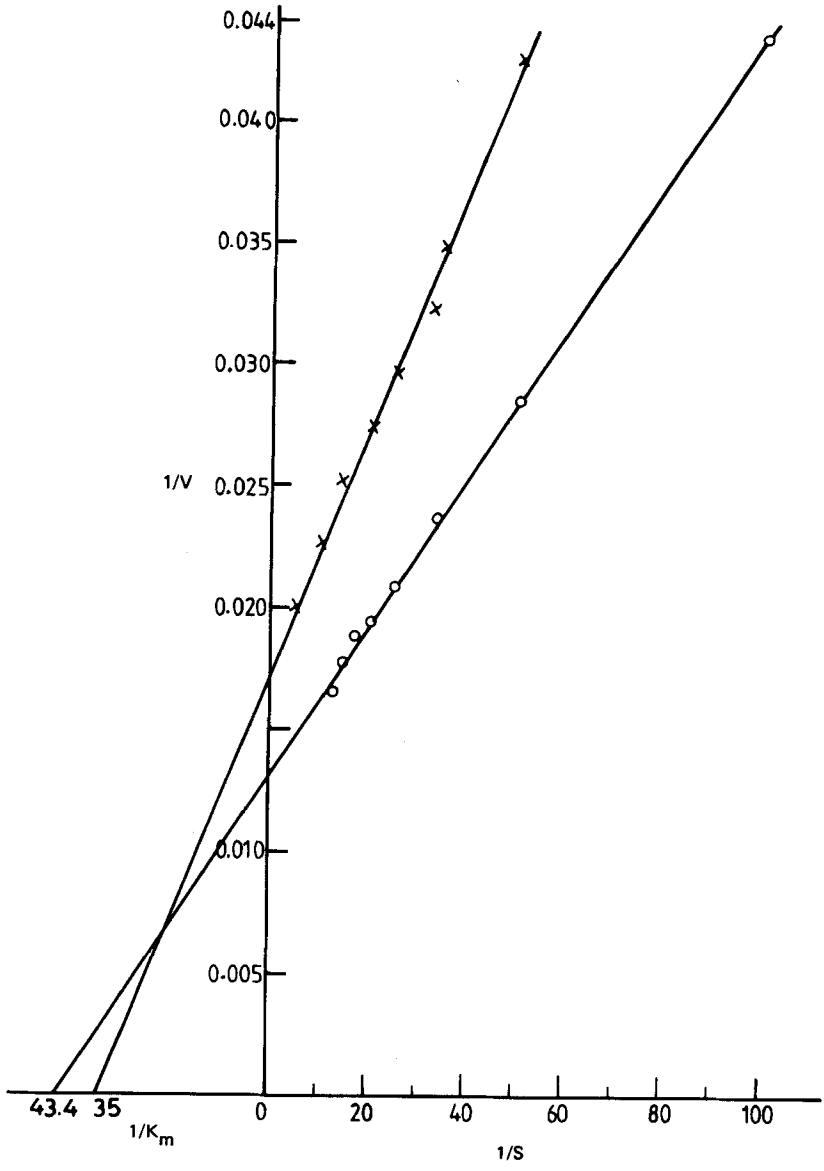

FIG. 2. K_m of free enzyme is 0.028 M (×). K_m of immobilized enzyme (through columns) is 0.023 M (○).

zyme is 0.023 M. This may be compared with the K_m value of the free enzyme, which is 0.028 M. Thus, the immobilized enzyme is more stable and the activity can be retained for a longer period.

CONCLUSIONS

Carboxypeptidase A has supplied useful data despite the fact that the enzymic approach has limited applicability. The gentle reaction conditions, the small amount of material required, and the ease of identification and determination of the released amino acids are advantages of carboxypeptidase which no chemical method is likely to equal. On the other hand, the difficulties in structural allocation when several amino acids split from multichain proteins sets definite limits to the usefulness of the enzyme. To some extent this problem could be solved by the use of immobilized enzyme columns and the right choice of flow rates. Work is in progress on finding better carriers for immobilization so that the half-life may be prolonged and the immobilized enzyme columns can be used for a larger number of runs. The use of this method for the sequential determination of unknown peptides is being explored.

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