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PURIFICATION AND CHARACTERIZATION OF A NEW TYPE OF ACID CARBOXYPEPTIDASE FROM ASPERGILLUS

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SUMMARY

1. This paper reports the purification and characterization of a new type of acid carboxypeptidase from Aspergillus. The highly purified enzyme was found to be essentially homogeneous by such criteria as sedimentation in the ultracentrifuge and disc electrophoreses on polyacrylamide gel at pH 9.5, 8.0 and 2.3.

2. The enzyme has 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, and pH 3.5 for Bz-Gly–Lys.

The K_m and v_{max} values for Z-Glu–Tyr at pH 3.1 and 30° are $1.25 \cdot 10^{-3}$ M and $1.9 \cdot 10^{4}$ μmoles tyrosine per min for $A_{280\ nm}$, the values for Z-Tyr–Leu at pH 3.5 and 30° are $1.0–10^{-3}$ M and $1.64 \cdot 10^{5}$ μmoles leucine/min for $A_{280\ nm}$, and the values for Bz-Gly-Lys at pH 3.5 and 30° are $4.0 \cdot 10^{-3}$ M and $5.5 \cdot 10^{2}$ μmoles lysine per min for $A_{280\ nm}$.

The specificity of the enzyme was investigated for a wider range of substrates at the acid end of the pH spectrum and the carboxypeptidase-like activity was confirmed.

3. According to the Yphantis' procedure, the molecular weight of the enzyme at 0.2 ionic strength was found to be 139 000. The sedimentation constant was determined to be 7.3 S. According to the gel filtration method, molecular weight values 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.

4. The enzymatic activity was inhibited by DFP at pH 6.0 and 0°, and was not affected by EDTA at pH 5.2 and 5°.

INTRODUCTION

While establishing purification procedures for acid proteinase[1] (aspergillopeptidase A, EC 3.4.4.17), it was found that a carboxypeptidase-like enzyme in the culture filtrates of Aspergillus would hydrolyze α-amino-substituted peptides such

Abbreviations: Z-, benzoxycarbonyl; Bz-, benzoyl; Ac-, acetyl; $TyrI_2$, 3,5-diiodo-L-tyrosyl; PCMB, *p*-chloromercuribenzoate; DFP, diisopropylfluorophosphate. The abbreviated designation of amino acid residue denotes the L-form, except where otherwise indicated.

as Z-Tyr–Leu at pH 3.5, Z-Glu–Tyr at pH 3.1 and Z-Gly–Pro–Leu–Gly at pH 3.2 (ref. 2).

We have now isolated the Z-Glu–Tyr, Z-Tyr–Leu, Bz-Gly–Lys and Z-Gly–Pro–Leu–Gly hydrolyzing enzyme in apparently pure form. The carboxypeptidase-like activity of the enzyme was confirmed with Z-Phe–Tyr–Leu, Z-Gly–Pro–Leu–Gly, and Z-Gly–Pro–Leu–Gly–Pro, Z-Tyr–Leu being the compound most rapidly broken down.

In addition, ARAI *et al.*[3] found that significant amounts of free leucine and phenylalanine were liberated by the action of *Aspergillus* acid carboxypeptidase[2] from the nonapeptide, (Ala, Arg, Asp, Gly, Val)–Gln–Tyr–Phe–Leu, which was isolated from the peptic hydrolyzate of soybean as the compound having a bitter taste.

These works from this and other laboratories have confirmed the earlier conclusions[2] that the highly purified exopeptidase of Aspergillus is one of a new type of carboxypeptidase with activity in the acidic pH range. The trivial name Aspergillus acid carboxypeptidase is suggested for this new type of carboxypeptidase.

METHODS AND MATERIALS

Enzyme substrates

Peptides were supplied by the Protein Research Foundation in the Institute for Protein Research, Osaka University.

Hammarsten milk casein was obtained from E. Merck.

To obtain a crude enzyme product

The microorganism used in this study was *Aspergillus saitoi* R-3813, which is now designated as ATCC-14332.

For the production of a new type of carboxypeptidase, cultivation of the Aspergillus was carried out in a submerged culture[1]. The culture filtrates were pooled and the pH was adjusted to 4–5. The culture filtrates were brought to 70% saturation with $(NH_4)_2SO_4$. The precipitate was filtered off and stored in the cold.

Column chromatography

Details are described in the legends to the figures.

Protein determination

Protein concentrations were usually estimated from the absorbance at 280 nm using a Hitachi Model 101 spectrophotometer.

Peptidase assay

The enzyme was assayed routinely with Z-Glu–Tyr ($5 \cdot 10^{-4}$ M) in 0.05 M sodium acetate buffer (pH 3.1). 0.5 ml of 10^{-3} M substrate solution was added to 0.5 ml of the enzyme solution in a 15–18-ml test tube at 30°. After 20 min the reaction was stopped by addition of 200 μl of 0.3 M NaOH. After 30 min or more, 200 μl of 2.5% acetic acid and furthermore 2 ml of 0.5 M sodium citrate buffer (pH 5.0) were added to the reaction mixtures. 1 ml of the freshly prepared ninhydrin reagent[4] was added and the mixture was heated at 100° for 15 min, and immediately cooled in an ice–water bath (0°) for 3–10 min. After addition of 0.6 ml of 60% ethanol, the absorbance

was measured at 570 nm in a Hitachi Model 101 spectrophotometer, and the amount of tyrosine liberated was determined from the standard amino acid solution. The tyrosine standard (10^{-4} M) was prepared by dissolving 18.12 mg of pure, dry tyrosine in 1 l of water containing 2 ml of 1 M HCl. Appropriate blanks were substituted, and the results expressed as μmoles of tyrosine liberated per min.

The concentration of dipeptides and tripeptides was $5 \cdot 10^{-5}$ M.

Definition of unit of acid carboxypeptidase activity

One unit of Z-Glu–Tyr hydrolase activity was defined as the amount of enzyme required to liberate 1 μmole tyrosine per min at pH 3.1 and 30°.

Specific activity was expressed as Z-Glu–Tyr hydrolase activity at pH 3.1 and 30° per min for unit absorbance at 280 nm of the enzyme.

Determination of kinetic parameters

To measure the K_m of the enzyme for Z-Glu–Tyr, Z-Tyr–Leu and Bz-Gly–Lys, a procedure enabling the estimation of a 2-fold higher concentration of the enzyme than was previously possible was developed and is described below. By this means, 50 points could be determined during the first 10 min of the incubation at 30°, and the initial rate could be determined with considerable precision. In all cases, satisfactory Michaelis–Menten kinetics were observed, and a plot of $1/v$ *vs.* $1/[S]$ permitted the fitting of unambiguous straight lines.

The values of the inhibition constant, K_i, for Z-Tyr–Leu-NH_2, Tyr–Leu and leucine as determined in this paper by an indirect method (Eqn. 1) are used.

$$\left(1 + \frac{[I]}{K_i}\right)\frac{1}{v} = \frac{1}{v_{app}} \qquad (1)$$

Acid proteinase assay

Endopeptidase activity was determined according to the previous method[1]. One unit of acid proteinase activity was defined as the amount of enzyme which yields the color equivalent to 1 μmole of tyrosine per min at pH 2.7 and 30°.

Thin-layer chromatography

The solvent system of thin-layer chromatography with Silica gel G was *n*-butanol–acetic acid–water (4:1:1, by vol.).

Release of C-terminal amino acid residues from Z-Gly–Pro–Leu–Gly

The acid carboxypeptidase at a concentration of $2.76 \cdot 10^{-3}$ absorbance at 280 nm was incubated at 30° with $5 \cdot 10^{-4}$ M substrate in 1 ml of 0.05 M acetate buffer (pH 3.2). The stopped reaction mixtures were applied to the column of the automatic amino acid analyzer, Hitachi Model KLA-2.

Disc electrophoresis procedure

Acrylamide gel electrophoreses were performed at 20° with the standard pore formulation of WILLIAMS AND REISFELD[5] at pH 9.5, 8.0 and those of MORIYA[6] at pH 2.3, and 5 mA constant for all gels.

Sedimentation analyses

Sedimentation analyses were performed with a Hitachi Model UCA-1A analytical ultracentrifuge. The partial specific volume, $\bar{v}$, of the acid carboxypeptidase was assumed to be 0.72 ml/g.

Molecular weight determination was performed with circular channel cells by the YPHANTIS' procedure[7]. The individual molecular weight values for three different concentrations (2.62, 5.23 and 7.85 units of absorbance at 280 nm) were obtained from the run at 11 937 rev./min and 20° in acetate buffer (pH 4.0, I 0.2).

Gel filtration for molecular weight studies

The following preparations were used at a concentration of either 5 mg/ml or 20 mg/ml: from Sigma Chemicals Co., bovine thyroglobulin, Type I (mol. wt., 165 000); Tokyo Kasei Chemicals Co., Tokyo, bovine γ-globulin, Fraction II (mol. wt., 150 000); Nutritional Biochemicals Co., U.S.A., bovine hemoglobin (mol. wt., 67 000); Sankyo Co. Tokyo, Taka-amylase A (mol. wt., 51 200); Wako Pure Chemicals Co. Tokyo, egg albumin (mol. wt., 45 000).

For gel filtration[8] on a column 2 cm × 70 cm, the eluting buffer was 0.05 M sodium acetate buffer (pH 3.0) with or without 0.2 M NaCl.

RESULTS

Purification of the acid carboxypeptidase from Aspergillus

The results of the purification are summarized in Table I. For all subsequent

TABLE I

PURIFICATION OF ASPERGILLUS ACID CARBOXYPEPTIDASE

Fraction	*Specific activity (units/$A_{280\ n}$)*	*Recovery (%)*
Culture filtrate	10	100
70% $(NH_4)_2SO_4$ precipitate	50	83
Sephadex G-100	430	72
Duolite CS-101, Peak 1	2300	27
Peak 2	1060	27
P-cellulose	4013	23
DEAE-cellulose, Peak 1A	5800	3.5
Peak 1B	4300	19
Sephadex G-100, Peak 1Ba	5200	5.4
Peak 1Bb	5100	11.4

purification steps fractions were assayed both with a carboxypeptidase substrate (Z-Glu–Tyr) at pH 3.1 and with an endopeptidase substrate (casein) at pH 2.7.

Sephadex G-100 was used for the initial step in the purification of the acid carboxypeptidase. The concentrated solution of the submerged filtrate was subjected to gel filtration on Sephadex G-100. As shown by Fig. 1, the acid carboxypeptidase activity was eluted as a single peak at this stage of the purification. The acid proteinase was retarded to a greater extent by Sephadex G-100.

TABLE II

RELEASE OF C-TERMINAL AMINO ACID RESIDUES FROM 500 μM Z-GLY-PRO-LEU-GLY AT PH 3.2 AND 30° BY ASPERGILLUS ACID CARBOXYPEPTIDASE

Time (h)	*Amino acid released (μmoles)*		
	Pro	*Leu*	*Gly*
1	0	0	0.425
3	0	Trace	0.525
5	0	Trace	0.525
20	0	0.03	0.525

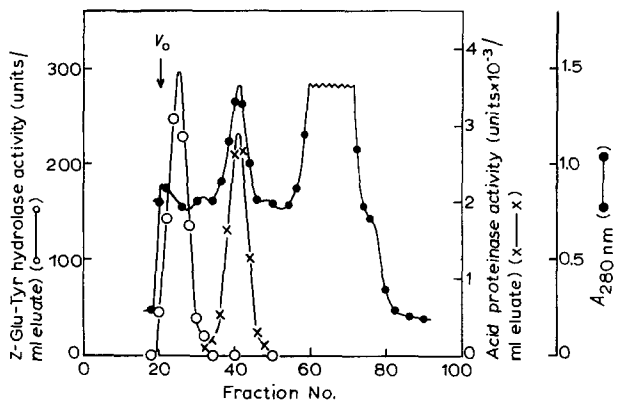


Fig. 1. Gel filtration of crude enzyme preparation from culture filtrate of Aspergillus on Sephadex G-100. Column: 2 cm × 58 cm. Eluent: 0.01 M acetic acid. Load: 3 ml of the concentrated enzyme filtrate. Flow rate: about 17 ml/h. Fraction volume: 2.7 ml. ●—●, absorbance at 280 nm; ○—○, Z-Glu–Tyr hydrolase activity at pH 3.1; ×—×, acid proteinase activity at pH 2.7.

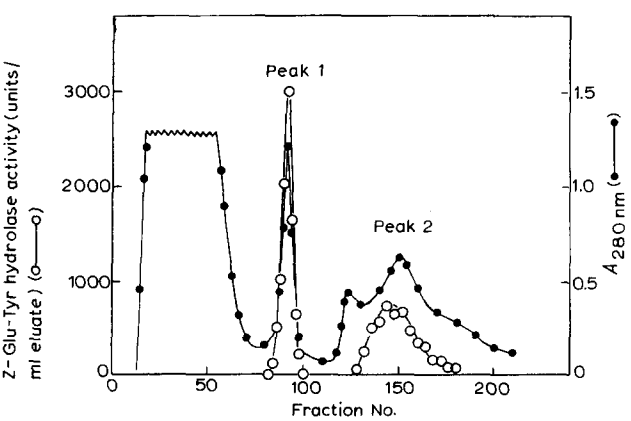


Fig. 2. Duolite CS-101 chromatography of Aspergillus acid carboxypeptidase from Sephadex G-100 run. The column (3 cm × 40 cm) was equilibrated with 0.01 M acetate buffer (pH 3.5). 500 ml of the enzyme solution were applied to the column. Elution was performed with 0.01 M acetate buffer (pH 3.5), with increasing concentration of salt and pH gradient using a mixing chamber of 500 ml. 0.2 M acetate buffer (pH 5.2) in the reservoir was tightly connected to the mixing chamber. Flow rate: 30 ml/h. Fraction volume: 10 ml. ●—●, absorbance at 280 nm; ○—○, Z-Glu–Tyr hydrolase activity at pH 3.1.

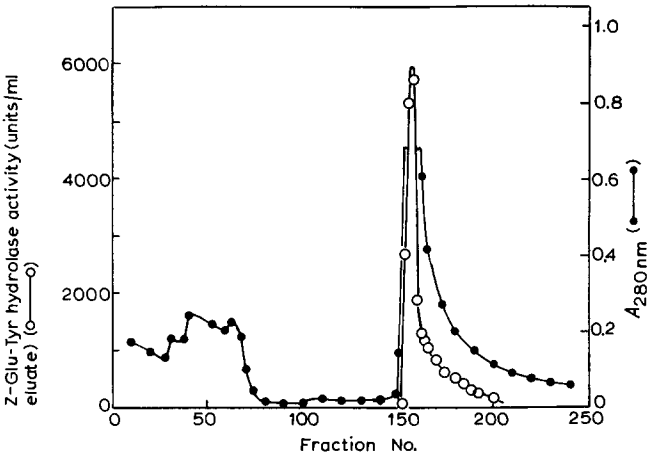


Fig. 3. P-cellulose chromatography of Aspergillus acid carboxypeptidase from Duolite CS-101 run. The column (2 cm × 50 cm) was equilibrated with 0.01 M acetate buffer (pH 4.0). 100 ml of the enzyme were applied to the column. Elution was performed with 0.01 M acetate buffer (pH 4.0), with increasing concentration of salt gradient using a mixing chamber of 500 ml. The buffer in the reservoir containing 0.05 M NaCl was tightly connected to the mixing chamber. Flow rate: 20 ml/h. Fraction volume: 5 ml. ●—●, absorbance at 280 nm; ○—○, Z-Glu–Tyr hydrolase activity at pH 3.1.

The active fraction eluted from Sephadex G-100 was run through a column of Duolite CS-101. As shown by Fig. 2, the acid carboxypeptidase activity was eluted into two components, the specific activity for the first (Peak 1) was 2300 units, and was about twice that of the second (Peak 2).

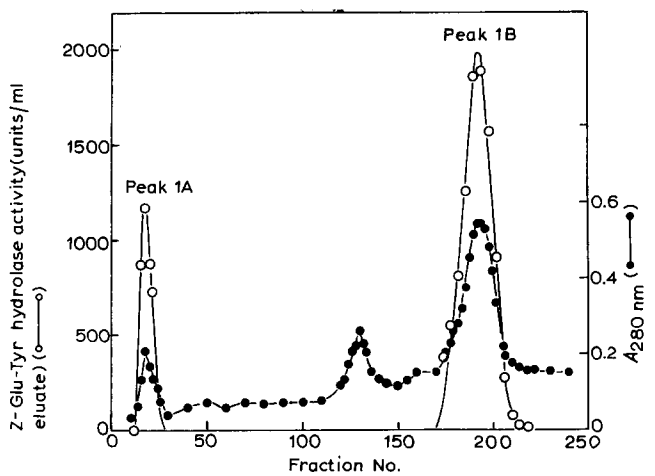


Fig. 4. DEAE-cellulose chromatography of Aspergillus acid carboxypeptidase from P-cellulose run. The column (2 cm × 40 cm) was equilibrated with 0.005 M acetate buffer (pH 5.0). 125 ml of the enzyme were applied to the column. Elution was performed with 0.005 M acetate buffer (pH 5.0), with increasing concentration of salt and pH gradient using a mixing chamber of 500 ml. 0.005 M acetate buffer (pH 3.0) in the reservoir containing 0.1 M NaCl was tightly connected to the mixing chamber. Flow rate: 30 ml/h. Fraction volume: 5 ml. ●—●, absorbance at 280 nm; ○—○, Z-Glu–Tyr hydrolase activity at pH 3.1.

Peak 1 was run through a column of P-cellulose. The result of the P-cellulose chromatography is shown in Fig. 3.

Chromatography on DEAE-cellulose was then performed. As shown by Fig. 4, the acid carboxypeptidase was eluted as two components. The specific activity for the fraction of Peak 1A was 5800 units, and for the second fraction (Peak 1B) 4300 units.

Further purification of the second peak (Peak 1B) was attempted using Sephadex G-100 with 0.01 M acetic acid. The final step of the gel filtration is shown in Fig. 5. The specific activity for the larger fraction (Peak 1Ba) was 5200 units and

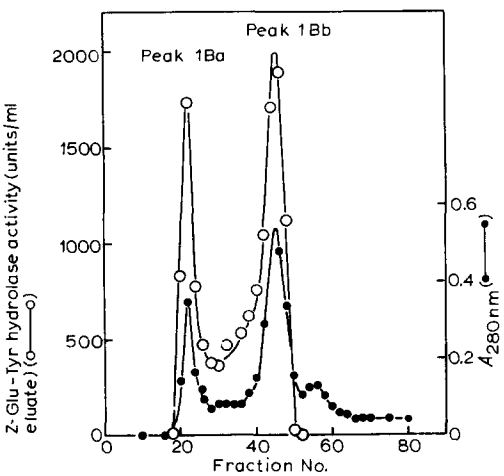


Fig. 5. Gel filtration of Aspergillus acid carboxypeptidase from DEAE-cellulose run. The column (2 cm × 67 cm) of Sephadex G-100 was equilibrated with 0.01 M acetic acid. Elution was performed with the same solution. Flow rate: 20 ml/h. Fraction volume: 3.4 ml. ●—●, absorbance at 280 nm; ○—○, Z-Glu–Tyr hydrolase activity at pH 3.1.

for the smaller one (Peak 1Bb) 5100 units per unit absorbance at 280 nm, respectively.

The highly purified fractions of Peak 1A (Fig. 4), Peak 1Ba and Peak 1Bb (Fig. 5) have the same K_m and v_{max} values towards Z-Glu–Tyr at pH 3.1 and 30° (Table III).

Portions from each highly purified fraction eluted from DEAE-cellulose (Fig. 4) and Sephadex G-100 (Fig. 5) were assayed for endopeptidase activity with milk casein at a wide range of pH. Perhaps the preparation did not have any endopeptidase activity.

TABLE III

KINETIC PARAMETERS OF CARBOXYPEPTIDASE ACTION OF THE THREE FRACTIONS OF ASPERGILLUS ACID CARBOXYPEPTIDASE TOWARDS Z-GLU–TYR AT pH 3.1 AND 30°

Enzyme preparation	$K_m \times 10^3$ (*M*)	$v_{max} \times 10^4$ (*μmoles/min per* $A_{280\,nm}$)
Peak 1A from DEAE-cellulose	1.25	1.88
Peak 1Ba from Sephadex G-100	1.25	1.90
Peak 1Bb from Sephadex G-100	1.25	1.68

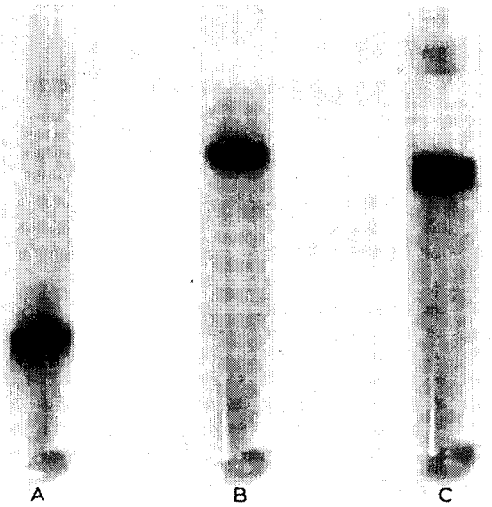


Fig. 6. Disc electrophoreses of Aspergillus acid carboxypeptidase. (A) Anodic run at pH 9.5 in 7.0 % polyacrylamide gel. (B) Anodic run at pH 8.0 in 7.5 % gel. (C) Cathodic run at pH 2.3.

Acrylamide gel electrophoreses of the acid carboxypeptidase

The highly purified product from the final Sephadex G-100 gel filtration of the larger component of the acid carboxypeptidase migrates as a single band on disc electrophoresis at pH 9.5, 8.0 and 2.3 (Fig. 6).

Ultracentrifugal analyses of the acid carboxypeptidase

Ultracentrifugation of the larger form of the acid carboxypeptidase at 60 000 rev./min in acetate buffer (pH 4.0, I 0.2) showed a single component (Fig. 7). The sedimentation constant gave a value $s^{\circ}_{20,w}$ of 7.3 S at zero concentration (Fig. 8).

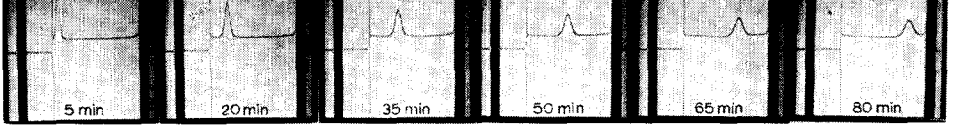


Fig. 7. Schlieren diagram of an ultracentrifuge run of Aspergillus acid carboxypeptidase. The concentration of enzyme was 10.39 units absorbance at 280 nm in acetate buffer (pH 4.0 and I 0.2). The photographs were taken at a phase-plate angle of 70° at 5, 20, 35, 50, 65 and 80 min after 60 000 rev./min were reached. The direction of sedimentation is towards the right.

Estimation of the molecular weight of the acid carboxypeptidase

Using the method described by YPHANTIS[7], the extrapolated mol. wt. value was 139 000.

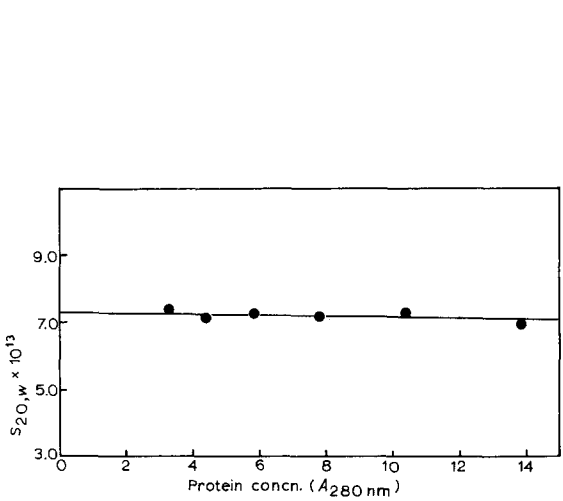
According to the gel filtration mol. wt. values of 155 000 for the larger form or polymer and 51 000 for the smaller form or monomer were obtained in the absence of NaCl, and in the presence of 0.2 M NaCl a value of 135 000 was obtained (Fig. 9).

When the smaller form (Peak 1Bb) of the acid carboxypeptidase was subjected

to gel filtration on Sephadex G-100 with 0.2 M NaCl in 0.01 M sodium acetate buffer, the enzymatic activity was eluted at the same volume peak as the larger form of the peptidase. It was assumed that part of the acid carboxypeptidase was dissociated as a subunit or monomer at low ionic strength.

Optimum pH of the acid carboxypeptidase

The effects of pH for α-amino substituted peptides on the activities of the acid carboxypeptidase are shown in Figs. 10A and 10B. The optimum pH with



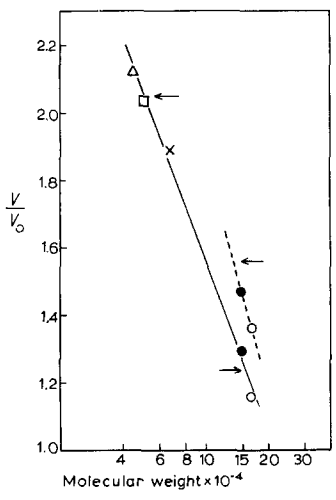


Fig. 8. Sedimentation constants, $s^\circ_{20,w}$, of Aspergillus acid carboxypeptidase as a function of protein concentration in acetate buffer (pH 4.0, I 0.2). The protein concentration was expressed as absorbance at 280 nm. The extrapolated value, $s^\circ_{20,w} = 7.3$ S.

Fig. 9. Molecular weight determination of Aspergillus acid carboxypeptidase with Sephadex G-200. The column (2 cm × 70 cm) was eluted 0.05 M acetate buffer (pH 3.1) in the absence (———) or presence of 0.2 M NaCl (— — —). △, egg albumin; □, Taka-amylase A; ×, bovine hemoglobin; ●, bovine γ-globulin; ○, bovine thyroglobulin. Molecular weights estimated for the larger form or polymer were 155 000 in the absence and 135 000 in the presence of 0.2 M NaCl. Molecular weight estimated for subunit or monomer was 51 000.

Z-Tyr–Leu as substrate was 3.5. The optimum with Z-Glu–Tyr was 3.1, and that with Z-Gly–Pro–Leu–Gly, 3.2. The pH optimum with Bz-Gly–Lys was estimated as 3.5. The amino acids released from synthetic substrates were identified by thin-layer chromatography.

Kinetic characteristics

Lineweaver–Burk plots of the acid carboxypeptidase catalyzed hydrolysis of Z-Glu–Tyr, Z-Tyr–Leu and Bz-Gly–Lys are shown in Figs. 11A and 11B. The K_m and v_{max} values for Z-Glu–Tyr at pH 3.1 and 30° are $1.25 \cdot 10^{-3}$ M and $1.9 \cdot 10^4$ μmoles tyrosine per min for $A_{280\,nm}$, the values for Z-Tyr–Leu at pH 3.5 and 30° are $1.0 \cdot 10^{-3}$ M and $1.64 \cdot 10^5$ μmoles leucine per min for $A_{280\,nm}$, and the values for Bz-Gly–Lys at pH 3.5 and 30° are $4.0 \cdot 10^{-3}$ M and $5.5 \cdot 10^2$ μmoles lysine per min for $A_{280\,nm}$.

The effects of substrate analogues and product on the Z-Tyr–Leu hydrolyzing activity of the acid carboxypeptidase at pH 3.5 and 30° are shown in Fig. 12. The kinetic plots of inhibition by Z-Tyr–D-Leu at two different concentrations showed non-competitive inhibition. Non-competitive inhibitions were observed with Z-Tyr–Leu-NH_2, Tyr–Leu and leucine when Z-Tyr–Leu was used as substrate. The K_i values for Z-Tyr–Leu-NH_2, Tyr–Leu, leucine and Z-Tyr–D-Leu at a concentration of

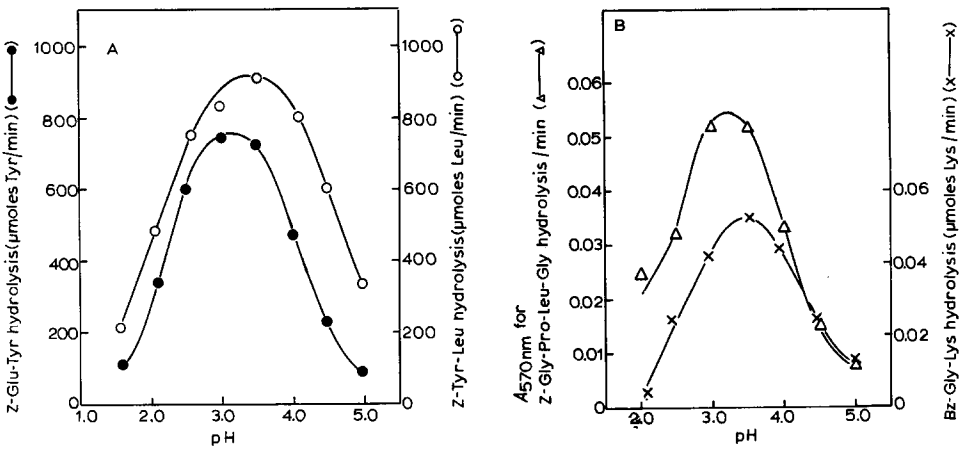


Fig. 10. Effect of pH on the activity of Aspergillus acid carboxypeptidase at 30°. The rates of hydrolysis of $5 \cdot 10^{-4}$ M Z-Glu–Tyr (●—●) in 0.05 M acetate buffer were measured by 30.7 Z-Glu–Tyr hydrolase activity units of solutions. For hydrolysis of $5 \cdot 10^{-4}$ M Z-Tyr–Leu (○—○) and $5 \cdot 10^{-4}$ M Z-Glu–Pro–Leu–Gly (△—△), 1.4 and 123 activity units of Z-Glu–Tyr hydrolase solutions were used, respectively. For hydrolysis of 10^{-3} M Bz-Gly–Lys (×—×), 290 activity units of Z-Glu–Tyr hydrolase solutions were used.

$2 \cdot 10^{-5}$ M were calculated as $4.4 \cdot 10^{-5}$ M, $6.3 \cdot 10^{-5}$ M, $2.2 \cdot 10^{-5}$ M and $5.7 \cdot 10^{-5}$ M, respectively.

Confirmation of carboxypeptidase activity

Several of Z-oligopeptides, including Z-Phe–Tyr–Leu, Z-Gly–Pro–Leu–Gly and Z-Gly–Pro–Leu–Gly–Pro, were hydrolyzed by the enzyme, liberating the carboxyterminal amino acid residue. Thin-layer chromatography confirmed that cleavage was at the peptide bond.

The determination and confirmation of the release of carboxyterminal amino acid residues from Z-Gly–Pro–Leu–Gly upon prolonged incubation (1–20 h) at pH 3.2 and 30° by the acid carboxypeptidase is shown in Table II.

When the carboxyterminal amino acid contained an amide group, as in the case of Z-Tyr–Leu-NH_2, the ability to serve as substrate was lost (Table IV). These findings indicated that the enzyme has a strict requirement for the free carboxyl amino acid in the carboxyterminal position.

On the basis of the results presented in this paper it is proved that the exopeptidase of Aspergillus is one of a new type of carboxypeptidase.

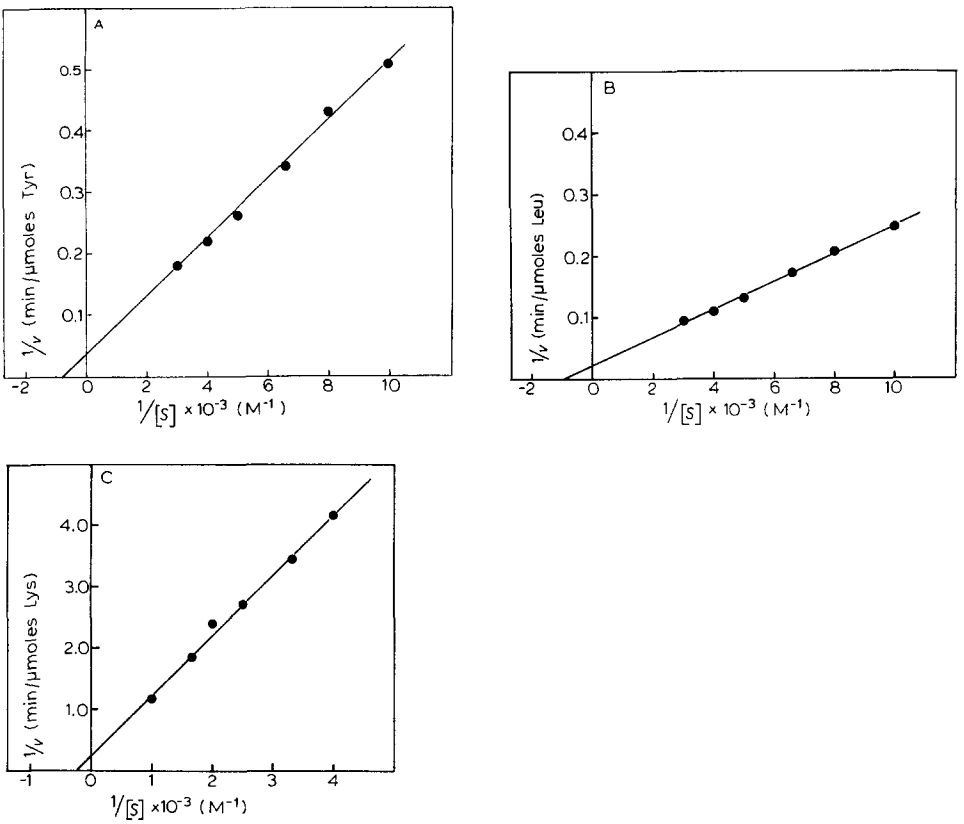


Fig. 11. Lineweaver–Burk plots of Aspergillus acid carboxypeptidase catalyzed hydrolyses of Z-Glu–Tyr at pH 3.1, Z-Tyr–Leu at pH 3.5 and Bz-Gly–Lys at pH 3.5 (30°). (A) Substrate, Z-Glu–Tyr ($1.0 \cdot 10^{-4}$–$3.33 \cdot 10^{-4}$ M) at pH 3.1. Concentration of the acid carboxypeptidase was $1.40 \cdot 10^{-3}$ units absorbance at 280 nm. The estimated values were $K_m = 1.25 \cdot 10^{-3}$ M, and $v_{max} = 1.9 \cdot 10^{4}$ μmoles tyrosine per min for $A_{280\ nm}$. (B) Substrate, Z-Tyr–Leu ($1.0 \cdot 10^{-4}$–$3.33 \cdot 10^{-4}$ M) at pH 3.5. Concentration of the enzyme was $2.78 \cdot 10^{-4}$ units absorbance at 280 nm. The estimated values were $K_m = 1.0 \cdot 10^{-3}$ M, and $v_{max} = 1.64 \cdot 10^{5}$ μmoles leucine per min for $A_{280\ nm}$. (C) Substrate, Bz-Gly–Lys ($0.125 \cdot 10^{-3}$–$1.0 \cdot 10^{-3}$ M) at pH 3.5. Concentration of the enzyme was $7.14 \cdot 10^{-2}$ units absorbance at 280 nm. The estimated values were $K_m = 4.0 \cdot 10^{-3}$ M, and $v_{max} = 5.5 \cdot 10^{2}$ μmoles lysine per min for $A_{280\ nm}$.

Specificity of the acid carboxypeptidase

The relative rates of hydrolysis of a series of peptides by the enzyme are shown in Table IV. The specificity of the acid carboxypeptidase displays the features typical of all pancreatic carboxypeptidases[13,14], hydrolysis of the specific substrate R-X–Y between X and Y (R = peptide residue, Z-, Bz-, Ac-). The amino acid in position Y must have a free carboxyl group; dipeptides (free amino group) are not hydrolyzed. The enzyme hydrolyzes most of the α-amino substituted peptides. The carboxypeptidase was inactive on a number of amides tried at pH 3.0. A peculiarity of its specificity, however, was its inability to hydrolyze the peptide bond of tripeptides.

Several qualitative conclusions can be drawn from the results. The acid carboxypeptidase splits off neutral and basic amino acids from the substrates and has the ability to hydrolyze the peptide bond . . .X–Pro in oligopeptides.

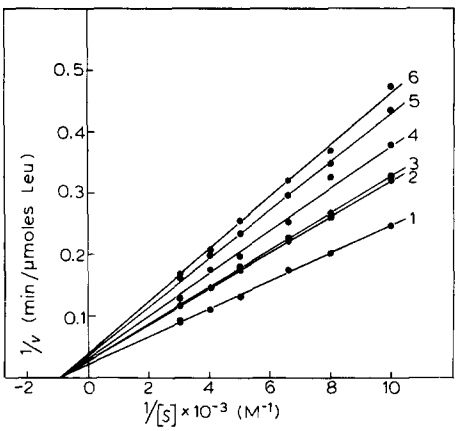


Fig. 12. Kinetics of inhibition of the hydrolysis of Z-Tyr–Leu of Aspergillus acid carboxypeptidase at pH 3.5 and 30° by Z-Tyr–Leu-NH_2, Tyr–Leu, leucine and Z-Tyr–D-Leu. The concentration of the enzyme was $2.78 \cdot 10^{-4}$ units absorbance at 280 nm. The K_i for Z-Tyr–Leu-NH_2, Tyr–Leu, leucine and Z-Tyr–D-Leu at the concentration of $2 \cdot 10^{-5}$ M are $4.4 \cdot 10^{-5}$ M, $6.3 \cdot 10^{-5}$ M, $2.2 \cdot 10^{-5}$ M and $5.7 \cdot 10^{-5}$ M. (1) Control; (2) $2 \cdot 10^{-5}$ M Tyr–Leu; (3) $2 \cdot 10^{-5}$ M Z-Tyr–D-Leu; (4) $2 \cdot 10^{-5}$ M Z-Tyr–Leu-NH_2; (5) 10^{-4} M Z-Tyr–D-Leu; (6) $2 \cdot 10^{-5}$ M leucine.

TABLE IV

COMPARATIVE RATE OF HYDROLYSIS FOR ASPERGILLUS ACID CARBOXYPEPTIDASE ON A RANGE OF PEPTIDES AND AMIDES

The rate of hydrolysis of Z-Glu–Tyr is arbitrarily taken to be 100. C-terminal amino acid residues of the substrates are expressed as R-X–Y.

Peptides	*Relative activity*	*Peptides*	*Relative activity*
α-Amino-substituted peptides		Amides	
(1) X = aromatic amino acid		Z-Tyr–Leu-NH_2*	0
Z-Tyr–Leu	2502	Z-Trp–Phe-NH_2*	0
Z-Tyr–D-Leu	0	Z-Gly–Phe-NH_2*	0
Z-Phe–Leu*	1968	Z-Gly–Leu-NH_2*	0
Z-Phe–Tyr*	916	Z-Ala–Phe-NH_2*	0
Z-Phe–Tyr–Leu*	1025	Tripeptides	
Ac-Phe–$TyrI_2$*	1	Gly–Gly–Gly	0
(2) X = acidic amino acid		Ala–Gly–Gly	0
Z-Glu–Phe*	184	Leu–Gly–Gly	0
Z-Glu–Tyr	100	Gly–Gly–Leu	0
(3) X = leucine		Dipeptides	
Z-Gly–Pro–Leu–Gly	30	Tyr–Leu	0
(4) X = glycine		Gly–Gly	0
Z-Gly–Leu	4	Gly–Leu	0
Z-Gly–Phe	2	Gly–Asp	0
Z-Gly–Trp	0.2	Leu–Gly	0
Z-Gly–Pro	0	Gly–D-Asp	0
Z-Gly–Pro–Leu–Gly–Pro	0.2		
Bz-Gly–Lys	1		
(5) X = valine			
Z-Val–Glu	0.4		
(6) X = proline			
Z-Gly–Pro–Leu	trace		

* Partially insoluble at pH 3.0.

Significant enzymatic hydrolysis has been found for substrates in which the penultimate position (X) of the carboxyterminal is occupied by an aromatic or acidic residue. It will be noted that, for substrates of the type Z-X–Y, a change in the X position from tyrosine to glycine, from phenylalanine to glutamic acid, or from glutamic acid to glycine leads to a marked decrease in the velocity rate. The favorable effect of the aromatic and carboxyl substituents at the side chain of the X residue is emphasized by the finding that where X is an aromatic amino acid residue and glutamic acid the values of the velocity rate are approximately one-sixhundredth and one-tenth that for X = glycine, resepctively. It may be concluded, therefore, that with small synthetic substrates of the type Z-X–Y, where the X–Y bond is broken, the acid carboxypeptidase exhibits a preference for aromatic or carboxyl in the X position. Only limited data are available on the effect of changing the X and Y groups on the side chain specificity of the enzyme, but it would appear that this conclusion holds for substrates in which the X group is changed from tyrosine or phenylalanine to glycine.

Thus Z-Tyr–Leu, Z-Glu–Tyr and Z-Gly–Pro–Leu–Gly are good substrates. Z-Phe–Tyr–Leu, Z-Phe–Leu, Z-Phe–Tyr and Z-Glu–Phe were cleaved very easily by the enzyme, however, they are extremely insoluble at the acid end of the pH range of carboxypeptidase activity (pH 2–4).

Stability

Dilute solutions of the enzyme in its impure or pure form were stable when stored at 4°. On freeze–drying of dilute or concentrated aqueous solutions extensive inactivation occurred. The acid carboxypeptidase was stable at temperatures of about 50° for 10 min, but rapid inactivation occurred at temperatures of about 60° for 10 min.

Judging from preliminary experiments, the acid carboxypeptidase can be inhibited by 0.25 μM DFP at pH 6.0 and 0°, whereas, treatment with 0.05 M EDTA or *o*-phenanthrolin at pH 5.2 resulted in no loss of activity. The thiol reagent, PCMB, inhibited the enzymatic activity when introduced at pH 5.0 at a final concentration of 10^{-4} M at 5°.

DISCUSSION

The work reported here shows that at least two different types of enzyme with proteolytic activity in the acid pH range are found in the culture filtrate of Aspergillus. One of these enzymes readily hydrolyzes carboxypeptidase substrates at the acid end of the pH range and differs markedly from the other in its caseinolytic activity. The other enzyme, which has caseinolytic activity at pH 2.7 and is designated as aspergillopeptidase A (ref. 1) does not hydrolyze either of the synthetic α-amino substituted peptides.

From the initial step of Duolite CS-101 in Fig. 2, two clearly separated peaks of the enzymatic active materials are visible. With regard to the specific activity for the second (Peak 2), the value is approximately one-half for the first (Peak 1). The presence in this stage of multiple forms of size isomeric proteins having two different specific activity values for the enzyme is not yet definitely established.

A possible identity of the second peak is an artifact resulting from digestion by acid proteinase, aspergillopeptidase A.

When the faster peak (Peak 1A) of the DEAE-cellulose step was rechromatographed on DEAE-cellulose, almost all the enzymatic activity was obtained in the same region of the chromatogram, indicating that a small section of the surface structure of the enzyme molecule was changed.

The enzyme has different gel chromatographic behavior on final Sephadex G-100 (Fig. 5). It was assumed that dissociation to monomer or subunit was observed at low ionic strength. The similar anomalous behavior on Sephadex gel filtration was observed with phaseolin[9-11] by WELLS.

When a mixture of the acid carboxypeptidase and blue dextran was subjected to gel filtration on Sephaex G-200 or G-100 with 0.01 M acetic acid or 0.05 M acetate buffer (pH 3.1) the acid carboxypeptidase was eluted at the void volume position of the blue dextran. The results showed that two components when present together at relatively high ionic strength (*i.e.* 0.2 M NaCl in 0.05 M sodium acetate buffer, pH 3.1), the elution profile of the acid carboxypeptidase can be separated from the void volume position of blue dextran. The effects of ionic strength indicated that, like the CM-cellulose complex[12] of chymotrypsin, the basis for the formation of the acid carboxypeptidase–blue dextran complex is primarily electrostatic.

The data in Figs. 11A and 11B indicated that the replacement of the Glu–Tyr of Z-Glu–Tyr by Tyr–Leu does not alter the K_m values significantly, while the replacement causes a remarkable increase in the velocity rate, $v_{max.}$

The slow hydrolysis of Bz-Gly–Lys or Z-Gly–Pro–Leu–Gly–Pro seems to imply that the specificity requirement of the acid carboxypeptidase may be caused in part by an important influence of the penultimate position (X) of the carboxyterminal of the peptides.

These observations were considered as suggesting that the Aspergillus acid carboxypeptidase differs from the known pancreatic carboxypeptidase A (ref. 13) and B (ref. 14). The enzymatic properties of Aspergillus acid carboxypeptidase towards Z-dipeptides indicate that this fungal enzyme is similar to French bean phaseolin[9-11], barley carboxypeptidase[15], citrus fruit carboxypeptidase[16] and *Penicillium janthinellum* peptidase B (ref. 17), but nevertheless shows important differences in the optimum pH for digestion of substrates. As regards specificity, partially purified carboxypeptidase C (ref. 16) differs from carboxypeptidase A chiefly in that carboxypeptidase C releases proline and splits off the basic amino acid at pH 5.3.

The only comparable fungal enzyme mentioned in the literature is Penicillum peptidase B (ref. 17), which has a much higher affinity for its substrate, the K_m being $5 \cdot 10^{-4}$ M for Z-Glu–Tyr at pH 4.7. Barley carboxypeptidase[15] has a lower affinity for its substrate, the K_m being $6.7 \cdot 10^{-3}$ M for Z-Phe–Ala at pH 5.2. The value of the acid carboxypeptidase is close to the K_m value of $1.5 \cdot 10^{-3}$ M for Z-Glu–Tyr at pH 3.5, obtained using the porcine pepsin[18] (EC 3.4.4.1).

According to the principles of the International Union of Biochemistry Commission on Enzymes of 1961, and to the report by HARTLEY[19], this acid carboxypeptidase is now considered to belong to a new class of enzymes, tentatively termed Aspergillus acid carboxypeptidase.

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