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SPECIFICITY AND MODE OF ACTION OF ACID CARBOXYPEPTIDASE FROM *ASPERGILLUS SAITOI*

EIJI ICHISHIMA AND TERUYOSHI ARAI

Laboratory of Microbiology and Enzymology, Tokyo Nōkō University, Fuchu, Tokyo 183 (Japan)

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SUMMARY

1. Acid carboxypeptidase isolated from culture filtrate of *Aspergillus saitoi* has been investigated for its use in carboxyterminal sequence determination of native insulin, angiotensin I and glucagon at pH 2.5 or 2.2.

The examination indicated that after 5 h incubation asparagine, alanine and lysine were liberated from native insulin. The acid carboxypeptidase catalyzed the release of leucine and histidine from the decapeptide angiotensin I. When $(Pro–Pro–Gly)_5$ and polylysine were incubated separately with the acid carboxypeptidase, no hydrolysis was found.

No release of free amino acids by autodigestion of the enzyme was detected after a prolonged period of incubation.

2. The pH dependence of log (V/K_m) and log V for the acid carboxypeptidase-catalyzed hydrolysis of a small synthetic peptide, Z-Tyr–Leu, has been determined. Bell-shaped pH–rate profiles were obtained. The main conclusions derived from analysis of the data were that two catalytically-active groups on the enzyme with $pK_{e1} \simeq 2.3$ and $pK_{e2} \simeq 4.9$ were important in the enzymatic action at the acid end of the pH range.

Competitive inhibition by small substrates was found with hydrocinnamic acid, indole-3-propionic acid and 4-phenylbutyric acid. The K_i value for hydrocinnamic acid inhibition was $4 \cdot 10^{-4}$ M.

INTRODUCTION

Although carboxypeptidases are well known and widely distributed in nature, there have been few reports on acid carboxypeptidase, *i.e.* an enzyme that removes the carboxyterminal amino acids at the acid end of the pH range. Previous work[1–6] resulted in the isolation and characterization of a new type of acid carboxypeptidase from *Aspergillus saitoi*[1–5], *Aspergillus oryzae*[5] and *Penicillium*[6].

The highly purified enzyme from *A. saitoi* exhibited homogeneity during ultracentrifugation and in polyacrylamide gel electrophoresis experiments[2]. According to the procedure of Yphantis, the molecular weight of the acid carboxypeptidase of *A. saitoi* at 0.2 ionic strength was found to be 139 000 (ref. 2). According to the gel-filtration method, molecular weight values of 155 000 for the larger form or polymer and 51 000 for the smaller form or monomer were obtained in the absence of NaCl, and a molecular weight value of 135 000 was obtained in the presence of 0.2 M NaCl (ref. 2). These two fractions having the different molecular weight values had the same K_m and V values toward Z-Glu–Tyr at pH 3.1 and 30 °C (ref. 2).

The acid carboxypeptidase had a pH optimum at pH 3.1 for Z-Glu–Tyr, pH 3.5 for Z-Tyr–Leu, pH 3.2 for Z-Gly–Pro–Leu–Gly, pH 3.3 for Z-Gly–Pro–Leu–Gly–Pro and 3.5 for Bz-Gly–Lys[2,3]. Previous studies had indicated that, with small synthetic substrates of the type R–X–Y where the X–Y bond was broken, the acid carboxypeptidase of *A. saitoi* exhibited a preference for aromatic or carboxyl groups in the X position.

EDTA and *o*-phenanthroline had no effect on the enzymatic activity at pH 5.2, suggesting that there was no requirement for metal ions[2]. DFP, tosyl-L-phenylalanine chloromethyl ketone, *p*-chloromercuribenzoate and monoiodoacetic acid were weak inhibitors of the enzyme[2,5]. The activity was apparently inhibited by indole-3-acetic acid, indole-3-propionic acid, hydrocinnamic acid, 4-phenylbutyric acid and 3-phenylpyruvic acid[5].

The present paper describes the specificity and mode of action of the acid carboxypeptidase in the hydrolysis of native insulin, angiotensin I, glucagon and Z-dipeptides.

MATERIALS

Acid carboxypeptidase

The acid carboxypeptidase from *A. saitoi* R-3813 was purified according to the previous paper[2]. The highly purified enzyme preparation did not appear to have any endopeptidase activity[2]. However, the original culture filtrate contained other trypsinogen-activating acid proteinase[7] (aspergillopeptidase A, EC 3.4.4.17) activity and it was only after four steps of purification that the unwanted proteinase was removed.

Materials

Bovine insulin was purchased from Shimizu Seiyaku Co. Crystalline bovine glucagon (Lot 98B-0200) was purchased from Sigma Chemical Co. Horse angiotensin I (No. 4007), $(Pro–Pro–Gly)_5$ (No. 4005), polylysine (No. 3056), Z-Glu–Tyr (No. 3018) and Z-Tyr–Leu were supplied by the Protein Research Foundation in the Institute for Protein Research, Osaka University.

Amino acid calibration mixture for the amino acid analyzer was purchased from Takara Kosan Co., Tokyo.

METHODS

Native bovine insulin (0.52 μmole, 3 mg) was dissolved in 30 ml dilute HCl, 5

munits of acid carboxypeptidase in 5 μl of sodium acetate buffer (pH 3.1) were added to the solution and the pH was adjusted to 2.5 with dilute HCl. The mixture was incubated at 30 °C for 1, 5 or 18 h. An equal volume of 10% trichloroacetic acid was added to inactivate the enzyme. After extraction with ether to remove trichloroacetic acid, the aqueous layer was evaporated to dryness *in vacuo*. The samples of hydrolyzate were investigated directly on the column of the automatic amino acid analyzer, Hitachi model KLA-3B.

Angiotensin I (0.55 μmole, 0.75 mg) was dissolved in 15 ml dilute HCl, and the pH was adjusted to 2.5 with dilute HCl. The reaction was started by addition of 5 munits of acid carboxypeptidase dissolved in 5 μl sodium acetate buffer (pH 3.1). The temperature of incubation was 30 °C for 1, 5 or 18 h. The amino acids liberated in the reaction mixture were analyzed by the same method as described above.

For Expt 1 of glucagon, 0.50 μmole glucagon (1.75 mg) was dissolved in 15 ml dilute HCl, and the pH was adjusted to 2.5 with dilute HCl. The reaction was started by addition of 5 munits of acid carboxypeptidase. The temperature of incubation was 30 °C for 1, 5 or 18 h. The reaction mixture was precipitated with an equal volume of 10% trichloroacetic acid to inactivate the enzyme. After extraction with ether to remove trichloroacetic acid, the aqueous layer was evaporated to dryness *in vacuo*. The hydrolyzates were subjected to quantitative amino acid analyses.

For further investigation (Expt 2) of glucagon hydrolysis, 0.50 μmole glucagon was dissolved in 1.5 ml 1/15 M sodium citrate buffer[8], pH 2.2, containing 20 ml/l thiodiglycol, 1 ml/l phenol and 4 ml/l surface active agent Brij-35. The citrate buffer described above had been prepared for quantitative amino acid analysis. The reaction was started by addition of 25 munits of acid carboxypeptidase dissolved in 25 μl 0.05 M acetate buffer, pH 3.1. The temperature was 30 °C. The samples were frozen and stored until they were subjected to quantitative amino acid analyses.

5 mg $(Pro–Pro–Gly)_5$ and 10 mg polylysine were dissolved separately in 30 ml dilute HCl at pH 2.5. The reaction was started by addition of 5 μl of the enzyme. The temperature of incubation was 30 °C for 1, 5 or 18 h.

Hydrolysis of Z-dipeptides was measured by the increase in ninhydrin color after hydrolysis at acidic pH and 30 °C, as described previously[2]. The values of initial velocities and Michaelis constants defined were calculated from the graphical determination of Lineweaver–Burk plots. Approximate apparent pK_a values of both the free enzyme and enzyme–substrate complex were obtained from the intercepts of these lines (Fig. 2), using the method of Dixon and Webb[9].

RESULTS

The results obtained from native insulin with acid carboxypeptidase are summarized in Table I. Free alanine was liberated very rapidly from the C-terminal of the B-chain of insulin. Evidence was also found for free lysine from the B-chain, which is the residue adjacent to alanine. The ability to liberate lysine from native insulin distinguished the acid carboxypeptidase from pancreatic carboxypeptidase A (EC 3.4.2.1) of animal origin[10,11]. The amino acids liberated from the A-chain of native insulin were asparagine and a trace of aspartic acid. The latter was shown to be a secondary product derived from asparagine in the experimental procedure. The examination indicated that after 5 h incubation the yields of asparagine, alanine and

TABLE I

RELEASE OF CARBOXYTERMINAL AMINO ACID RESIDUES FROM 0.52 μmole NATIVE BOVINE INSULIN (A) AND 0.55 μmole ANGIOTENSIN I (B) AT pH 2.5 AND 30 °C BY ACID CARBOXYPEPTIDASE FROM *Aspergillus saitoi*

Substrate	*Time (h)*	*Amino acid released (μmoles)*					
		Asn	*Asp*	*Lys*	*Ala*	*His*	*Leu*
A. Native bovine insulin	1	0.043	Trace	Trace	0.159		
	5	0.059	Trace	0.011	0.328		
	18	0.043	Trace	<0.011	0.346		
B. Angiotensin I	1					Trace	0.280
	5					0.034	0.398
	18					0.107	0.363

lysine were 11, 63 and 2%, respectively. As the average rate of recovery of amino acids in the precipitation and extraction procedure was found to be about 70%, the 63% yield of alanine obtained showed that 1 mole alanine was liberated from a mole of insulin by the action of acid carboxypeptidase.

C-terminal leucine bound to histidine of the decapeptide angiotensin I was released rapidly, followed by slow liberation of histidine (Table I). The basic residue histidine was hydrolyzed, indicating the ease of hydrolysis by acid carboxypeptidase of the peptide linkages on either side of the residue. The third residue, phenylalanine was not released from the digestion product of angiotensin I.

The subsequent hydrolysis of the first 12 C-terminal residues from glucagon is shown in Table II. In Expt 1, oxidation of methionine and tryptophan in the reaction mixtures was apparently observed (Table II). In sodium citrate buffer (pH 2.2) containing thiodiglycol, these amino acids were completely recovered. The yields of

TABLE II

RELEASE OF CARBOXYTERMINAL AMINO ACID RESIDUES FROM 0.5 μmole GLUCAGON BY ACID CARBOXYPEPTIDASE FROM *Aspergillus saitoi*

Amino acid	*Amino acid released (μmoles)*				
	*Expt 1**			*Expt 2***	
	1 h	*3 h*	*18 h*	*5 h*	*18 h*
Thr + Gln	0.047	0.101	0.387	0.445	0.825
Asn	0.076	0.187	0.423	0.246	0.444
Met		Trace	0.146	0.293	0.455
Leu		0.132	0.341	0.307	0.500
Trp		Trace	0.184	0.290	0.472
Val		Trace	0.037	0.053	0.149
Phe			0.018	0.051	0.135
Asp	Trace	0.022	0.066	0.092	0.170
Ala			0.010	0.100	0.253
Arg				Trace	0.037

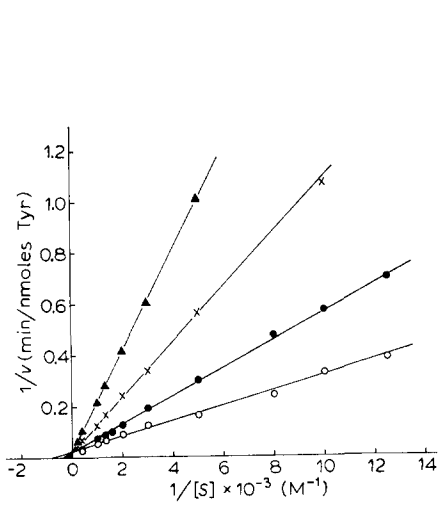
* In Expt 1, the reaction mixture was precipitated with trichloroacetic acid and then the aqueous layer was extracted with ether.

** In Expt 2, the sodium citrate buffer of pH 2.2 with thiodiglycol was used in the incubation.

methionine, tryptophan and leucine in Expt 1 were 32, 40 and 68%, respectively. Citrate buffer containing thiodiglycol was found to be effective for C-terminal analysis using the acid carboxypeptidase. Traces of arginine, which occupies position 12 and/or 13 from the C-terminus, could be first detected after a period of 5 h incubation with thiodiglycol.

No release of free amino acids by incubation of $(Pro–Pro–Gly)_5$ or polylysine with the acid carboxypeptidase was detected after 1, 5 and 18 h incubation at 30 °C.

The effects of the substrate analogue, hydrocinnamic acid, on the Z-Glu–Tyr hydrolyzing activity of the acid carboxypeptidase at pH 3.1 and 30 °C are shown in Fig. 1. The kinetic plots of incubation by hydrocinnamic acid at three different concentrations showed competitive inhibition. The value of the inhibition constant, K_i, for hydrocinnamic acid as determined by the Dixon plots[9] was $4 \cdot 10^{-4}$ M. Competitive inhibitions were also observed with indole-3-propionic acid and 4-phenyl-butyric acid when Z-Glu–Tyr was used as substrate.



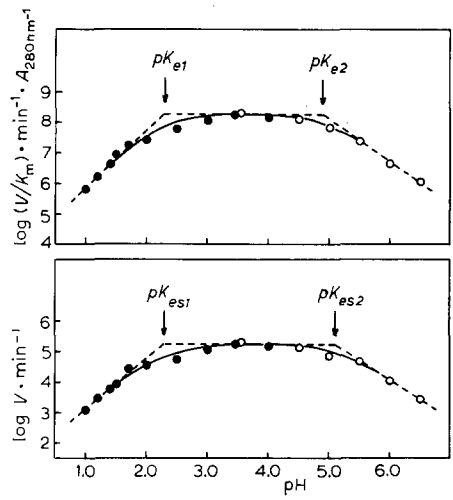


Fig. 1. Kinetics of inhibition of the hydrolysis of Z-Glu–Tyr of acid carboxypeptidase from *Aspergillus saitoi* at pH 3.1 and 30 °C by hydrocinnamic acid. The concentration of the enzyme was $2.42 \cdot 10^{-3}$ units absorbance at 280 nm. ○—○, control; ●—●, $2.5 \cdot 10^{-4}$ M hydrocinnamic acid; ×—×, 10^{-3} M hydrocinnamic acid; ▲—▲, $2 \cdot 10^{-3}$ M hydrocinnamic acid.

Fig. 2. The pH dependence of log (V/K_m) and log V for Z-Tyr–Leu hydrolysis over the pH range 1.0–6.5 at 30 °C by acid carboxypeptidase of *Aspergillus saitoi*. Sodium acetate buffer (●) and Sörensen's citrate buffer (○) were used in the determination.

The variation of log (V/K_m) and log V as a function of pH for the acid carboxypeptidase from *A. saitoi* catalyzed hydrolysis of Z-Tyr–Leu over the pH range 1.0–6.5 at 30 °C is shown in Fig. 2. The pH–rate profiles of both log (V/K_m) and log V for the hydrolysis of Z-Tyr–Leu were bell-shaped with a maximum at pH 3.5. The ionization of the enzyme with pK_{e1} of 2.3 and pK_{e2} of 4.9 units characterized these pH profiles. Between pH 2 and 3, and pH 5 and 6, log V changed, as the groups in the enzyme–substrate complex ionized with pK_{es1} of 2.3 and pK_{es2} of 5.1. pK_a of 2.3 could represent participation of an ionized carboxyl group. The $pK_a \simeq 4.9$ could be in accord with the ionization of a carboxyl group or an imidazole group.

DISCUSSION

In the previous paper[2], incubation of the acid carboxypeptidase from *A. saitoi* with small synthetic peptides indicated that the acid carboxypeptidase released acidic, neutral and basic amino acid residues. In the present paper, three different large peptides, native insulin, angiotensin I and glucagon, have been degraded demonstrating the combined activity and specificity of the pancreatic carboxypeptidases A[12] and B[13], but showing important differences in the optimum pH for digestion of substrates.

With regard to specificity for insulin hydrolysis, the acid carboxypeptidase differed from carboxypeptidase A chiefly in that the acid carboxypeptidase released lysine at pH 2.5 (Table I). Further confirmation that the enzyme could release basic residues as well as neutral and acidic amino acids was obtained from experiments with angiotensin I (Table I) and glucagon (Table II) hydrolyses. The slow liberation of lysine had been suggested by the effect of the adjacent proline residue involving the imino function, since the previous study had shown that the acid carboxypeptidase hydrolyzed a small amount of leucine from Z-Gly–Pro–Leu[2]. It was also found in the present paper that in a peptide with sequence $(Pro–Pro–Gly)_5$ glycine was not liberated. Since no liberation of phenylalanine had been suggested by the effect of the adjacent proline residue involving the imino function, it was suggested that the acid carboxypeptidase catalyzed the hydrolysis of the decapeptide angiotensin I to the vasopressive octapeptide angiotensin II, and free leucine and histidine.

A previous study[3] showed that in Z-Gly–Pro–Leu–Gly–Pro the –Gly–Pro bond was cleaved very slowly by the acid carboxypeptidase .Preliminary experiments had indicated that a small amount of free proline was liberated from the urea-denaturated pepsinogen by the acid carboxypeptidase[14,15]. These results indicated that the –X–Pro bond was cleaved by the action of the acid carboxypeptidase. A detailed account of release C-terminal proline will appear elsewhere.

The specificity of the acid carboxypeptidase from *A. saitoi* was similar to citrus carboxypeptidase C[16], but showing important differences in the optimum pH for digestion of substrates.

A large number of compounds inhibited the acid carboxypeptidase activity[2,5]. The present work indicated that common structural requirements for a competitive inhibitor were a free carboxyl group and an aromatic function (Fig. 1). All of these inhibitors except hydrocinnamic acid were known as competitive inhibitors of pancreatic carboxypeptidase A[12].

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