A 155K Acid Carboxypeptidase O from *Aspergillus oryzae*

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Received September 3, 1985

When *Aspergillus oryzae* IAM 2640 was grown in liquid medium, a high molecular weight acid CPase was produced exclusively in the medium. The enzyme was purified and characterized. The purified enzyme was homogeneous by PAGE at pH 9.4, by isoelectric focusing, and by FPLC, and its molecular weight was 155K by gel filtration. When the 155K enzyme was electrophoresed in SDS-polyacrylamide gel, a single band with a molecular weight of 73K was obtained. The enzyme was inhibited by TPCK and PMSF but not IAA or PCMB. The K_m and k_{cat} values of the enzyme for Z-Glu-Tyr, angiotensin, and bradykinin at pH 3.7 and 30°C were 0.48 mM, 86 sec⁻¹, 0.1 mM, 0.34 sec⁻¹, and 0.06 mM, 14.7 sec⁻¹, respectively. The enzyme hydrolyzed proangiotensin and bradykinin sequentially at pH 3.7 from their C-terminus. The enzyme shared an antigen with low molecular weight *A. oryzae* acid CPase O-1.

Acid CPases release most amino acid residues, including proline, from the C-terminal of peptides and proteins at acidic pHs. Acid CPases have been isolated and purified from various sources such as *Aspergillus saitoi*,^{1~5)} *A. oryzae*,^{3~6)} *Penicillium janthinellum*,^{7~11)} *P. roqueforti*,¹²⁾ baker's yeast,¹³⁾ *Neurospora crassa*,¹⁴⁾ *Picnoporus sanguineus*,¹⁵⁾ citrus fruit and leaves,^{16,17)} barley,¹⁸⁾ French beans,¹⁹⁾ and wheat bran.²⁰⁾

Acid CPases of *A. oryzae* are produced extracellularly on both solid bran and liquid cultures. We reported the purification and some properties of acid CPase O-1 and O-2 from *A. oryzae* IAM 2640.⁶⁾ The two enzymes are identical in most properties and have a molecular weight of about 63K, but their action on substrates with Pro-X bonds and their isoelectric points differed.

We found that when *A. oryzae* IAM 2640 was grown in a liquid medium of 5% rice bran, the 155K molecular weight acid CPase was

produced exclusively.

MATERIALS AND METHODS

Materials. *N*-Acylpeptides, proangiotensin, angiotensin, and bradykinin were purchased from the Peptide Institute, Inc., Osaka. An isoelectric focusing column and Ampholine were obtained from LKB-Produkter AB, Sweden. All other chemicals were of reagent grade.

Organism. *A. oryzae* IAM 2640 was used as the source of acid CPase. The strain was isolated by Sakaguchi and Yamada²¹⁾ from *sake-koji* at Kyoto.

Acid CPase assay. The enzyme was assayed routinely with Z-Glu-Tyr in 50 mM sodium acetate buffer. One katal of the 155K acid CPase O was defined as the amount of enzyme required to liberate one mol of tyrosine from Z-Glu-Tyr per sec at 30°C and pH 3.7, according to the IUPAC and IUB recommendation.²²⁾

Protein measurement. Protein was measured by the absorbance at 280 nm ($E_{1\text{cm},280\text{nm}}^{1\%} = 18.8$) using a Shimadzu UV 100-02 spectrophotometer.

Submerged culture. For large-scale cultivation we used a 30-liter jar fermentor (Marubishi Co., Tokyo). One kg of

Abbreviations: CPase, carboxypeptidase; FPLC, fast protein liquid chromatography; C-, carboxy-; PAGE, polyacrylamide gel electrophoresis; Z-, benzyloxycarbonyl-; IAA, monoiodoacetic acid; PCMB, *p*-chloromercuribenzoate; TPCK, tosyl-phenylalanine chloromethyl ketone; PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetate; S-PI, *Streptomyces* pepsin inhibitor. The abbreviations of amino acid residues denote the L-forms.

rice bran and 100 g of KH_2PO_4 were added to 19 liters of tap water and the mixture was adjusted to pH 4.5 with conc. HCl. The medium was sterilized at 121°C for 30 min and inoculated with 400 ml of seed culture. Cultivation was at 30°C with aeration of 10 liters/min and agitation of 250 rpm for 0~24 hr and 400 rpm for 24~72 hr. Seed cultures were cultivated in a 3-liter Sakaguchi flask containing the same medium inoculated with one platinum loop of spores for 2 days on a reciprocal shaker at 30°C.

Purification of the enzyme. All steps were done at 4°C. The culture broth was filtered through a Toyo Glass filter type GC-90 and the culture filtrate was adjusted to pH 5.2 with 2N NaOH. An equal volume of cold ethanol (about 0°C) was added to the culture filtrate. The resulting precipitate was collected by centrifugation at $8,000 \times g$, and lyophilized. The crude enzyme precipitate was extracted three times with 600 ml of distilled water. The supernatant was then dialyzed against 0.005 M acetate buffer (pH 5.0). Next the enzyme solution was put on DEAE-Sephadex A-50 column (4 × 50 cm) that had been equilibrated with 0.005 M acetate buffer (pH 5.0). The enzyme was eluted with an NaCl gradient (0~0.2 M) in 0.005 M acetate buffer, pH 5.0, using a 500-ml mixing chamber. The active fraction was then put on a column (2 × 60 cm) of CM-Sephadex C-50 that had been equilibrated with 0.01 M acetate buffer, pH 4.0. The enzyme was eluted with an NaCl gradient (0~0.1 M) in 0.01 M acetate buffer, pH 4.0, using a 500-ml mixing chamber. The active fraction was dialyzed against 0.01 M acetate buffer, pH 4.0, and the dialyzed enzyme solution was put on a column (2 × 70 cm) of SP-Sephadex C-50. Elution was done with 0.01 M acetate buffer (pH 4.0), by an increasing pH gradient, using a 500-ml mixing chamber. The reservoir, with 0.01 M acetate buffer (pH 5.0), was tightly connected to the mixing chamber. Pooled active fractions were frozen and stored at -25°C. About 150 mg of the purified enzyme was obtained.

Enzyme characterization. Isoelectric focusing²³⁾ was done on an LKB column (110 ml) containing 1% carrier ampholite (Ampholine) with a pH from 3.5 to 5.0. The phoresis was at 450 V for 48 hr at about 4°C.

PAGE was done by the standard pore formation of Davis at pH 9.4.²⁴⁾ SDS-PAGE was run as described by Weber and Osborn,²⁵⁾ using gel with a crosslinkage of 10%. Protein bands in the gel were stained with Coomassie Brilliant Blue R-250, and carbohydrate bands with periodic acid-Schiff reagent prepared by the method of Zacharius *et al.*²⁶⁾

The antiserum to *A. oryzae* acid CPase O-1, that was produced on solid bran culture was prepared as described previously.⁶⁾

The molecular weight was measured by the gel filtration method described previously.⁶⁾ The SDS-polyacrylamide gel method of Weber and Osborn²⁵⁾ was also used. Proteins of known molecular weight served as reference markers.

The CD spectrum was measured with a JASCO J-20A automatic recording spectropolarimeter at room temperature and a 1 mm pathlength quartz cell in the ultraviolet region, extrema between 200 and 250 nm.

FPLC was done on a column of Mono Q. After injection of 500 μl of the samples, elution was done at a flow rate of 2.0 ml/min with 0~0.2 M NaCl containing 0.01 M acetate buffer (pH 5.0).

Sequential release of C-terminal amino acid residues from peptides and measurement of kinetic parameters were done as previously described.⁶⁾

RESULTS

In a 30-liter jar fermentor, the maximum specific activity (1 nkat/ml/ $A_{280\text{nm}}$) was obtained after 72 hr of inoculation at 30°C. When cultivation was done for 96 hr, ninhydrin-positive materials increased and the specific activity decreased. When the aeration was changed from 10 liter/min and 400 rpm to 5 liter/min and 300 rpm, the enzyme production was not affected. The CPase activity from DEAE-Sephadex, CM-Sephadex, and the final SP-Sephadex were each eluted as a

TABLE I. PURIFICATION OF 155 K *A. oryzae* ACID CPASE O

Fraction	Total activity (n kat)	Volume (ml)	Specific activity (kat/kg protein)	Yield (%)	Purification (-fold)
Culture filtrate	165,850	15,500	0.00161	100	1
Extract from ethanol precipitate	143,350	1,600	0.0587	86	36
DEAE-Sephadex eluate	126,592	920	0.206	76	128
CM-Sephadex eluate	101,360	1,120	0.343	61	213
SP-Sephadex eluate	74,825	275	0.502	45	312

TABLE II. SOME ENZYMATIC AND PHYSICOCHEMICAL PROPERTIES OF 115K *A. oryzae* ACID CPASE O

Properties	Acid CPases from <i>A. oryzae</i>		
	O	O-1 ^a	O-2 ^a
Culture method	Submerged	Koji	Koji
Molecular weight by			
Gel filtration	155K	63K	63K
SDS-PAGE	73K	61K	—
Isoelectric point	4.37	4.08	4.18
$E_{1\text{cm}, 280\text{nm}}^{1\%}$	18.8	18.9	18.6
CD λ_{max} nm	210	—	—
$[\theta]_{\text{max}}$	9800	—	—
Specific activity ^b	0.502	0.394	0.393
Optimum pH for			
hydrolysis of Z-Glu-Tyr	3.7	3.7	3.7
pH stability	5~6	5~6	5~6
Residual activity (%) at			
pH 3.0 and 50°C	84	0	0
pH 4.5 and 60°C	63	0	0
Inhibitor	PMSF (40)	PMSF (77)	PMSF (78)
(Inhibition %) ^c	IAA (16)	IAA (100)	IAA (100)

^a Data from ref. 6.^b Standard substrate used was Z-Glu-Tyr. Specific activity towards Z-Glu-Tyr is expressed as katal/kg protein.^c The enzyme was incubated with 1 mM inhibitor at pH 3.7 for 10 min and the residual activity was measured at pH 3.7.

single peak when the enzyme was purified from submerged culture broth. Purified enzyme was stable for at least six months at -25°C . Results of the purification are summarized in Table I.

The purified enzyme preparation gave a single band on PAGE at pH 9.4 and SDS-PAGE as shown in Fig. 1. The enzyme gave a single peak on isoelectric focusing with an isoelectric point of 4.37. Further experiment with FPLC also showed a single symmetrical peak of the enzyme. We concluded that the enzyme was homogeneous.

Based on Sephadex G-200 gel filtration studies, the molecular weight of the enzyme from submerged culture broth was about 155K in the presence of 0.2M NaCl. When the purified enzyme was electrophoresed in SDS polyacrylamide gel with or without mercaptoethanol, a single band with molecular weight 73K was obtained. Carbohydrate staining of the gel showed a band with exactly the same migration rate as that of the protein

FIG. 1. PAGE and SDS-PAGE of the Final Preparation of 155K *A. oryzae* Acid CPase O.

1, PAGE was done in 7.5% gel at pH 9.4; 2 and 3, SDS-PAGE was done in 10% gel with an enzyme preparation treated with 1% SDS in the presence of 1% mercaptoethanol at room temperature overnight.

Slots 1 (15.3 μg protein) and 2 (10.2 μg protein) stained with Coomassie Brilliant Blue, slot 3 (10.2 μg protein) stained with periodic acid-Schiff reagent.

FIG. 2. Immunodiffusion Cross-reactivity of Antiserum Raised against Acid CPase O-1 and 155K Acid CPase O. Center well, antiserum to acid CPase O-1; 1 and 4, 155K acid CPase O; 2 and 5, acid CPase O-1; 3 and 6, acid CPase O-2.

TABLE III. RELATIVE RATE OF HYDROLYSIS FOR *A. oryzae* ACID CPASES ON A RANGE OF PEPTIDES

Peptide ^a	Relative activity ^b (%)		
	Acid CPases from <i>A. oryzae</i>		
	O	O-1 ^c	O-2 ^c
Z-Glu-Tyr	100	100	100
Z-Tyr-Leu	233	98	97
Z-Tyr-D-Leu	0	0	0
Z-Gly-Pro-Leu-Gly	11	65	61
Z-Val-Glu	23	—	—
Bz-Gly-His-Leu	7.8	0.4	0.5
Z-Gly-Leu	6.7	4	4
Z-Gly-Lys	2.6	7	8
Z-Gly-Phe	7.5	Trace	Trace
Z-Gly-Pro-Leu-Gly-Pro	0.2	Trace	Trace
Z-Gly-Pro	Trace	Trace	Trace
Z-Gly-Pro-Leu	0.03	4	2
Z-Pro-Pro	0	0	0
Tyr-Leu	0.08	—	—
Glu-Glu	0.04	—	—

^a Concentration of each substrate was 5×10^{-4} M.

^b The enzyme reaction was done at pH 3.7 and 30°C. Relative activities were expressed as percentages of the activity toward Z-Glu-Tyr.

^c Data in ref. 6.

—, not measured.

band. The enzyme must therefore be a glycoprotein.

Some catalytic and physical properties of

TABLE IV. RELEASE OF C-TERMINAL AMINO ACID RESIDUES FROM PROANGIOTENSIN (18.5 nmol) AND BRADYKININ (22 nmol) AT pH 3.7 AND 30°C BY 155K *A. oryzae* ACID CPASE O

Released amino acid from:	Released amino acid by 155K <i>A. oryzae</i> acid CPase O ^a (nmol) ^b				
	Incubation time (hr)				
	1	3.5	6	24	48
Proangiotensin ^c					
Leu	17.9	18.2	18.1	18.0	18.5
His	12.4	16.9	17.4	17.4	19.5
Phe	0.6	4.0	6.3	15.6	18.6
Pro	0	0	0	1.5	5.9
Ile	0	0	0	0.2	0.9
Tyr	0	0	0	0	0.4
Val	0	0	0	0	0.3
Arg	0	0	0	0	trace
Asp	0	0	0	0	trace
Bradykinin ^d					
Arg	21.6	21.8	21.9	22.3	22.3
Phe	0.9	4.5	7.4	24.4	39.6
Pro	0	1.4	3.4	14.1	24.4
Ser	0	1.4	2.9	12.1	20.5
Gly	0	0	0	0	0.1

^a 50 pkat of 155K *A. oryzae* acid CPase O was used.

^b The released amino acids were analyzed on an automatic amino acid analyzer by the previously described method.⁽⁶⁾

^c The amino acid sequence of proangiotensin is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu.

^d The amino acid sequence of bradykinin is Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg.

the enzyme are summarized in Table II. The optimum pH of the enzyme for Z-Glu-Tyr was 3.7. At pH 2.5~3.5, the enzyme had still above 50% activity of the maximum activity. Enzymatic activity was inhibited by TPCK, ZPCK, and PMSF but not by IAA, PCMB, EDTA, or S-PI. NaCl at high concentration (20%) caused about 30% inhibition of the enzyme activity measured with Z-Glu-Tyr as a substrate.

Immunodiffusion studies showed a spur formation between the 155K acid CPase O and low molecular acid CPase O-1 (Fig. 2).

The relative hydrolysis rates of a series of peptides by the enzyme are shown in Table III. The enzyme splits off neutral and basic amino

TABLE V. KINETIC PARAMETERS 155K *A. oryzae* ACID CPASE O TOWARD ANGIOTENSIN, BRADYKININ, AND Z-Glu-Tyr AT 3.7 AND 30°C

Substrate Subsite ^a	CPase O			CPase O-1 ^b			CPase O-2 ^b		
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)
Angiotensin -His ⁶ -Pro ⁷ -Phe ⁸	0.34	0.1	3.4	1.7	0.2	8.5	0.15	0.09	1.7
Bradykinin -Pro ⁷ -Phe ⁸ -Arg ⁹	14.7	0.06	245	19	0.05	380	24	0.07	367
Synthetic peptide Z-Glu-Tyr	86	0.48	179.2	53	0.5	106	51	0.5	102

^a Residues in peptide are numbered according to Schechter and Berger.²⁷⁾^b Data in ref. 6.

↓, indicates split bond.

acids from the substrate but not D-amino acids in the terminal position. Z-Tyr-Leu was the best substrate for the enzyme in the experiment. The substrate specificity for *N*-acyl-peptides of the enzyme resembles that of the acid CPase from *A. saitoi*.¹⁾

The data in Table IV indicate that the 155K acid CPase O from *A. oryzae* removes neutral and basic amino acids as well as proline from the C-terminal position of proangiotensin and bradykinin at pH 3.7. No indication of endopeptidase activity could be detected, nor was any release of free amino acids by autodigestion of the enzyme detected after a prolonged period of incubation. When proangiotensin with proline at the penultimate position was used as a substrate, the C-terminal phenylalanine was released slowly by the enzyme. A small amount of proline, which occupies the fourth position from the C-terminus, could first be detected after the incubation for 24 hr. On the other hand, with bradykinin, proline at the third position from the C-terminus was first detected after 3.5 hr.

Kinetic parameters of the 155K acid CPase O of angiotensin, bradykinin, and Z-Glu-Tyr are summarized in Table V. Initial rates towards angiotensin and bradykinin were measured at pH 3.7. To obtain the k_{cat} values for hydrolysis of the C-terminal peptide bond of the substrates, the released C-terminal

amino acid was analyzed by an automatic amino acid analyzer. No amino acids other than the C-terminal amino acid from the substrate were observed. The 155K acid CPase O and low molecular weight acid CPases from *A. oryzae* had similar values of K_m and k_{cat} in the hydrolysis of the C-terminal of bradykinin and Z-Glu-Tyr at pH 3.7 and 30°C.⁶⁾ When proline was present at the penultimate position from the C-terminus as in angiotensin, the value of k_{cat} of the 155K acid CPase O was larger than that of low molecular weight acid CPase O-2 but smaller than that of acid CPase O-1.⁶⁾

DISCUSSION

In our previous paper we reported that *A. oryzae* IAM 2640 produced two forms of acid CPase in solid bran medium; the enzymes were termed *A. oryzae* acid CPase O-1 and O-2, both with molecular weights of 63K. In this paper we found that an 155K molecular weight acid CPase was produced exclusively in the liquid medium of 5% rice bran by *A. oryzae* IAM 2640. When *A. oryzae* IAM 2640 was grown in liquid medium at pH 4.5, little acid proteinase was produced; consequently, acid CPase could be easily purified to homogeneity. The 155K acid CPase O and low molecular weight acid CPases had the same pH stability

and pH optimum for Z-Glu-Tyr, but they were distinguishable by their molecular weight and certain enzymatic and physicochemical properties.⁶⁾ To examine whether 155K acid CPase O was the same structure as low molecular weight acid CPase, an immunological study was done. 155K acid CPase O shared an antigen with low molecular weight acid CPase O-1. Thus these enzymes are at least partly homologous.

When the purified 155K acid CPase O was electrophoresed in SDS polyacrylamide gel with or without mercaptoethanol, a single band with molecular weight around 73K was obtained, indicating that the enzyme from submerged culture broth was composed of two similar monomers. Since no mercaptoethanol was required, the dimer formation was noncovalent and did not involve the cysteine residues. Our previous study⁶⁾ showed that the value of k_{cat} for angiotensin markedly differs between acid CPase O-1 and O-2. The value of 155K acid CPase was intermediate between the value of acid CPase O-1 and O-2.

155K acid CPase O seems to be less dependent on the sulfhydryl group than is low molecular weight acid CPase; concentrations of PCMB and IAA that inhibit 80~100% of low molecular weight acid CPase activity produce only 1~15% inhibition of 155K acid CPase O activity. The active center of 155K acid CPase O may be slightly different structurally.

Acknowledgments. This research was supported in part by a Grant-in-Aid for Research Council from the Ministry of Agriculture, Forestry and Fisheries of Japan, as an original and creative research project in biotechnology. We thank Dr. Y. Narahashi of Riken (The institute of Physical and Chemical Research) for the operation of the CD.

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