Biochimica et Biophysica Acta, 397 (1975) 443—448

BBA 67582

ACTION OF CRYSTALLINE ACID CARBOXYPEPTIDASE FROM *PENICILLIUM JANTHINELLUM*

SADAJI YOKOYAMA, AKIRA OOBAYASHI, OSAMU TANABE and EIJI ICHISHIMA*

*Central Research Laboratories, Takara Shuzo Co., Ltd, Setahashimoto, Ootsu, Shiga 520-21 and * Laboratory of Enzymology and Microbiology, Tokyo Nōkō University, Fuchu, Tokyo 183 (Japan)*

(Received April 1st, 1975)

Summary

Acid carboxypeptidase (EC 3.4.12.-) crystallized from culture filtrate of *Penicillium janthinellum* has been investigated for its use in carboxy-terminal sequence determination of Z-Gly-Pro-Leu-Gly, Z-Gly-Pro-Leu-Gly-Pro, angiotensin I, native lysozyme, native ribonuclease T_1, and reduced *S*-carboxymethyl-lysozyme.

The examination indicated that proline and glycine were liberated from Z-Gly-Pro-Leu-Gly-Pro. At high enzyme concentration, the enzyme catalyzed complete sequential release of amino acids from the carboxy-terminal leucine to the amino-terminal aspartic acid of angiotensin I.

The enzyme released the carboxy-terminal leucine from native lysozyme, however, no release of the carboxy-terminal threonine from native ribonuclease T_1 was observed after a prolonged period of incubation with the enzyme. The sequence of the first nine carboxy-terminal residues of denatured lysozyme, leucine, arginine, *S*-carboxymethyl-cysteine, glycine, arginine, isoleucine, tryptophane, alanine, and glutamine, could be deduced unequivocally from a time release plot of an incubation mixture with the enzyme.

Introduction

Several species of the genus *Penicillium* have been found to be a rich source of acid carboxypeptidase (EC 3.4.12.-) [1,2]. Some differences were observed among the acid carboxypeptidase obtained from these strains in the pH optima for hydrolysis of several peptides, effects of some inhibitors on enzyme activity, and molecular weight [1,2].

Penicillium janthinellum acid carboxypeptidase with a molecular weight

Abbreviations: Z-, benzyloxycarbonyl-; Cm-, carboxymethyl-; RNAase, ribonuclease.

of 51 000 was purified from koji culture and submerged culture to yield a crystalline protein which was demonstrated by disc electrophoresis to be homogeneous [3,4]. The crystals of the acid carboxypeptidase suspended in 0.05 M sodium acetate buffer (pH 3.7) were completely stable for twelve months at 5°C [3,4].

Acid carboxypeptidases have been isolated from *Aspergillus* [5—9], citrus fruit [10] and leaves [11], barley [12], cotton seeds [13,14], French beans [15,16], and yeast [17,18].

In this communication, we describe the mode of action of the acid carboxypeptidase from *P. janthinellum* IFO-8070 in the hydrolysis of Z-Gly-Pro-Leu-Gly, Z-Gly-Pro-Leu-Gly-Pro, angiotensin I, native lysozyme, native RNAase T_1, and denatured lysozyme.

Materials and Methods

Acid carboxypeptidase

The acid carboxypeptidase from *P. janthinellum* IFO-8070 was purified and crystallized according to previous papers [3,4]. The crystallized enzyme preparation was disc-electrophoretically homogeneous at pH 9.4, and stored, suspending in 0.05 M sodium acetate buffer (pH 3.7) at 5°C [3,4]. The assay method of acid carboxypeptidase is described in the preceeding paper [2]. One unit (katal) of acid carboxypeptidase was defined as the amount of enzyme required to liberate 1 mol of tyrosine from Z-Glu-Tyr per s at pH 3.7 and 30°C.

Materials

Z-Gly-Pro-Leu-Gly and Z-Gly-Pro-Leu-Gly-Pro were supplied by the Protein Research Foundation, Osaka. Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) was purchased from Nakarai Kagaku Co., Kyoto. Hen egg-white lysozyme (EC 3.2.1.17) (Grade 1, No. L-6876, 3 × crystallized, dialyzed, Lot 11 C-8150) was purchased from Sigma Chemical Company, St. Louis, U.S.A. RNAase T_1 of *Aspergillus oryzae* was a kind gift of Dr A. Endo, Sankyo Co.

Amino acid calibration mixture for the amino acid analyzer was purchased from Kokusai Shiyaku Co. Tokyo.

Methods

Carboxymethylated reduced lysozyme was prepared according to the procedure of Anfinsen and Canfield [19]. Hen lysozyme was reduced with 2-mercaptoethanol and then carboxymethylated with monoiodoacetic acid. The product was precipitated with ethanol and centrifuged. The precipitate were dissolved in distilled water, then lyophilized.

0.5 μmol of Z-Gly-Pro-Leu-Gly and Z-Gly-Pro-Leu-Gly-Pro were dissolved in 15 ml distilled water, separately, and the acid carboxypeptidase in minimum amount of sodium acetate buffer was added to the solution. The mixture was incubated at 30°C for intimated time. 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. Samples of the hydrolyzate were investigated on the column of a Hitachi model KLA-3B automatic amino acid analyzer.

Angiotensin I (0.41 μmol, 0.53 mg) was dissolved in 15 ml distilled water, and incubated with the acid carboxypeptidase at 30°C for the intimated time. The amino acid liberated in the reaction mixture were analyzed by the same method described above.

0.5 μmol of native egg white lysozyme, RNAase T_1, and carboxymethylated reduced lysozyme were dissolved in 15 ml distilled water, separately, and the acid carboxypeptidase was added to the solution. The mixture was incubated at 30°C for the intimated time. An equal volume of 10% trichloroacetic acid was added to inactivate the enzyme and to precipitate the residual protein. After filtration of the precipitate with Toyo No. 2 filter paper, trichloroacetic acid was removed by the extraction with ether. The aqueous layer was evaporated to dryness in vacuo, and was investigated by the same method described above.

Results

The results obtained from N-substituted peptides Z-Gly-Pro-Leu-Gly, and Z-Gly-Pro-Leu-Gly-Pro with the acid carboxypeptidase are summarized in Table I. Free glycine was liberated very rapidly from the C-terminus of Z-Gly-Pro-Leu-Gly. Experiments with Z-Gly-Pro-Leu-Gly-Pro indicate that the enzyme sequentially release proline, glycine, and leucine from the carboxy-terminus.

C-terminal leucine and histidine of angiotensin I were sequentially released by 0.834 nkat of the enzyme, but only trace amount of phenylalanine which is adjacent to imino acid proline was released (Table II).

TABLE I

RELEASE OF CARBOXYTERMINAL AMINO ACID RESIDUES FROM 0.5 μmol Z-Gly-Pro-Leu-Gly (A) AND Z-Gly-Pro-Leu-Gly-Pro (B) AT 30°C BY CRYSTALLINE ACID CARBOXYPEPTIDASE FROM *PENICILLIUM JANTHINELLUM*

Substrate	Enzyme (nkat)	Incubation		Amino acid released (μmol)		
		(h)	(pH)	Leu	Gly	Pro
A. Z-Gly-Pro-Leu-Gly	0.0834	3	4.0	—	0.136	—
	0.0834	5	4.0	—	0.162	—
	0.0834	20	4.0	—	0.186	—
	0.834	1	4.5	—	0.253	—
	0.834	3	4.5	—	0.422	—
	0.834	5	4.5	—	0.534	—
	0.834	20	4.5	—	0.510	—
	8.34	44	5.2	trace	0.494	—
	83.4	15	5.2	trace	0.525	—
	83.4	23	5.2	trace	0.540	—
	83.4	61	5.2	0.034	0.525	—
B. Z-Gly-Pro-Leu-Gly-Pro	0.834	2	5.0	—	—	—
	0.834	13	5.0	—	trace	trace
	0.834	40	5.0	—	0.045	0.044
	83.4	1	5.2	—	0.088	0.091
	83.4	3	5.2	—	0.152	0.165
	83.4	10	5.2	trace	0.302	0.295
	83.4	24	5.2	trace	0.322	0.302
	83.4	48	5.2	0.027	0.337	0.332

TABLE II

RELEASE OF CARBOXYTERMINAL AMINO ACID RESIDUES FROM 0.41 μmol ANGIOTENSIN I* AT 30°C BY CRYSTALLINE ACID CARBOXYPEPTIDASE FROM *PENICILLIUM PANTHINELLUM*

Enzyme (nkat)	Incubation		Amino acid released (μmol)								
	(h)	(pH)	Asp	Arg	Val	Tyr	Ile	Pro	Phe	His	Leu
0.834	0.5	4.8	—	—	—	—	—	—	—	0.161	0.207
0.834	1.0	4.8	—	—	—	—	—	—	—	0.169	0.276
0.834	5.0	4.8	—	—	—	—	—	—	trace	0.289	0.498
8.34	1.0	4.8	—	—	—	—	—	—	trace	0.289	0.372
83.4	3.0	5.2	—	—	0.303	0.321	0.337	0.321	0.339	0.634	0.396
83.4	15.0	5.2	0.112	0.106	0.362	0.378	0.392	0.398	0.368	0.855	0.421

* Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu.

TABLE III

RELEASE OF CARBOXYTERMINAL AMINO ACID RESIDUES FROM 0.5 μmol NATIVE EGG WHITE LYSOZYME (A) AND NATIVE RIBONUCLEASE T_1 (B) AT 30°C BY CRYSTALLINE ACID CARBOXYPEPTIDASE FROM *PENICILLIUM JANTHINELLUM*

Substrate	Enzyme (nkat)	Incubation		Amino acid released (μmol)		
		(h)	(pH)	Thr	Arg	Leu
A. Egg White Lysozyme	0.834	5	3.5		—	—
	0.834	22	3.5		—	—
-Cys-Arg-Leu	83.4	1	5.2		—	0.346
|	83.4	10	5.2		trace	0.342
	83.4	24	5.2		trace	0.354
B. RNAase T_1	83.4	1	5.2	—		
	83.4	24	5.2	—		
-Cys-Thr						
|						

TABLE IV

RELEASE OF CARBOXYTERMINAL AMINO ACID RESIDUES FROM 0.5 μmol CARBOXYMETHYLATED REDUCED LYSOZYME* AT pH 5.2 AND 30°C BY 8.34 nKATAL CRYSTALLINE ACID CARBOXYPEPTIDASE FROM *PENICILLIUM JANTHINELLUM*

Incubation time	Amino acid released (μmol)									
	Asp	Val	Gln	Ala	Trp	Ile	Gly	CM-Cys	Arg	Leu
10 min	—	—	—	—	—	—	—	trace	0.100	0.140
30 min	—	—	—	—	—	—	trace	+**	0.360	0.294
1 h	—	—	—	—	—	trace	0.112	+**	0.476	0.436
3 h	—	—	—	—	trace	0.138	0.330	+**	1.030	0.450
5 h	—	—	trace	0.184	0.336	0.316	0.454	+**	0.968	0.480

* -Thr-Asp-Val-Gln-Ala-Trp-Ile-Arg-Gly-(CM-Cys)-Arg-Leu-OH (from the ref. 25).
** Present, but not quantitatively determined.

On the other hand, by the incubation with 83.4 nkat of the enzyme, all amino acid residues of angiotensin I were liberated (Table II). It was confirmed that no indication of endopeptidase activity could be detected.

C-terminal leucine was released by the acid carboxypeptidase from the carboxy-terminus of native lysozyme, but no amount of the penultimate amino acid arginine bound to cysteine was released (Table III). Similarly, from native RNAase T_1, no release of C-terminal threonine bound to cysteine was observed (Table III).

Sequential C-terminal proteolysis of carboxymethylated reduced lysozyme could be obtained using 8.34 nkat of the acid carboxypeptidase, as shown in Table IV. The first nine residues, leucine, arginine, *S*-carboxymethylcysteine, glycine, arginine, isoleucine, tryptophane, alanine, and glutamine, were liberated from the C-terminus. The experiments showed that about 1 mol of leucine was liberated per mole of carboxymethylated reduced lysozyme by the acid carboxypeptidase.

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

Discussion

In 1964, Shaw [20] reported some properties of an dipeptidase-like enzyme (peptidase B) of *Penicillium janthinellum*. Peptidase B hydrolyzed Z-Glu-Tyr optimally at pH 4.7, and not inhibited by sulfhydryl reagents [20].

In the previous papers [1—4], we reported some enzymatic properties of acid carboxypeptidase from several *Penicillium* molds and culture conditions. Furthermore, low molecular weight (51 000) of acid carboxypeptidase from *P. janthinellum* IFO-8070 was purified and crystallized, which had the optimum pH of 3.7 toward Z-Glu-Tyr, and was inhibited by monoiodoacetic acid, *p*-chloromercuribenzoate, hydrocinnamic acid and diisopropylfluorophosphate [1,2].

Hofmann et al. [21,22] purified a carboxypeptidase (penicillocarboxypeptidase-S) from the culture filtrate of *P. janthinellum* by affinity chromatography on phenylbuthylamine Sepharose followed by isoelectric focusing. Penicillocarboxypeptidase S had a pH optimum between 4 and 4.5 toward Z-Glu-Tyr and a molecular weight of about 48 000.

In the present paper, synthetic N-substituted peptides Z-Gly-Pro-Leu-Gly, Z-Gly-Pro-Leu-Gly-Pro, decapeptide angiotensin I, native lysozyme, and carboxymethylated reduced lysozyme have been degraded demonstrating the combined activity of pancreatic carboxypeptidase A (EC 3.4.12.2) and B (EC 3.4.12.3), but showing important difference in the release of C-terminal proline from Z-Gly-Pro-Leu-Gly-Pro.

It was shown that the acid carboxypeptidase of *Aspergillus saitoi* exhibited a preference for aromatic, carboxyl or leucyl groups in the penultimate position to the C-terminal of substrates [5]. The acid carboxypeptidase of *P. janthinellum* IFO-8070 hydrolyzed the C-terminal glycine from Z-Gly-Pro-Leu-Gly, but only trace of leucine was released, which was adjacent to imino acid proline.

Carboxypeptidase A are known to have the ability to remove the C-terminal leucine and threonine from native lysozyme [23] and RNAase T_1 [24],

respectively. It is noteworthy that the acid carboxypeptidase of *P. janthinellum* IFO-8070 could release the C-terminal leucine from native lysozyme, but could not release the C-terminal threonine which is adjacent to cysteine residue composing S-S bridge in native RNAase T_1. These phenomena seem to suggest that the penultimate position is very important on the binding site in the enzyme reaction of the *Penicillium* acid carboxypeptidase.

Ichishima et al. [9] have recently found that the sequence of the first four C-terminal residues of carboxymethylated reduced lysozyme, leucine, arginine, *S*-carboxymethyl-cysteine, and glycine could be deduced unequivocally from a time release plot of an incubation mixture with the *Aspergillus* acid carboxypeptidase.

Experiments with the carboxymethylated reduced lysozyme indicate that the crystalline acid carboxypeptidase of *P. janthinellum* sequentially released free nine amino acids from the C-terminal leucine to glutamine. These experiments also reveal that the enzyme preparation was free of any contaminating endopeptidase activity.

The broad specificity of the *Penicillium* acid carboxypeptidase mentioned above, make the enzyme applicable to sequence determination of peptides and proteins.

References

1 Yokoyama, S. and Ichishima, E. (1972) Agric. Biol. Chem. Tokyo 36, 1259—1261
2 Yokoyama, S., Oobayashi, A., Tanabe, O., Sugawara, S., Araki, E. and Ichishima, E. (1974) Appl. Microbiol. 27, 953—960
3 Yokoyama, S., Oobayashi, A., Tanabe, O. and Ichishima, E. (1974) Appl. Microbiol. 28, 742—747
4 Yokoyama, S., Oobayashi, A., Tanabe, O. and Ichishima, E. (1975) Agric. Biol. Chem. Tokyo 39, 1211—1217
5 Ichishima, E. (1972) Biochim. Biophys. Acta 258, 274—288
6 Ichishima, E., Sonoki, S., Hirai, K., Torii, Y. and Yokoyama, S. (1972) J. Biochem. Tokyo 72, 1045—1048
7 Ichishima, E. and Arai, T. (1973) Biochim. Biophys. Acta 293, 444—450
8 Ichishima, E., Yamane, A., Nitta, T., Kinoshita, M., Nikkuni, S., Oka, T. and Yokoyama, S. (1973) Appl. Microbiol. 26, 327—331
9 Arai, T. and Ichishima, E. (1974) J. Biochem. Tokyo 76, 765—769
10 Zuber, H. (1964) Nature 201, 613
11 Sprössler, B., Heilmann, H.-D., Grampp, E. and Uhlig, H. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1524—1530
12 Visuri, K., Mikola, J. and Enari, T.-M. (1969) Eur. J. Biochem. 7, 193—199
13 Ihle, J.W. and Dure, L.S., III (1972) J. Biol. Chem. 247, 5034—5040
14 Ihle, J.W. and Dure, L.S. III (1972) J. Biol. Chem. 247 5041—5047
15 Wells, J.R.E. (1965) Biochem. J. 97, 228—235
16 Carey, W.F. and Wells, J.R.E. (1972) J. Biol. Chem. 247, 5573—5579
17 Hayashi, R., Moor, S. and Stein, H. (1973) J. Biol. Chem. 248, 2296—2302
18 Kuhn, R.W., Walsh, K.A. and Neurath, H. (1974) Biochemistry 13, 3871—3877
19 Canfield, R.E. and Anfinsen, C.B. (1963) J. Biol. Chem. 238, 2684—2690
20 Shaw, R. (1964) Biochim. Biophys. Acta 92, 558—566
21 Jones, S.R. and Hofmann, T. (1972) Can. J. Biochem. 50, 1297—1310
22 Hui, A., Rao, L., Kurosky, A., Jones, S.R., Mains, G., Dixon, J.W., Szewczuk, A. and Hofmann, T. (1974) Arch. Biochem. Biophys. 160, 577—587
23 Thompson, A.R. (1952) Nature 169, 495—496
24 Takahashi, K. (1962) J. Biochem. Tokyo 52, 72—81
25 Canfield, R.E. and Liu, A.K. (1965) J. Biol. Chem. 240, 1997—2002