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ENZYMATIC PROPERTIES OF AN ACID CARBOXYPEPTIDASE FROM *ASPERGILLUS NIGER* VAR. *MACROSPORUS*

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Summary

Acid carboxypeptidase (peptidyl-L-amino-acid hydrolase, EC 3.4.16.1) isolated from *Aspergillus niger* var. *macrosporus* was investigated in regard to its kinetic parameters for two synthetic substrates. The optimal pH of peptidase activity toward Z-Glu-Tyr-OH was pH 3.0. K_m and k_{cat} values were $4.0 \cdot 10^{-3}$ M and 270 s^{-1} at pH 3.0 and 30°C. The optimal pH of esterase activity toward Bz-Arg-OEt was pH 5.2. K_m and k_{cat} for esterolytic activity were $6.1 \cdot 10^{-4}$ M and 1500 s^{-1} at pH 5.0 and 30°C. The enzyme released expected amino acids sequentially from the carboxyl ends of *S*-β-aminoethylated ribonuclease A and the B-chain of oxidized insulin, demonstrating carboxypeptidase activity of the enzyme.

The enzyme was inactivated by diisopropylphosphorofluoridate and phenylmethanesulfonyl fluoride. In the reaction with [^{14}C]diisopropylphosphorofluoridate, the amount of [^{14}C]diisopropylphosphoryl group incorporated into the enzyme in complete inactivation was estimated as 2 mol/mol enzyme.

Introduction

Acid carboxypeptidases originally named by Zuber and Matile [1] are exopeptidases which have the ability to release most amino acids including proline from the carboxyl termini of polypeptide chains sequentially in an acidic pH. Because of their broad specificities, they have been regarded as very useful tools for the determination of the carboxyl terminal structure of proteins [2,3,4,5].

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Abbreviation: AE-, *S*-β-aminoethyl.

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As reported in our previous paper [6], an acid carboxypeptidase was highly purified on a large scale from *Aspergillus niger* var. *macrosporus*. The purified enzyme was an acidic glycoprotein with a molecular weight of 136 000 and appeared to be composed of two identical monomers. About 22% of the mass of the enzyme consisted of carbohydrate.

The present paper reports the determination of kinetic parameters for two synthetic substrates, the elucidation of the mode of action of the enzyme toward proteins and the results of active site titration.

Materials and Methods

Materials. The acid carboxypeptidase preparation was purified as described in our previous paper [6]. Bovine β-trypsin purified from twice-crystallized bovine trypsin by the method of Schroeder and Shaw [7] and *p*-nitrophenyl *p'*-guanidinobenzoate were generous gifts from Dr. K. Kasai of Hokkaido University, Sapporo. Z-Glu-Tyr-OH and Bz-Arg-OEt were purchased from Protein Research Foundation, Minowa, Osaka. [^{14}C]Diisopropylphosphorofluoridate and Aquasol-2 were purchased from New England Nuclear. Diisopropylphosphorofluoridate was a product of BDH Chemicals, Ltd. Phenylmethanesulfonyl fluoride was from Sigma Chemical Co.

Proteins. *S*-β-Aminoethylated ribonuclease A (AE-RNAase A) was prepared by the method of Plapp et al. [8] from bovine pancreatic ribonuclease A (Sigma Chemical Co.) which had been purified according to the method of Hirs et al. [9] in our laboratory. When the AE-RNAase A preparation was digested with porcine carboxypeptidase A according to the method of Ambler [10], it gave expected carboxyl terminal structure of ribonuclease A (—His-Phe-Asp-Ala-Ser-Val-OH). Crystalline zinc bovine insulin was obtained from Connaught Medical Research Laboratory, University of Toronto. The B-chain of oxidized insulin was prepared according to Craig et al. [11] and Griffin et al. [12]. The B-chain gave the expected amino acid composition and a single NH_2-terminal residue (phenylalanine).

Incorporation of [^{14}C]diisopropylphosphorofluoridate into the acid carboxypeptidase. [^{14}C]Diisopropylphosphorofluoridate (100 μCi/9.8 · 10^{-7} mol in 0.5 ml propylene glycol from New England Nuclear) was diluted with 1.0 ml isopropanol containing 5.4 · 10^{-5} mol cold diisopropylphosphorofluoridate. The mixture was used as a stock solution for the experiments. The specific activity of the [^{14}C]diisopropylphosphorofluoridate solution was determined to be 1812 cpm/nmol by reacting bovine β-trypsin (0.81 mol of active site per mol β-trypsin specimen was titrated by *p*-nitrophenyl *p'*-guanidinobenzoate [13]) as described by Kuhn et al. [14].

2.6 · 10^{-8} mol of the acid carboxypeptidase in 2.5 ml of 0.05 M sodium phosphate buffer, pH 6.1, was mixed with 50 μl of the [^{14}C]diisopropylphosphorofluoridate solution described above and incubated at 30°C. At certain time intervals, 2.0-ml aliquots of the reaction mixture were withdrawn and applied to a column of Bio-Gel P-6 (1.46 × 45 cm) equilibrated with 0.05 M sodium acetate buffer, pH 5.0. The break-through fractions containing protein were collected. The protein concentration was determined from the absorbance at 280 nm and the remaining enzyme activity was assayed toward Bz-Arg-OEt,

0.5 ml each of these was then dissolved in 10 ml Aquasol-2 and the individual radioactivities were counted using a Packard Tri-Carb liquid scintillation counter Model 574.

Digestion of oxidized insulin B-chain and AE-RNAase A with the acid carboxypeptidase. The B-chain of oxidized insulin (1.34 μmol) in 2.0 ml of 0.05 M sodium acetate buffer, pH 3.0 or 0.82 μmol of the B-chain in 2.0 ml of 0.05 M sodium acetate buffer, pH 5.0 were mixed with 0.1 ml of the enzyme solution at 30°C. After incubation for 0.5, 1, 2 and 22 h, 0.5 ml of the reaction mixture was withdrawn and the reaction was stopped by heating in a boiling water bath for 10 min. The solutions were dried in a desiccator and the released amino acids were determined by amino acid analysis using a JEOL /Type JLC-6AH) amino acid analyzer. In digestion of AE-RNAase A, 0.75 μmol of the substrate in 1.5 ml of 0.05 M sodium acetate buffer, pH 3.0 was incubated at 30°C with 50 μl of the enzyme solution for 0.5, 1, 2 and 4 h. At these time intervals, 0.3 ml each of the mixture was withdrawn, treated and analyzed as described above.

Determination of protein concentrations. The acid carboxypeptidase concentration was determined using the value of $E^{1\%}_{280\,nm} = 14.0$ [6]. In the case of digestion of proteins by the acid carboxypeptidase, substrate concentrations were determined by performing amino acid analysis with aliquots of individual specimens after acid hydrolysis.

Enzymatic assays. Peptidase activity toward Z-Glu-Tyr-OH and esterase activity toward Bz-Arg-OEt were assayed according to the procedures described in our previous paper [6].

Results and Discussion

pH-Activity profiles and some kinetic studies on peptidase and esterase activities of the acid carboxypeptidase. Fig. 1 shows pH-activity profiles of the acid carboxypeptidase-catalyzed hydrolysis of Z-Glu-Tyr-OH and Bz-Arg-OEt, respectively. The pH optima toward Z-Glu-Tyr-OH and Bz-Arg-OEt are 3.0 and 5.2, respectively. Table I summarizes kinetic parameters toward the two synthetic substrates obtained by Lineweaver-Burk plots [15]. The pH optima and these kinetic parameters are very similar to those of the acid carboxypeptidase from *A. saitoi* [16,17].

Mode of action of the acid carboxypeptidase on AE-RNAase A and B-chain of oxidized insulin. Fig. 2 shows the time courses for the release of amino acids from AE-RNAase A by the acid carboxypeptidase. The enzyme hydrolyzed AE-RNAase A at pH 3.0 and 30°C, releasing valine, serine, alanine, aspartic acid

TABLE I

KINETIC PARAMETERS TOWARD THE TWO SYNTHETIC SUBSTRATES OF THE ACID CARBOXYPEPTIDASE

Substrates	K_m (M)	k_{cat} (s^{-1})	pH	Temperature (°C)
Z-Glu-Tyr-OH	$4.0 \cdot 10^{-3}$	270	3.0	30
Bz-Arg-OEt	$6.1 \cdot 10^{-4}$	1500	5.0	30

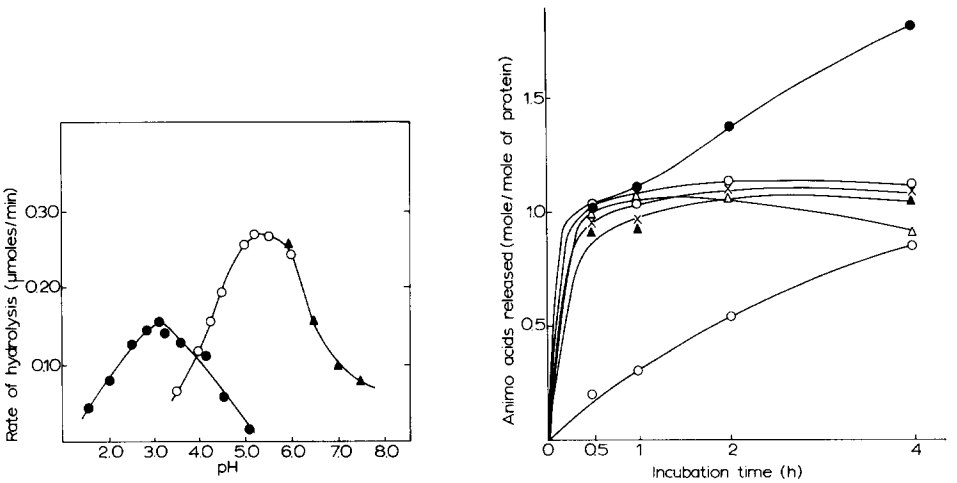

Fig. 1. pH-Activity profiles of acid carboxypeptidase-catalyzed hydrolysis of Z-Glu-Tyr-OH and Bz-Arg-OEt. Peptidase activity toward Z-Glu-Tyr-OH (●———●) was measured in 0.05 M sodium acetate buffer at 30°C and at an enzyme concentration of $2.5 \cdot 10^{-8}$ M. Esterase activity toward Bz-Arg-OEt was measured in 0.05 M sodium acetate buffer (○———○) and in 0.05 M sodium phosphate buffer (▲———▲) at 30°C and at an enzyme concentration of $1.4 \cdot 10^{-8}$ M.

Fig. 2. Time course of release of amino acids from AE-RNAase A by acid carboxypeptidase. AE-RNAase A (0.75 μmol) was digested at pH 3.0 and 30°C with $0.87 \cdot 10^{-3}$ μmol enzyme (AE-RNAase A/acid carboxypeptidase = 910/1, mol/mol). ●———●, Val; ○———○, Ser; X———X, Ala; ▲———▲, Asp; △———△, Phe; ○———○, His.

and phenylalanine rapidly in nearly stoichiometric amounts in the first 30 min. Thereafter, histidine was released slowly, followed by a further release of valine. After 4 h incubation, trace amounts of proline and tyrosine could be detected. Up to 4 h of digestion, no other amino acids could be detected.

Table II summarizes the release of amino acids from the B-chain of oxidized insulin by the enzyme. When the B-chain was subjected to the enzyme digestion at pH 3.0 and 30°C, alanine was released rapidly in a stoichiometric amount. However, it was after a very prolonged incubation that the following lysine was released. Thereafter, the immediate release of seven additional amino acids expected from the carboxyl terminal sequence, occurred in amounts nearly equivalent to that of lysine. This indicates that the sequence of $-\overset{28}{\text{Pro}}\text{-}\overset{29}{\text{Lys}}$-OH of the B-chain was very resistant to the action of the enzyme. With digestion occurring at pH 5.0 which is near to the optimum pH of esterase activity of the enzyme toward Bz-Arg-OEt, the enzyme exhibited nearly the same mode of action on the B-chain as at pH 3.0. Even after 22 h of extensive digestion by the enzyme specimen at either pH 3.0 or 5.0, the release of amino acids not expected from the carboxyl terminal sequence could not be detected, showing that endopeptidase action is absent in the enzyme specimen. The optimum pH for the rapid release of alanine from the carboxyl terminal of B-chain was found to be very near to the optimum pH for a synthetic peptide of Z-Glu-Tyr-OH.

Thus, these results of digestion experiments confirm that the acid carboxypeptidase is able to release sequentially most types of amino acids, including

TABLE II

DIGESTION OF OXIDIZED INSULIN B-CHAIN BY THE ACID CARBOXYPEPTIDASE

In digestion at pH 3.0, the B-chain of oxidized insulin (1.34 μmol) was incubated at 30°C with $0.42 \cdot 10^{-3}$ μmol enzyme (B-cahin/acid carboxypeptidase = 3200/1, mol/mol). At pH 5.0, 0.82 μmol of the B-chain was incubated at 30°C with the same amount of the enzyme (B-chain/acid carboxypeptidase = 1950/1, mol/mol). The released amino acids are expressed as mol/mol of the B-chain.

	Time	-Leu	-Val	-Cya *	-Gly	-Glu	-Arg	-Gly	-Phe	-Phe	-Tyr	-Thr	-Pro	-Lys	-Ala	-OH
Digestion at pH 3.0	0.5 h													+	0.91	
	1													+	0.97	
	2									+	+	+	+	0.026	1.02	
	22	+	+			0.16	0.18	0.29		0.46	0.22	0.24	0.21	0.25	0.98	
Digestion at pH 5.0	0.5 h													+	1.00	
	1													+	0.96	
	22	+	+			+	0.14	0.24		0.85	0.45	0.51	0.45	0.55	1.00	

* Cya: Cysteic acid.

TABLE III

SECOND-ORDER RATE CONSTANTS ($M^{-1} \cdot min^{-1}$) FOR INACTIVATION OF SERINE ENZYMES BY DIISOPROPYLPHOSPHOROFLUORIDATE AND PHENYLMETHANESULFONYL FLUORIDE

Enzymes	Chymotrypsin *	Trypsin *	Carboxypeptidase ** Y	Acid carboxypeptidase *** from *A. niger*
Reaction conditions	pH 7.0, 25°C	pH 7.2, 25°C	pH 7.0, 25°C	pH 5.0, 30°C
Inhibitors				
Diisopropylphosphorofluoridate	$2.7 \cdot 10^3$	$3.0 \cdot 10^2$	$5.6 \cdot 10^3$	$1.09 \cdot 10$
Phenylmethanesulfonyl fluoride	$1.5 \cdot 10^4$	$2.7 \cdot 10^2$	$9.7 \cdot 10^2$	4.8

* Ref. 18.

** Ref. 19.

*** These values were calculated by dividing the apparent first-order rate constants obtained from the plots in Fig. 3 by the reagent concentrations.

proline from the carboxyl termini of polypeptide chains at acidic pH, as reported on acid carboxypeptidases from several sources.

Inactivation of the acid carboxypeptidase by diisopropylphosphorofluoridate and phenylmethanesulfonyl fluoride. The time courses for the inactivation for the acid carboxypeptidase by diisopropylphosphorofluoridate, and phenylmethanesulfonyl fluoride are shown in Fig. 3. In the case of inactivation by diisopropylphosphorofluoridate, both esterase and peptidase activities decreased at comparable rates and were almost completely lost after 90 min of incubation. The reactions apparently followed first-order kinetics in a large excess of reagents over the enzyme. The second-order rate constants, which were calculated by dividing the apparent first-order rate constant by the

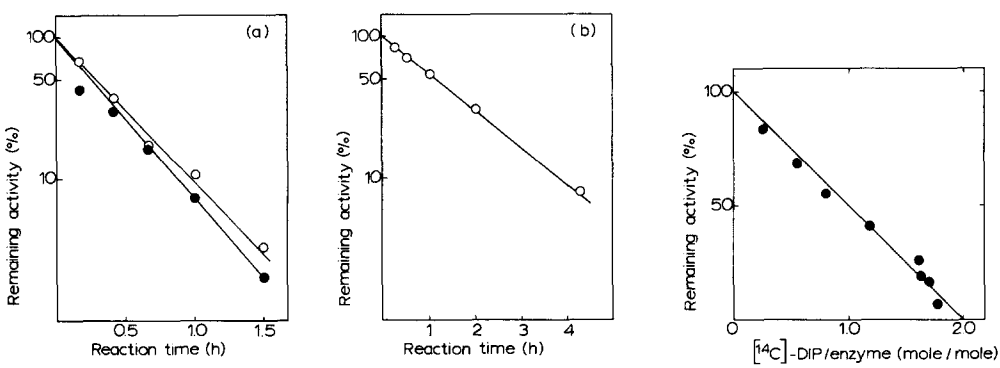

Fig. 3. Inactivation of acid carboxypeptidase by diisopropylphosphorofluoridate and phenylmethanesulfonyl fluoride. (a) The enzyme ($9.0 \cdot 10^{-7}$ M) in 0.05 M sodium acetate buffer, pH 5.0, was incubated with $3.6 \cdot 10^{-3}$ M diisopropylphosphorofluoridate at 30°C. At appropriate time intervals peptidase (●——●) and esterase (○——○) activities were measured. (b) The enzyme ($8.0 \cdot 10^{-7}$ M) in 0.05 M sodium acetate buffer, pH 5.0—4.5% isopropanol was incubated with $2.1 \cdot 10^{-3}$ M phenylmethanesulfonyl fluoride at 30°C. At appropriate time intervals, the esterase (○——○) activity was measured.

Fig. 4. Stoichiometry of incorporation of [^{14}C]diisopropylphosphorofluoridate into the acid carboxypeptidase. DIP, diisopropylphosphoryl group.

reagent concentrations, are shown in Table III. In the table, the values reported on three serine proteases are also included for comparison. The inactivation rates of the acid carboxypeptidase by these reagents were much less than those of chymotrypsin, trypsin and carboxypeptidase Y.

Incorporation of [^{14}C]diisopropylphosphorofluoridate into the acid carboxypeptidase. The enzyme was incubated with [^{14}C]diisopropylphosphorofluoridate in a 71-fold molar excess over the enzyme at pH 6.1 and 30°C. The incorporation of radioactivity into the enzyme occurred with a decrease in the enzyme activity in parallel. Fig. 4 shows the plotting of the remaining activities vs. the molar ratios of incorporated [^{14}C]diisopropylphosphoryl group/enzyme. Extrapolation to zero activity corresponded to the incorporation of 2.0 [^{14}C]diisopropylphosphoryl group per enzyme molecule, assuming the molecular weight of 136 000. The finding that the observed points fell on a straight line suggests that the two diisopropylphosphorofluoridate-reactive sites are equivalent and independent. If the enzyme had been first inactivated by phenylmethanesulfonyl fluoride, there was no incorporation of [^{14}C]diisopropylphosphoryl group. Also, little of the radioactive group was incorporated into the enzyme in solution containing 8 M urea under the same conditions as in the experiments described above.

These results indicate the existence of two diisopropylphosphorofluoridate-reactive active sites per molecule of native acid carboxypeptidase. The observation supports the conclusion that the enzyme is composed of two identical monomers as reported in our previous paper [6].

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