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THE SUBSTRATE SPECIFICITY OF PYRROLIDONE CARBOXYLYL PEPTIDASE FROM *BACILLUS AMYLOLIQUEFACIENS* *

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Summary

The present study was undertaken to investigate the substrate specificity of pyrrolidone carboxylyl peptidase (L-pyroglutamyl-peptide hydrolase, EC 3.4.11.8) isolated from the cells of *Bacillus amyloliquefaciens* with twenty L-pyroglutamyl amino acids, most of which were newly synthesized, and with several peptide hormones containing pyroglutamyl residue in their amino termini. All the dipeptides tested, except for L-pyroglutamyl-L-proline and -D-amino acids, were hydrolyzed at measurable rates with the maximum V values of 4.0—38.7 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, and the K_m values were 0.09—0.3 mM. Of these substrates, dipeptides containing L-threonine, L-glutamic acid and L-methionine were hydrolyzed most rapidly, and the dipeptides which contained hydrophobic L-amino acids were split at rather slower rate (V: 4.0—15.2 $\mu mol \cdot mg^{-1} \cdot min^{-1}$), whereas they had rather high affinity to the enzyme (K_m: 0.09—0.14 mM). On the other hand, the enzyme was very slightly active toward L-pyroglutamyl-D-amino acids and was completely inert toward L-pyroglutamyl-L-proline, both of which were found to competitively inhibit the enzyme reaction. Several peptide hormones containing L-pyroglutamic acid were also subjected to the enzyme digestion. The enzyme split bradykinin potentiator, neurotensin, luteinizing hormone releasing hormone, thyrotropic

* Supplementary data to this article are deposited with, and can be obtained from Elsevier/North-Holland Biomedical Press, BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to BBA/DD/109/68807/570 (1979) 140—148. The information includes analytical data and some properties of Z-L-pyroglutamyl-amino acids and L-pyroglutamyl-amino acids.

Abbreviations used follow the tentative rules and recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature for amino acids and peptides. The constituent amino acids of the peptides used here were all of the L-configuration, unless otherwise specified.

hormone releasing hormone, frog litorin, Glu(OMe)^2-litorin and physalaemin, liberating pyroglutamic acid and the des-pyroglutamyl peptides having the corresponding new amino terminal amino acids, but was inactive toward uperolein and thyrotropic hormone releasing hormone analogue, piperidone-carbonyl-His-Pro-NH_2.

Introduction

The activity of pyrrolidone carboxylyl peptidase (L-pyroglutamyl-peptide hydrolase or L-pyrrolidonyl peptidase: EC 3.4.11.8) has been detected in various bacteria [1—8], plants and animal tissues [9—11]. The enzyme seems useful for structure analysis of proteins and peptides, since amino termini of some proteins, peptides and hormones have been found to be blocked by L-pyroglutamyl residue [1,12—16], and the determination of their amino acid sequences cannot be performed by an usual stepwise degradation procedure [17], unless the amino terminal pyroglutamyl residue was removed.

Quite recently, we have succeeded in obtaining an electrophoretically homogeneous preparation of pyrrolidone carboxylyl peptidase from the lysozyme lysate of *Bacillus amyloliquefaciens* cells and characterized its enzymatic and physicochemical properties [8]. The enzyme was fairly stable as compared with the other pyrrolidone carboxylyl peptidase so far reported [1—4,6,7] and active toward pyroglutamyl derivatives of β-naphthylamine, α- and β-naphthols and 4-methyl umbelliferon as well as pyroglutamyl-alanine but was completely inert toward amino acid-β-naphthylamides, acyl amino acids, amino acid esters, di- and oligopeptides used as the substrates for usual endo- and exopeptidases [8]. We have also reported that pyroglutamyl-4-methylcoumarinylamide and pyroglutamyl-*p*-nitroanilide were very sensitive fluorogenic and chromogenic substrates of this enzyme, respectively. The use of the former substrate made it possible to detect 0.1 ng of the enzyme per ml of sample [18].

In order to clarify further the substrate specificity of this enzyme, we synthesized a number of pyroglutamyl-amino acids and examined their susceptibilities to the enzyme. Several pyroglutamyl peptide hormones available were also subjected to the enzyme digestion.

The present paper deals with kinetic analysis of pyrrolidone carboxylyl peptidase-catalyzed hydrolysis of the dipeptides and the susceptibility of the peptide hormones to the enzyme. It was found from the present results that the enzyme is active toward pyroglutamyl-amino acids or -peptides of L-configuration, except for pyroglutamyl-Pro bond. Pyroglutamyl derivatives of D-amino acids as well as of proline were found to be competitive inhibitors for the enzyme.

Materials and Methods

Enzyme. The pyrrolidone carboxylyl peptidase from *B. amyloliquefaciens* was purified to homogeneity by the method previously described [8] and the enzyme activity was assayed by the method of Szewczuk and Mulczyk [4].

Peptides and chemicals. Bradykinin potentiator, neurotensin, luteinizing

hormone releasing hormone (LHRH), thyrotropic hormone releasing hormone (TRH) and a TRH analogue, piperidonecarbonyl-His-Pro-NH_2, were chemically synthesized and kindly supplied by Dr. M. Fujino of Takeda Chemical Industries Co., Osaka, Japan. Frog litorin, Glu(OMe)[2]-litorin, physalaemin and uperolein were generous gifts from Prof. T. Nakajima of Hiroshima University. The following amino acid derivatives and chemicals were obtained from Protein Research Foundation, Minoh, Japan: Z-pyroglutamic acid, Ser(OBzl), Thr(OBzl), Asp(OBzl), Glu(OBzl), Lys(z), Arg(NO_2)OBzl-2 Tos, *N*,*N*'-dicylohexylcarbodiimide and *N*-hydroxysuccinimide. D-Ala, D-Leu, β-Ala, trifluoroacetic acid, phenylisothiocyanate and other reagents for sequence determination study were purchased from Nakarai Chemicals Co., Kyoto, Japan, and pyroglutamyl-Pro was a product of Vega-Fox Biochemicals, U.S.A. The other peptides were synthesized as described below. Z-Pyroglutamyl-*N*-hydroxysuccinimide ester was synthesized according to the method of Yanaihara et al. [19] by the condensation of Z-pyroglutamate and *N*-hydroxysuccinimide in the presence of *N*,*N*'-dicyclohexylcarbodiimide.

Synthesis of L-pyroglutamyl amino acids. Z-Pyroglutamyl-D-Ala: to 640 mg of Z-pyroglutamyl-*N*-hydroxysuccinimide (2 mmol) in 10 ml of tetrahydrofuran, 178 mg D-alanine (2 mmol) dissolved in the mixture of 10 ml of water and 280 μl of triethylamine was added dropwise and allowed to stir overnight at room temperature. The reaction mixture was concentrated in vacuo to half volume and the remaining aqueous solution was adjusted to pH 9.0 with triethylamine. After removal of unreacted materials by extraction with ethylacetate, aqueous layer was acidified with 1 N citric acid. The resulted precipitate was extracted with ethylacetate, the organic layer being washed with 1 N citric acid and with saturated sodium chloride solution and then concentrated in vacuo. The product (Z-pyroglutamyl-D-Ala) was crystallized from methanol; yield, 65%.

Pyroglutamyl-D-Ala: Z-pyroglutamyl-D-Ala, 200 mg, dissolved in 25 ml of methanol was hydrogenated in the presence of palladium black; yield, 95%.

Other pyroglutamyl-amino acids were synthesized in essentially the same way as that described above. In case of preparing dipeptides composed of hydrophobic amino acids, these amino acids were directly coupled with Z-pyroglutamyl-*N*-hydroxysuccinimide and the desired products were obtained with high yield. For hydrophilic amino acids, on the other hand, their derivatives in which the functional groups other than those in α-carbon were blocked with benzyl or carbobenzoxy group were used. Tyrosine was also converted to Tyr(OBzl) and the coupled with Z-pyroglutamyl-*N*-hydroxysuccinimide as mentioned above. All the compounds synthesized were analyzed for carbon, hydrogen and nitrogen, and the purities were confirmed by thin layer chromatography. The solvent system used was *n*-butanol/acetic acid/water (4 : 1 : 5, by vol.) or *n*-butanol/pyridine/acetic acid/*t*-butanol/water (15 : 10 : 3 : 4 : 12, by vol.). The chromatograms were sprayed first with 0.2% ninhydrin in acetone, followed by $CeSO_4$ treatment. Analytical data and properties of Z-pyroglutamyl-amino acids and those of the final products, pyroglutamyl-amino acids, obtained by the hydrogenation of the above compounds are in the BBA Data Bank (see footnote p. 140).

Hydrolysis of dipeptides. Hydrolysis of various synthetic dipeptides was

measured by the ninhydrin method as follows: the reaction mixture was composed of 0.1 M Tris-HCl buffer of pH 8.0, 10 mM 2-mercaptoethanol, varied concentrations of peptide substrates and a suitable amount of the enzyme and was incubated at 30° C. At various intervals of time, 150-μl aliquots of the reaction mixture were withdrawn and put into a test tube which contained 300 μl of 0.1 N HCl to stop the enzyme reaction. The extent of hydrolysis was followed by the ninhydrin method of Yemm and Cocking [20]. The Michaelis constant, K_m, and maximum V values of the enzyme reaction were estimated by the Lineweaver-Burk plot. The inhibition constant, K_i, was also determined from the Lineweaver-Burk plot with a constant concentration of the inhibitor.

Hydrolysis of peptide hormones

(A) Edman method. Hydrolysis of peptides by pyrrolidone carboxylyl peptidase was followed by detection of new amino terminal amino acid as phenylthiohydantoin derivatives by Edman procedure [17]. About 0.3 μmol peptide was dissolved in 30 μl of 20 mM phosphate buffer, pH 7.8, containing 5 mM 2-mercaptoethanol and 1 mM ethylenediaminetetraacetate (EDTA) and then mixed with 20 μl of the enzyme (2.7 units/ml). After incubation at 30° C for 6 h, the peptide was precipitated by the addition of 1 ml acetone and washed twice with acetone. After being dried under N_2 gas, the precipitate was dissolved in 200 μl of dimethylarylamide buffer, pH 9.5, and subjected to coupling with phenylisothiocyanate and degradation with trifluoroacetic acid [21]. The phenylthiohydrantoin-amino acid formed was analyzed spectrophotometrically and identified by thin layer chromatography using a Kiesel gel 60 F254 plate (Merck). The solvent systems used were (A) a mixture of $CHCl_3$/CH_3OH (9 : 1, by vol.), (B) $CHCl_3$/HCOOH (100 : 5, by vol.) and (C) heptan/propionic acid/ethylenechloride (58 : 17 : 25, by vol.).

(B) Amino acid analysis after separation of the digestion products by thin layer chromatography on cellulose. TRH and its analogue, piperidonecarbonyl His-Pro-NH_2 were treated with the enzyme as mentioned above, and the digests were spotted on a cellulose plate (Merck) and developed using the solvent system of *n*-butanol/formic acid/water (70 : 15 : 15, by vol.) for 4 h at room temperature. A part of the plates was sprayed first with Rydon-Smith reagent [22] and then with pauli reagent [23] to detect digestion products. The areas containing pyroglutamic acid and the des-pyroglutamyl peptide, His-Pro-NH_2, in the untreated cellulose plate were collected and extracted twice with 0.5 ml each of deionized water and 0.1 M acetic acid. The extracts were concentrated in vacuo, dissolved in 6 N HCl and hydrolyzed for 24 h at 110° C in evacuated sealed tubes. The glutamic acid and histidine formed were determined by the method of Spackman et al. [24] using a Nippon Denshi JLC-6AM automatic amino acid analyzer.

(C) Dansylation method. The stocked solution of the enzyme (5.0 units/ml) was diluted 2.5 fold with 50 mM ammonium formate buffer (pH 7.0) containing 0.2 mM EDTA and 1 mM 2-mercaptoethanol. To 50 μl of this enzyme solution was added 10—13 μg of peptides dissolved in 10 μl of deionized water. After incubation for 6 h at 30° C, the digest was lyophilized and then subjected to dansylation procedure [25]. The dansylated peptide was hydrolyzed at 105° C with 6 N HCl for 18 h and the resulted dansyl amino acid was identified

by two-dimensional chromatography on polyamide layer sheet (Cheng Chin Trading Co., Taiwan). The solvent systems used were 1.5% formic acid for the first run and benzene/acetic acid (9 : 1, by vol.) followed by ethyl acetate/methanol/acetic acid (20 : 1 : 1, by vol.) for the second run.

Results and Discussion

Hydrolysis of L-pyroglutamyl-amino acids

Table I summarizes the kinetic parameters of pyrrolidone carboxylyl peptidase-catalyzed hydrolysis of dipeptides at pH 8.0. The dipeptides containing neutral, basic and acidic amino acids with L-configuration, except for proline, were hydrolyzed at measurable rates. This indicates that the enzyme is useful in determining the amino acid sequence of peptide chain in which the amino terminus is blocked by pyroglutamyl residue. This result also indicates that substrate specificity of this enzyme is distinguishable from those of *Pseudomonas fluorescens* and *Klebsiella cloacae*. The enzyme from *P. fluorescens* was shown to be inert toward pyroglutamyl-Lys as well as pyroglutamyl-Pro, though the comprehensive studies have not yet been done on the specificity of the enzyme [3]. The enzyme from *K. cloacae* was reported to be active toward pyroglutamyl derivatives of Ala, Val and Pro, but no report was available for the susceptibility of other dipeptides to this enzyme [6].

The relative rate of hydrolysis of the peptides by the enzyme from *B. amyloliquefaciens* varies in a decreasing order: Thr > Glu > Met > Ala: The former three pyroglutamyl amino acids were hydrolyzed more rapidly than pyroglutamyl-Ala, which was the most sensitive dipeptide substrate of the enzyme from *P. fluorescens* [3].

Of sixteen dipeptides with L-configuration tested, only pyroglutamyl-Pro was not split under the condition used. We tried to prepare pyroglutamyl derivatives of cysteine and cystine but failed to obtain the pure preparations of them. On the other hand, the hydrolytic activity of the enzyme toward pyro-

TABLE I

KINETIC CONSTANTS OF PYRROLIDONE CARBOXYLYL PEPTIDASE-CATALYZED HYDROLYSIS OF VARIOUS DIPEPTIDES

L-Pyr-	K_m (mM)	V *	L-Pyr-	K_m (mM)	V *
Gly	0.20	15.3	L-Met	0.13	26.0
L-Ala	0.25	22.5	L-Asp	0.29	11.3
L-Leu	0.14	11.0	L-Glu	0.23	34.3
L-Ile	0.14	10.9	L-His	0.23	13.3
L-Phe	0.10	13.4	L-Arg	0.29	14.0
L-Val	0.09	4.0	L-Lys	0.20	18.9
L-Trp	0.11	13.2	L-Pro	2.2 **	—
L-Tyr	0.20	15.2	β-Ala	/	+
L-Thr	0.24	38.7	D-Ala	9.2 **	±
L-Ser	0.15	15.2	D-Leu	3.3 **	±

* μmol substrate hydrolyzed · mg^{-1} · min^{-1}.
** K_i values.

glutamyl derivatives of D-Ala and D-Leu was very slight or almost negligible, indicating the strict stereospecificity of this enzyme on penultimate amino acid residue, as was the case of the enzyme from *P. fluorescens* [26].

The inhibitory effect of these poor substrates was examined. The reaction system was composed of pyroglutamyl-4-methylcoumarinylamide as substrate, inhibitor (pyroglutamyl-D-Ala or -D-Leu) and the enzyme in 0.1 M Tris-HCl buffer, pH 8.0. The liberation of 4-methyl-7-aminocoumarin was followed fluorometrically as described previously [18]. The Lineweaver-Burk plot is shown in Fig. 1, indicating that pyroglutamyl-D-Ala and -D-Leu show a competitive type of inhibition with K_i values of 9.2 mM and 3.3 mM, respectively. These values are about 10-fold greater than the K_m values of the corresponding L-peptides.

The inhibitor effect of pyroglutamyl-Pro was also tested using 6.5 mM concentration against various concentrations (0.2—0.8 mM) of substrate. Pyroglutamyl-Pro also showed a competitive type of inhibition for the enzyme reaction with a K_i value of 2.2 mM.

The values of K_m for pyroglutamyl derivatives of acidic, basic and neutral L-amino acids were almost similar each other (Table I). This result suggests that the presence of charged group at penultimate amino acid in peptide substrates does not affect the affinity to the enzyme. The K_m values of these dipeptides were almost equivalent to or somewhat lower than those for pyroglutamyl

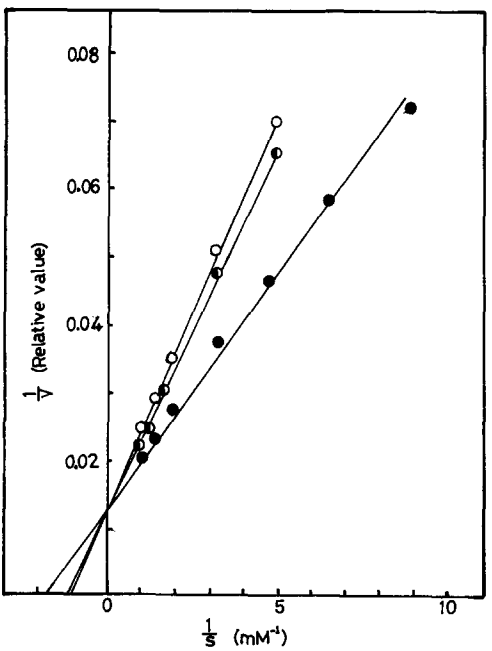

Fig. 1. Inhibition of pyrrolidone carboxylyl peptidase-catalyzed hydrolysis of L-pyroglutamyl-4-methylcoumarinylamide by L-pyroglutamyl-D-amino acids. The reaction mixtures were composed of 0.09—0.80 mM substrate, 6.5 mM pyroglutamyl-D-Ala or 1.66 mM pyroglutamyl-D-Leu and 0.22 μg/ml of the enzyme in 0.1 M Tris-HCl buffer, pH 8.0. The liberation of 4-methyl-7-aminocoumarin was followed fluorometrically at 30°C [18]. ●, without inhibitor; ○, with pyroglutamyl-D-Ala; ◐, with pyroglutamyl-D-Leu.

derivatives of β-naphthylamine (0.13 mM), 7-amino-4-methylcoumarine (0.33 mM) and *p*-nitroaniline (0.69 mM) previously reported by us [8,18].

There is a tendency that the K_m value becomes smaller with increase in hydrophobicity of amino acid, for example, pyroglutamyl derivatives of Phe, Leu, Ile, Trp and Val give small K_m values, whereas the V values are rather low, except for pyroglutamyl-Met.

Pyroglutamyl-β-Ala was also susceptible to the enzyme action, but we failed to obtain the accurate value of kinetic constants, because of the low color development of β-alanine in ninhydrin reaction and of very small K_m value of the substrate.

Hydrolysis of peptide hormones

Eight peptide hormones and an analogue were subjected to enzyme digestion and the new amino terminal amino acids formed were estimated by the methods of Edman and/or dansylation. The results are summarized in Table II. The yields of the corresponding new amino terminal amino acids were about 60% or less, somewhat lower than those we expected, as judged by the Edman method. Particularly, the yield from TRH was only 5%. However, when the digestion products were checked by Rydon-Smith and pauli reagents, after being separated by thin layer chromatography on cellulose plate, most of the TRH was found to be split into pyroglutamic acid and the des-pyroglutamyl peptide, His-Pro-NH_2. Both compounds were extracted from the cellulose plate, and their amounts were determined by amino acid analysis, after acid hydrolysis. The yields of glutamic acid and histidine were calculated to be more than 60% (Table II, B). Thus, it is possible to conclude that pyrrolidone carboxylyl peptidase from *B. amyloliquefaciens* can easily hydrolyze bradykinin potentiator, neurotensin, LHRH and TRH. A TRH analogue, piperidonecarbonyl His-Pro-NH_2 was completely resistant to the enzyme action.

TABLE II

SUSCEPTIBILITY OF SEVERAL PEPTIDE HORMONES TO PYRROLIDINE CARBOXYLYL PEPTIDASE FROM *B. AMYLOLIQUEFACIENS*

Substrate	Primary structure in amino terminal region	New amino terminal amino acid found after digestion	Analytical method [a]
Bradykinin potentiator	Pyr-Trp-Pro------	Trp	A (60) [b]
Neurotensin	Pyr-Leu-Tyr------	Leu	A (30) [b], C
LHRH	Pyr-His-Trp------	His	A (30) [b], C
TRH	Pyr-His-Pro-NH_2	His	A (5) [b], B (60) [c], C
Litorin	Pyr-Gln-Trp------	Glu	C
Glu(OMe)[2]-litorin	Pyr-Glu(OMe)-Trp-	Glu	C
Physalaemin	Pyr-Ala-Asp------	Ala	C
Uperolein	Pyr-Pro-Asp------	None	C
TRH anlogue	Kpc-His-Pro-NH_2 [d]	None	C, B

[a] A: Edman method: B: amino acid analysis after separation of the digestion products by thin layer chromatography and acid hydrolysis; C: dansylation method.

[b] Yield of amino terminal amino acid, %.

[c] Yield of pyroglutamic acid.

[d] Piperidonecarbonyl His-Pro-NH_2.

It remains unclear why the yields of new terminal amino acids were low, as judged by the Edman method, but it seems likely that the precipitation of the des-pyroglutamyl peptides by acetone, after the enzyme treatment, is not quantitative and that, in the case of TRH, cyclization of the des-pyroglutamyl peptide, His-Pro-NH_2, would occur.

Frog litorin, Glu(OMe)^2-litorin, physalaemin and uperolein were also subjected to the enzyme digestion, and the amino terminal amino acids newly formed were qualitatively identified by dansylation method [25], since the amounts of peptide hormones available were quite small. Glutamic acid was detected as the amino terminal amino acid of des-pyroglutamyl peptides from litorin and Glu(OMe)^2-litorin, and alanine was identified to be that from physalaemin. These results confirm those of sequence studies of these peptide hormones recently performed by Nakajima et al. [27]. On the other hand, no enzymatic hydrolysis was observed for uperolein, the amino terminal sequence of which was pyroglutamyl-Pro-Asp-Pro- (Nakajima, T., personal communication).

Recently, Iwanaga and the co-workers have shown that several pyroglutamic acid-containing proteins and peptides, bovine cold insoluble globulin, fibrinopeptide B, kininogen, pyroglutamyl-Gln-Trp-OH and pyroglutamyl-Asn-Trp-OH were split into pyroglutamic acid and des-pyroglutamyl proteins (or peptides) by the pyrrolidone carboxylyl peptidase prepared by us (Iwanaga, S. et al. unpublished data). On the other hand, Nagata and Maruo (University of Tokyo) have found, by using this enzyme, that the amino terminal sequence of wheat germ agglutinin is pyroglutamyl arginine (Nagata, Y., personal communication).

These results lead us to conclude that the pyrrolidone carboxylyl peptidase from *B. amyloliquefaciens* is very useful for the removal of pyroglutamic acid residue which blocks amino termini of proteins and peptides.

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