Purification and Properties of Acid Carboxypeptidase II from *Aspergillus oryzae*

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Acid carboxypeptidase II from *Aspergillus oryzae* was purified from the rivanol non-precipitated fraction. The purified enzyme was homogeneous on polyacrylamide gel disc electrophoresis. The optimum activity of the enzyme lay at pH 3.0 for carbobenzoxy-L-glutamyl-L-tyrosine. The enzyme was inhibited by diisopropylphosphorofluoridate and SH reagents such as *p*-chloromercuribenzoate and moniodoacetate, but not by such metal chelating agents as ethylenediaminetetraacetate, α , α' -dipyridyl and *o*-phenanthroline. The molecular weight of the enzyme was estimated to be about 105,000.

In previous papers^{1,2)} peptidases from *Aspergillus oryzae* or *Asp. sojae* were found to play an important role in digestion of the peptides to yield free amino acids. The acid carboxypeptidase I from *Asp. oryzae* was purified from the rivanol non-precipitated fraction and its properties were described in a previous paper.³⁾ This paper deals with the purification and some properties of acid carboxypeptidase II as a part of the systematic studies on peptidases from *Asp. oryzae*.

MATERIALS AND METHODS

To 1 ml of 5×10^{-4} M solution of Cbz-Glu-Tyr (Protein Research Foundation, Osaka, Japan) in 0.05 M acetate buffer (pH 3.0) was added 50 μ l of enzyme solution. After incubation at 30°C for 20 min, reaction was stopped by the addition of 0.5 ml of ninhydrin solution. Then acid carboxypeptidase II activity was determined by the same method as re-

ported in the previous papers.^{3,4)} A unit of enzyme activity was defined as the enzyme quantity which liberates 1 μ mole of amino acid (as glutamic acid) from the substrate (Cbz-Glu-Tyr) per min under these conditions. All the other experimental methods were described in detail in the previous paper.³⁾

RESULTS

Column chromatography on DEAE-Sephadex A-50

The fractions of acid carboxypeptidase II from DEAE-cellulose chromatography reported previously³⁾ were adjusted to pH 6.0 and charged on a column (3 $\times$ 30 cm) of DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M phosphate buffer at pH 6.0. After the column had been washed with the same buffer, elution of the adsorbed protein was performed by a linear gradient of NaCl concentration. The reservoir was filled with 2 liters of 0.4 M NaCl in 0.01 M acetate buffer (pH 5.5) and the mixing chamber contained 2 liters of the same buffer. Figure 1 illustrates the elution pattern of acid CPase II. Fractions 198 to 248 were pooled, adjusted to pH 5.5 and desalted by dialysis against distilled water.

Abbreviations used in this paper: Cbz, carbobenzoxy; Aoc, *t*-amyloxycarbonyl; Bz, benzoxy; PyroGlu, pyroglutamic acid; β -NA, β -naphthylamide; amino acids, first three letters abbreviation; Gln, glutamine; Asn, asparagine; approx., approximately; mol. wt., molecular weight; CPase, carboxypeptidase; DFP, diisopropylphosphorofluoridate; EDTA, ethylenediaminetetraacetate.

FIG. 1. Chromatography of Acid CPase II on DEAE-Sephadex A-50.

See the text for experimental details. ○—○: peptidase activity. ●—●: O.D. at 280 $m\mu$. ×—×: NaCl concentration. 730 ml of acid CPase II solution containing 143 mg of protein (specific activity 1.18 units/mg protein) was used.

FIG. 2. Chromatography of Acid CPase II on SE-cellulose.

See the text for experimental details. ○—○: peptidase activity. ●—●: O.D. at 280 $m\mu$. ×—×: NaCl concentration. 700 ml of acid CPase II solution containing 31 mg of protein (specific activity 2.78 units/mg protein) was used.

Column chromatography on SE-cellulose

The dialyzed solution of acid CPase II from DEAE-Sephadex A-50 was adjusted to pH 3.5 and put onto the column (3×13 cm) of SE-cellulose (Serva) equilibrated with 0.01 M acetate buffer (pH 3.5). Linear gradient elution was performed by introducing 0.1 M NaCl in 0.01 M acetate buffer (pH 4.5) through a 2-liter mixing chamber containing the same buffer. The flow rate was adjusted to 100 ml/hr and 15-ml fractions were collected. A typical elution pattern of acid CPase II is

shown in Fig. 2. Fractions 94 to 150 were pooled and immediately adjusted to pH 5.5.

Gel filtration on Sephadex G-200

The above enzyme preparation obtained from SE-cellulose chromatography was concentrated to 3 ml by a Diaflo UM-1 (type; 401, Amicon Co.) and applied to a column (2.8×118 cm) of Sephadex G-200 equilibrated with 0.1 M acetate buffer at pH 5.5. Elution was performed with the same buffer and 15-ml fractions were collected. Figure 3 shows a

TABLE I. PURIFICATION OF ACID CARBOXYPEPTIDASE II.

Procedure	Total volume ml	Total activity Units	Specific activity U/mg protein	Yield %
Crude enzyme solution	10,100	5030	0.0695	100
Batchwise treatment with Amberlite IRC-50	10,200	2200	0.128	96.5
Fractionation with ammonium sulfate	235	1210	0.202	53.0
Rivanol precipitation	235	552	0.269	24.2
DEAE-cellulose chromatography	730	169	1.18	7.40
DEAE-Sephadex A-50 chromatography	700	142	2.78	6.23
SE-cellulose chromatography	740	121	4.55	5.30
Gel filtration on Sephadex G-200	146	38.7	9.55	1.70

FIG. 3. Gel Filtration of Acid CPase II through Sephadex G-200.

See the text for experimental details.

○—○: peptidase activity. ●—●: O.D. at 280 mμ.

typical elution pattern of acid CPase II. Pooled fractions of 20 to 29 were frozen and stored at -15°C . A summary of the purification procedure is shown in Table I.

Determination of molecular weight by gel filtration

Figure 4 is plots of elution volume (V_e) against logarithms of molecular weights of standard proteins and acid CPase II obtained through a Sephadex G-200 (pH 5.5) column. The molecular weight of acid CPase II determined by gel filtration method was calculated to be 105,000.

Disc electrophoresis

To check the purity of the enzyme, the final

FIG. 4. Plots of Elution Volume, V_e , against Logarithms of Molecular Weights for Proteins on a Sephadex G-200 Column.

Column (2.8×118 cm) was equilibrated with 0.1 M acetate buffer at pH 5.5. 0.1 M acetate buffer (pH 5.5) was applied for the elution of proteins.

A: trypsin. B: ovalbumin. C: human albumin. D: intestinal alkaline phosphatase. E: human albumin dimer. F: blue dextran. G: acid CPase II.

enzyme preparation was submitted to polyacrylamide gel disc electrophoresis at pH 9.4 as reported in the previous paper.³⁾ A single protein band corresponding to acid CPase II was found as shown in Fig. 5.

Action on various peptides

The hydrolytic action of the acid CPase II on various peptides was examined by the ninhydrin method. To 0.5 ml of purified enzyme solution in 0.1 M acetate buffer at pH 3.0 ($27.8 \mu\text{g}$ protein/ml, specific activity; 9.55 units/mg protein) was added 0.5 ml of

TABLE II. ACTION ON VARIOUS SYNTHETIC PEPTIDES

Peptide	Δ O.D.	Peptide	Δ O.D.	Peptide	Δ O.D.	Peptide	Δ O.D.
Gly-Gly	0	Glu-Pro	0	Leu-Gly-Glu	0	Cbz-Gln-Pro	0.018
Gly-Leu	0	Glu-Tyr	0.026	Leu-Gly-Gln	0	Cbz-Gln-Gly	0.328
Leu-Gly	0	Glu-Ser	0	Leu-Gly-Asp	0	Aoc-Gln-Pro	0.024
Gly-Phe	0	Glu-Anilide	0	Leu-Gly-Asn	0	Bz-Gly-Lys	0.063
Gly-Glu	0	Try-Glu	0	Glu-Ala-Gly	0	Cbz-Gly-Gly-Phe	0.214
Gly-Asp	0	Arg-Glu	0	Phe-Gln-Gly	0.140	Bz-Arg-NH ₂	0.021
Gly-Gln	0	Met-Glu	0	γ -Glu-CySH-Gly	0.055	Cbz-Pro-Leu-NH ₂	0.038
Gly-Asn	0	Glu-Try	0.026	PyroGlu-Ala	0.034	Cbz-Gly-Ala-NH ₂	0.023
Pro-Gln	0	Glu-Glu	0	PyroGlu-Aln-Ala	1.976	Cbz-Gly-Phe-NH ₂	0.029
Try-Glu	0	Glu-Lys	0	Cbz-Ala-Glu	1.501	Cbz-Gly-Leu-NH ₂	0.004
Tyr-Gln	0	Glu-Tyr-Glu	0.659	Cbz-Gly-Glu	0.353	Cbz-Ala-Glu-Dibenzylester	0.059
His-Glu	0.002	Glu-Try-Glu	0.393	DiCbz-Lys-Glu	1.071	Leu- β -NA	0 ^{a)}
Ala-Glu	0	Glu-Val-Phe	0.286	Cbz-Glu-Tyr	1.479	PyroGlu- β -NA	0 ^{a)}
Lys-Glu	0	Glu-Glu-Glu	0.874	Cbz-Glu-Gly	0.051	Glu- β -NA	0 ^{a)}
Phe-Glu	0	Gly-Gly-Gly	0	Cbz-Glu-Val	0	Poly-Glu (Approx. mol. wt. 72,000)	0.348
Glu-Ala	0	D,L-Ala-Gly-Gly	0.048	Cbz-Gly-Phe	1.137	Poly-Lys (Approx. mol. wt. 2600)	0
Glu-Val	0	Leu-Gly-Gly	0.039	Cbz-Gly-Try	0.203	Poly-Pro (Approx. mol. wt. 3000)	0
Gly-Gly	0	Gly-Gly-Glu	0	Cbz-Gly-Leu	0.555	Ileu-Gln-Asn-CySH-Pro-Leu-Gly-NH ₂	0.130
Gln-Gly	0	Glu-Gly-Phe	0.078	Cbz-Glu-Pro	0.048	Milk casein (pH 3.0 for 3 hr)	0.081 ^{b)}
Glu-Asp	0	Glu-Ala-Ala	0.206				

^{a)} Δ O.D. at 535 m μ measured with the reagent of serum leucine aminopeptidase assay (Iatron Co.).¹⁶⁾^{b)} Δ O.D. at 660 m μ measured by Anson's method.

each 5×10^{-4} M solution for di- and tri-peptides and 10^{-3} M solution for other peptides. After incubation at 30°C for 30 min, reaction was stopped by the addition of 0.5 ml of ninhydrin reagents, followed by heating at 100°C for 15 min and the optical density at $570\text{ m}\mu$ was measured by the same method as reported in the previous paper.³⁾ The results are summarized in Table II. Acid CPase II hydrolyzed the peptides that possess masked or bulky N-terminal. Two-tenth ml of enzyme solution ($27.8\text{ }\mu\text{g}$ protein/ml) in 0.1 M acetate buffer (pH 3.0) was added to 1 mg of α -Glu-Val-Phe (Cyclo Chemical Corp.). After incubation for 5 and 24 hr at 30°C , $10\text{-}\mu\text{l}$ aliquots of the reaction mixture were spotted on a thin-layer of Kieselgel G (E. Merck AG, Darmstadt) and developed at 25°C for 3 hr by the ascending method using a mixture of *n*-butanol, acetic acid and water (4:1:1) as the solvent. The thin-layer was then dried and detection of separated components was carried out by spraying of 0.25% solution of ninhydrin dissolved in ethanol. Based on the results shown in Fig. 6, the enzyme hydrolyzed the tripeptide from C-terminal and was considered to be CPase.

Time courses of hydrolysis of two peptides

Time courses of acid CPase II action on two peptides are shown in Fig. 7. Activity ratio for Cbz-Glu-Tyr (pH 3.0) and Cbz-Ala-Glu (pH 4.0) was 4.18:1.

Effect of pH on acid CPase II

The effect of pH on acid CPase II activity was studied with some peptides. Purified acid CPase II was found to be most active at pH 3.0 for Cbz-Glu-Tyr (Fig. 8). The enzyme was stable in the pH range of 5 to 6 (Fig. 9).

Effect of temperature on acid CPase II

The activity of purified acid CPase II at various temperatures was determined with

FIG. 5. Polyacrylamide Gel Electrophoresis of Purified Acid Carboxypeptidase II from *Aspergillus oryzae* at pH 9.4 for 45 min.

The operation was done in a cold room (4°C) for 45 min at 4 mA per gel column according to the method of Davis¹⁷⁾ for pH 9.4 gel.

FIG. 6. Thin-layer Chromatogram of Products from α -Glu-Val-Phe Hydrolyzed by Acid CPase II and Authentic Peptides and Amino Acids.

p: purple, spot 1: Phe and α -Glu-Val, spot 2: Val, spot 3: α -Glu-Val-Phe, spot 4: Glu.

See the text for experimental details.

Cbz-Glu-Tyr on 10-min incubation. The enzyme was found to be most active at 50°C as shown in Fig. 10. Heat stability test showed that the enzyme lost a half of the initial activity at 60°C after 10 min and lost its activity almost completely at 70°C on 10-min incubation (Fig. 11). Calcium ions showed no

FIG. 7. Time Courses of Hydrolysis of Peptides by Acid CPase II.

The reaction mixture consisted of 100 μ l of the purified enzyme (69.5 μ g) and 10 ml of Cbz-Glu-Tyr in 0.05 M acetate buffer at pH 3.0 (●—●) or Cbz-Ala-Glu in 0.05 M acetate buffer at pH 4.0 (○—○). Hydrolysis at 30°C was determined with 1 ml aliquots taken at various time.

FIG. 8. Effect of pH on Acid CPase II.

The reaction mixtures containing 0.2 ml of the purified enzyme, 0.3 ml of the buffer and 0.5 ml of the substrate were incubated for 10 min at 30°C. Buffers used were 0.05 M acetate buffer (pH 3.0 to 6.0), 0.25 M Tris-HCl buffer (pH 6.5 to 9.0) and 0.5 M $\text{CH}_3\text{COONa-HCl}$ buffer (pH 1.0 to 4.0). Substrate used; ●—●: Cbz-Glu-Tyr. ○—○: Cbz-Ala-Glu. ■—■: α -Glu-Tyr-Glu (unit/ml $\times 10^{-1}$). ●---●: Cbz-Glu-Tyr (Enzyme with less activity was used in this experiment.)

protection of the enzyme against heat denaturation.

FIG. 9. pH Stability of Acid CPase I.

To 25 μ l of 0.25 M buffer of various pH values, 100 μ l of purified and dialyzed enzyme solution (protein; 2.78 μ g) was added and the mixture was incubated at 37°C for 2 hr. The activities remaining after the treatments are expressed as percentages of the initial activity. Buffers used were; ●—●: acetate buffer (pH 3.5 to 6.0), ○—○: tris-HCl buffer (pH 6.5 to 9.0).

FIG. 10. Effect of Temperature on Activity of Acid CPase II.

The incubation mixtures contained 100 μ l of the purified enzyme solution (2.78 μ g) and 1 ml of 5×10^{-4} M Cbz-Glu-Tyr in 0.05 M acetate buffer (pH 3.0). Hydrolysis was assayed after 10 min at various temperatures.

Effect of metal ion on the enzyme activity

As shown in Table III, Co^{2+} , Mn^{2+} , Ni^{2+} , Pb^{2+} , Cu^{2+} and Fe^{2+} ions inhibited the enzyme to some extent at 10^{-3} M. Remarkable in-

FIG. 11. Heat Stability of Acid CPase II.

The incubation mixtures consisting of 2.78 μ g of the purified enzyme and 100 μ l of 0.05 M acetate buffer at pH 5.0 with or without 10^{-2} M of $\text{Ca}(\text{CH}_3\text{COO})_2$, were incubated at various temperatures for 10 min. The activities remaining after the preincubation are expressed as percentages of the initial activity. ●—●: without $\text{Ca}(\text{CH}_3\text{COO})_2$. ○—○: $\text{Ca}(\text{CH}_3\text{COO})_2$ added.

TABLE III. EFFECT OF VARIOUS METAL SALTS ON ACID CARBOXYPEPTIDASE II

The assay system contained 0.2 ml of metal salt (pH 5.5), 0.3 ml of dialyzed enzyme (2.78 μ g) and 0.5 ml of 10^{-3} M Cbz-Glu-Tyr in 0.1 M acetate buffer (pH 3.0). Hydrolysis was assayed after 10 min at 30°C with preincubation for 10 min at 30°C. Enzyme activities were assayed by ninhydrin method and are expressed as percentages of the activity in the absence of metal salts.

Metal salts added	Concentration (M)	Relative activity (%)
None		100
CoSO_4	10^{-3}	67.0
MnCl_2	10^{-3}	67.0
NiSO_4	10^{-3}	79.3
ZnSO_4	10^{-3}	87.1
MgSO_4	10^{-3}	89.8
CaCl_2	10^{-3}	93.5
FeSO_4	10^{-3}	79.4
$\text{Pb}(\text{CH}_3\text{COO})_2$	10^{-3}	77.7
CuSO_4	10^{-3}	68.7
HgCl_2	4×10^{-3}	11.6

inhibition of the enzyme was observed by incubation with 4×10^{-3} M Hg^{2+} ions.

TABLE IV. EFFECT OF VARIOUS REAGENTS ON THE ACID CARBOXYPEPTIDASE II

The assay system contained 0.2 ml of substances listed below (pH 5.5), 0.3 ml of dialyzed enzyme (2.78 μ g) and 0.5 ml of 10^{-3} M Cbz-Glu-Tyr in 0.1 M acetate buffer (pH 3.0). Hydrolysis was assayed after 10 min at 30°C with preincubation for 20 min at 30°C.

Inhibitor	Concentration M	Remaining activity %
None		100
EDTA	5×10^{-2}	85.6
EDTA	10^{-2}	95.4
EDTA	5×10^{-3}	94.6
EDTA	4×10^{-3}	90.4
EDTA	10^{-3}	97.4
EDTA (Dialysis for 12 hr)	10^{-3}	102.0
α, α' -Dipyridyl	4×10^{-3}	114.3
<i>o</i> -Phenanthroline	4×10^{-3}	119.0
<i>p</i> -Chloromercuribenzoate	10^{-3}	13.8
Monoiodoacetate	4×10^{-3}	45.0
Sodium lauryl sulfate	10^{-3}	10.2
Potato inhibitor	0.025 %	82.0
Sodium hexametaphosphate	4×10^{-3}	88.6
Thiourea	10^{-3}	81.2
β -Mercaptoethanol	4×10^{-3}	89.5
Sodium thioglycolate	4×10^{-3}	68.5
DFP	10^{-2}	10.4

Effect of various reagents on the enzyme activity

Table IV shows the results of experiments undertaken in an attempt to find out some informations about the active site of acid CPase II. Metal chelating agents such as EDTA, α, α' -dipyridyl and *o*-phenanthroline did not show any inhibitory effect on the peptidase activity. The enzyme activity was inhibited by DFP, sodium lauryl sulfate and by specific sulfhydryl reagents like *p*-chloromercuribenzoate and monoiodoacetate.

DISCUSSION

The molecular weight of *Asp. oryzae* acid CPase II was determined to be about 105,000 by gel filtration method. It is noteworthy that this value is larger than the molecular weights of all other known CPase except for

the acid CPase from *Asp. saitoi* (trimer; 155,000⁸⁾) and the acid CPase I from *Asp. oryzae* (120,000³⁾). For example, the molecular weights of bovine CPase A α ⁶⁾, spiny pacific dogfish CPase A,⁷⁾ CPase B,⁸⁾ fungal CPase of *Phymatotrichum omnivorum*⁹⁾ and *Asp. saitoi*⁵⁾ (monomer) and barley CPase¹⁰⁾ were 34,600, 35,000, 51,000 and 90,000 respectively.

Since the optimum pH for Cbz-Glu-Tyr lies at pH 3.0, the CPase II from *Asp. oryzae* was named acid CPase. This value is much lower compared with the optimum pH of CPases from other sources except that of *Asp. saitoi*.¹¹⁾ The optimum pHs are 5.2 with Cbz-Gly-Ala for barley CPase,¹⁰⁾ 7.0 with Cbz-Gly-Phe for *Phymatotrichum omnivorum* CPase,⁹⁾ 5.3 with Cbz-Leu-Phe for citrus fruit CPase C,¹²⁾ 6.0 to 6.2 for brewer's yeast peptidase α , β ¹³⁾ and 3.1 with Cbz-Glu-Tyr for *Asp. saitoi* acid CPase.¹¹⁾

The acid CPase II from *Asp. oryzae* was inhibited by SH reagents, like yeast peptidase α , β ¹³⁾ and barley CPase,¹⁰⁾ but not inhibited by metal chelating agents, like barley CPase,¹⁰⁾ yeast peptidase β ,¹³⁾ CPase C¹²⁾ and the acid CPase from *Asp. saitoi*.⁵⁾ Also, acid CPase II from *Asp. oryzae* was inhibited by DFP, like yeast peptidase β ,¹³⁾ CPase C¹²⁾ and the acid CPase from *Asp. saitoi*.⁵⁾

Acid CPase II degraded poly- α ,L-glutamic acid as CPase B¹⁴⁾ yeast proteinase B¹⁵⁾ and acid CPase I³⁾ and also degraded the peptides that possess bulky N-terminus as acid CPase I.³⁾

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