Short Communication

Mode of Action of a New *Aspergillus oryzae* Carboxypeptidase O-1

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The serine carboxypeptidases (EC. 3.4.16.1) release most carboxyterminal amino acids from peptides and proteins. However, the rate of hydrolysis by acid carboxypeptidases from *Aspergillus saitoi*^{1~3)} and *Penicillium janthinelum*,⁴⁾ yeast carboxypeptidase Y,⁵⁾ and serine carboxypeptidase from *Phaseolus vulgaris*⁶⁾ and *Citrus natsudaidai*⁷⁾ is influenced by the amino acid residue occupying the penultimate position of carboxyterminus. When the penultimate amino acids such as aromatic amino acid or glutamic acid are replaced with glycine, the rate of hydrolysis is decreased. In particular, the presence of proline as the penultimate amino acid greatly decreases the activity of serine carboxypeptidases.

This paper describes the unique specificity of a new *Aspergillus oryzae* carboxypeptidase O-1 for the substrate having a structure of -Pro-X (X=amino acid).

The enzyme was isolated from solid culture extract of *Aspergillus oryzae* IAM 2640,^{8,9)} and assayed at pH 3.7 according to the method reported previously.¹⁾ The enzyme was purified by precipitation with 90% saturation of ammonium sulfate from the extract, gel chromatography with Sephadex G-100 and ion exchange chromatographies with DEAE-Sephadex, CM-cellulose and SP-Sephadex. In the final SP-Sephadex chromatography, the carboxypeptidase activity was eluted separating into two peak fractions (Fig. 1). The first and second peak fractions were tentatively named as *Aspergillus oryzae* carboxypeptidase O-1 and O-2, respectively. The recovery yield of the total activity was about 40% and the

FIG. 1. SP-Sephadex C-50 Chromatography of *Aspergillus oryzae* Carboxypeptidases.

The column (1×30 cm) was equilibrated with 0.01 M acetate buffer (pH 4.0). Eighty ml of the enzyme solution was applied to the column. Elution was performed with 0.02 M acetate buffer (pH 5.5), with an increasing concentration of salt and pH gradient using a mixing chamber of 300 ml. Acetate buffer (0.02 M, pH 5.5) in the reservoir was tightly connected to the mixing chamber. Flow rate, 10 ml; fraction volume, 4 ml; ●—●, absorbance at 280 nm; ○—○, Z-Glu-Tyr hydrolase activity at pH 3.7.

degree of purification, about 270-fold for both O-1 and O-2. The specific activity was 0.394 kat/kg for O-1 and 0.393 kat/kg for O-2, respectively. Both the enzymes examined by polyacrylamide disc gel electrophoresis at pH 9.4 and isoelectric focusing were homogeneous. The isoelectric point was 4.08 for O-1 and 4.18 for O-2, respectively.

The two enzymes appeared to be identical in the specificity for *N*-acyl-peptides, the optimum pH (pH 3.7) for Z (benzyloxycarbonyl)-Glu-Tyr, molecular weight (63,000) estimated by the methods of gel filtration, pH- and thermal stabilities, and sensitivity for various inhibitors such as tosyl-phenylalanine chloromethyl ketone, phenylmethane sulfonylfluoride, moniodoacetate, and *p*-chloromercuri benzoate. But their mode of action for the substrate having -Pro-X-

TABLE I. RELEASE OF CARBOXYLTERMINAL AMINO ACID RESIDUES FROM PROANGIOTENSIN (15.5 nmol) AND BRADYKININ (16.0 nmol) AT pH 3.7 AND 30°C BY *Aspergillus oryzae* CARBOXYPEPTIDASE O-1 AND O-2

Amino acid released from	Amino acid (nmol) released by							
	Carboxypeptidase O-1				Carboxypeptidase O-2			
	Incubation time (hr)				Incubation time (hr)			
	0.5	1	3.5	6	0.5	1	3.5	6
Proangiotensin*								
Leu		9.6	15.6	15.8		7.3	15.5	15.1
His		9.3	16.5	18.4		6.7	14.9	15.0
Phe		6.8	14.2	13.2		0.4	2.1	4.6
Pro		0	0.8	2.0		0	0	0.2
Ile		0	0.7	1.9		0	0	0.2
Tyr		0	0.6	1.6		0	0	0.1
Val		0	0	0		0	0	0
Arg		0	0	0		0	0	0
Asp		0	0	0		0	0	0
Bradykinin**								
Arg	10.2	15.2		15.1	13.3	15.3		15.2
Phe	1.6	12.3		23.3	0	1.1		3.3
Pro	0	0.7		10.9	0	0		0.5
Ser	0	0.6		9.8	0	0		0.3
Gly	0	0		0.4	0	0		0

* Forty-four p kat of the carboxypeptidase O-1 and 38 p kat of the carboxypeptidase O-2 were used. The amino acid sequence of proangiotensin is Asp-Arg-Val-Tyr-Ile-His-Pro⁷-Phe-His-Leu.

** Twenty-nine p kat of the carboxypeptidase O-1 and 38 p kat of the carboxypeptidase O-2 were used. The amino acid sequence of bradykinin is Arg-Pro-Pro-Gly-Phe-Ser-Pro⁷-Phe-Arg.

TABLE II. KINETIC PARAMETERS OF *Aspergillus oryzae* CARBOXYPEPTIDASE O-1 AND O-2 TOWARD ANGIOTENSIN, BRADYKININ AND Z-Glu-Tyr AT pH 3.7 AND 30°C

Substrate -P ₂ -P ₁ -P ₁ '	Carboxypeptidase O-1			Carboxypeptidase O-2		
	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	1.70	0.2	8.5	0.15	0.09	1.7
Bradykinin -Pro ⁷ -Phe ⁸ -Arg ⁹	19	0.05	380	24	0.07	367
Synthetic peptide Z -Glu -Tyr	53	0.5	106	51	0.5	102

bond differed from each other.

Table I shows the action mode of carboxypeptidase O-1 and O-2 on proangiotensin (angiotensin I) and bradykinin. Carboxypeptidase O-1 readily released Phe⁸ adjacent

to proline present as the penultimate amino acid in proangiotensin. Carboxypeptidase O-2 also split Phe⁸ but not so readily as O-1. Similar results were obtained on bradykinin, that is, when proline was the penultimate

amino acid (Pro⁷), the carboxyterminal amino acid (Phe⁸) newly formed was readily released by carboxypeptidase O-1. The release of Phe⁸ by O-2 was not so rapid as by O-1.

In order to make clear the action mechanism, kinetic studies for angiotensin (angiotensin II), bradykinin, and Z-Glu-Tyr were done. The values of K_m and k_{cat} were graphically determined according to the Lineweaver-Burk plots. The initial rate of hydrolysis was determined by an automatic amino acid analyzer, Hitachi model 835. Amino acids other than the carboxyterminal amino acid from the substrate used in the kinetic studies were not observed.

Carboxypeptidase O-1 and O-2 showed similar values of K_m and k_{cat} in the hydrolysis of bradykinin (-Phe⁸-Arg⁹) and Z-Glu-Tyr at pH 3.7 and 30°C (Table II). On the other hand, the values of k_{cat} for angiotensin (-Pro⁷-Phe⁸) markedly differs between the two enzymes. When proline was the penultimate carboxyterminal amino acid, carboxypeptidase O-1 showed a remarkable large value of k_{cat} as compared with O-2. It seems that the carboxypeptidase O-2 closely resembles several serine carboxypeptidases from various sources.¹⁻⁷⁾ On the other hand, regarding carboxypeptidase O-1 in the present paper, we suggest it may be a new carboxypeptidase.

Nakagawa and Kaiser¹⁰⁾ reported that the yeast carboxypeptidase might form an acyl-

enzyme, using *p*-nitrophenyl trimethylacetate as substrate. A similar reaction mechanism may occur for carboxypeptidases from *Aspergillus oryzae*. A remarkably large k_{cat} value of carboxypeptidase O-1 on angiotensin (-Pro⁷-Phe⁸) may be attributed to the rate constant for deacylation.

It is certain that *Aspergillus oryzae* carboxypeptidase O-1 is useful in the determination of carboxyterminal amino acid sequence.

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REFERENCES

- 1) E. Ichishima, *Biochim. Biophys. Acta*, **258**, 274 (1972).
- 2) E. Ichishima and T. Arai, *Biochim. Biophys. Acta*, **293**, 444 (1973).
- 3) T. Arai and E. Ichishima, *J. Biochem.*, **76**, 765 (1974).
- 4) S. Yokoyama, A. Oobayashi, O. Tanabe and E. Ichishima, *Biochim. Biophys. Acta*, **397**, 443 (1975).
- 5) R. Hayashi, "Methods in Enzymology," Vol. 45, ed. by L. Lorand, Academic Press Inc., New York, N.Y., 1976, pp. 568~587.
- 6) W. F. Carey and J. R. E. Wells, *J. Biol. Chem.*, **274**, 5573 (1972).
- 7) Y. Kubota, S. Shoji, T. Funakoshi and H. Ueki, *J. Biochem.*, **74**, 757 (1973).
- 8) E. Ichishima, S. Sonoki, K. Hirai, Y. Torii and S. Yokoyama, *J. Biochem.*, **72**, 1045 (1972).
- 9) T. Nomi, S. Mikami, A. Yamane, M. Takeuchi and E. Ichishima, *Curr. Microbiol.*, **1**, 25 (1978).
- 10) Y. Nakagawa and E. T. Kaiser, *Biochem. Biophys. Res. Commun.*, **61**, 730 (1974).