Biochimica et Biophysica Acta, 525 (1978) 417—422

BBA 68518

SUCCESSIVE CLEAVAGE OF N-TERMINAL Arg[1]-Pro[2] AND Lys[3]-Pro[4] FROM SUBSTANCE P BUT NO RELEASE OF Arg[1]-Pro[2] FROM BRADYKININ, BY X-Pro DIPEPTIDYL-AMINOPEPTIDASE

TAKESHI KATO [a], TOSHIHARU NAGATSU [a], KATSUHIKO FUKASAWA [b], MINORU HARADA [b], IKUKO NAGATSU [c] and SHUMPEI SAKAKIBARA [d]

[a] *Laboratory of Cell Physiology, Department of Life Chemistry, Graduate School at Nagatsuta, Tokyo Institute of Technology, Yokohama 227,* [b] *Department of Oral Biochemistry, Matsumoto Dental College, Shiojiri 399-07,* [c] *Department of Anatomy, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi 470-11, and* [d] *Peptide Institute, Protein Research Foundation, Minoh, Osaka 562 (Japan)*

(Received February 13th, 1978)

Summary

X-Pro dipeptidyl-aminopeptidase (EC 3.4.14.1) purified homogeneously from the human submaxillary gland was proved to hydrolyze N-terminal dipeptide Arg[1]-Pro[2] and subsequent dipeptide Lys[3]-Pro[4] from substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 *). K_m and V values of hydrolysis of substance P were 2.0 mM and 3.6 μmol/min per mg protein, respectively. In contrast, the N-terminal Arg-Pro of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) was not cleaved by the enzyme.

Introduction

X-Pro dipeptidyl-aminopeptidase (EC 3.4.14.1), which had been discovered by using a new chromogenic substrate, Gly-Pro β-naphthylamide, by Hopsu-Havu and Glenner in rat liver and kidney [1], has been purified and characterized from porcine kidney [2—4], lamb kidney [5], and human submaxillary gland [6]. The enzyme purified from the human submaxillary gland cleaves the peptide chain between R_2 and R_3 of a peptide substrate with a general structure H-R_1-R_2-R_3. R_1 can be an amino acid with a free amino terminal such as Gly, Ala, Arg, Lys, Glu and Asp [7]. Free amino terminal is essential [2,5,8]. The presence of Pro in the penultimate position (R_2) is substantial, but Ala [2—5,8] and Hyp [8] can give a weak activity. R_3 can be β-naphthylamine [1], *p*-nitroaniline [7], 4-methyl-coumarin [9], and amino acid or peptide [2—6] except Pro or Hyp [4,10].

The enzyme was found in various connective tissues such as bovine or human

* The final -NH_2 is an amide group [19]

dental pulp, dental follicles, gingiva, and rat granuloma [11,12]. Since the enzyme preferentially hydrolyzes Gly-Pro sequence which is rich in the collagen molecule, it may work on the degradation of peptides with the N-terminal Gly-Pro sequence derived from collagen in the connective tissues. However, since X-Pro sequence can be found also in various biologically active peptides, the enzyme could have some physiological role in the degradation of bioactive peptides.

Substance P is a putative peptide neurotransmitter in the brain [13,14] with a sequence of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 [15]. We have found the enzyme activity in the human and rat brain by a highly sensitive fluorescence assay [9] using a newly synthesized fluorogenic substrate, 7-(Gly-Pro)-4-methyl-coumarinamide. Therefore, substance P in the brain could be hydrolyzed by the enzyme, liberating first N-terminal Arg^1-Pro^2 and subsequently Lys^3-Pro^4.

The present report describes detailed kinetic studies on the degradation of substance P by pure X-Pro dipeptidyl-aminopeptidase. Another active peptide, bradykinin, with a sequence of Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg [16] has also been examined.

Materials and Methods

Substance P and bradykinin were obtained from Peptide Institute. Arg-Pro *p*-nitroanilide and Lys-Pro *p*-nitroanilide were synthesized in our laboratory (Peptide Institute) by the procedure described previously [7]. X-Pro dipeptidyl-aminopeptidase in the human submaxillary gland was purified to a nearly homogeneous form from the materials obtained at autopsy by a procedure described previously. The purity was approx. 95%, as judged by polyacrylamide gel electrophoresis.

The incubation mixture (total volume 500 µl) contained 375 µl 0.1 M Tris-maleate buffer (pH 7.0), 3.6 µg X-Pro dipeptidyl-aminopeptidase, and 2.0 mM substrate (substance P, bradykinin, Arg-Pro *p*-nitroanilide, or Lys-Pro *p*-nitroanilide). Incubation was carried out at 37° C for 2 h. The reaction was terminated by adding 700 µl 0.3% sulfosalicylic acid. The mixture was centrifuged, and the peptide product in the supernatant was analyzed by an amino acid analyzer (JLC-6AH fully automatic amino acid analyzer with JLC-DK dual channel integrator, JEOL Ltd, Tokyo) by the procedure of Spackman et al. [17].

The column for basic amino acids (0.8 × 15 cm), packed with JEOL LC-R-2 resin was used for the analysis. The temperature of the column was maintained at 52° C, and the flow rate through the column was 60.9 ml/h. Buffer used was 0.12 M citrate buffer (pH 5.29)/0.01% *n*-caprylic acid/0.04% Brij-35. Rate of enzymatic hydrolysis of *p*-nitroanilides of Arg-Pro, Lys-Pro and Gly-Pro was measured by the photometric assay of *p*-nitroaniline formed [7], and the value was used to calculate the amount of Arg-Pro and Lys-Pro in the amino acid analyzer.

The product from substance P or bradykinin was also identified by paper chromatography. *n*-Butanol/acetic acid/water (2 : 1 : 1, v/v) or *n*-butanol/formic acid/water (20 : 6 : 5, v/v) was used as solvent for paper chromatog-

raphy. The chromatograms were developed with 0.2% ninhydrin in acetone/pyridine (94 : 6, v/v).

Results

Substance P was proved to be hydrolyzed to release N-terminal Arg^1-Pro^2 and successively Lys^3-Pro^4 by X-Pro dipeptidyl-aminopeptidase by paper chromatography and the amino acid analyzer. Since authentic samples of Arg-Pro and Lys-Pro were unstable for synthesis, due to their conversion to diketopiperazines, *p*-nitroanilides of Arg-Pro and Lys-Pro were synthesized and used as authentic samples to identify and to quantitate the amount of Arg-Pro and Lys-Pro released during the enzymatic hydrolysis of substance P, as described in Materials and Methods.

In paper chromatography (Fig. 1), the spot corresponding to Arg-Pro or Lys-Pro was observed from *p*-nitroanilide of Arg-Pro or Lys-Pro as substrate, and the two spots corresponding to Arg-Pro and Lys-Pro were observed from substance P as substrate. The spot corresponding to Arg-Pro first appeared from substance P in the beginning (5 min and 20 min) of incubation, and later the spot corresponding to Lys-Pro was also observed at 120 min incubation. Therefore, the results of the paper chromatogram indicated successive cleavage of

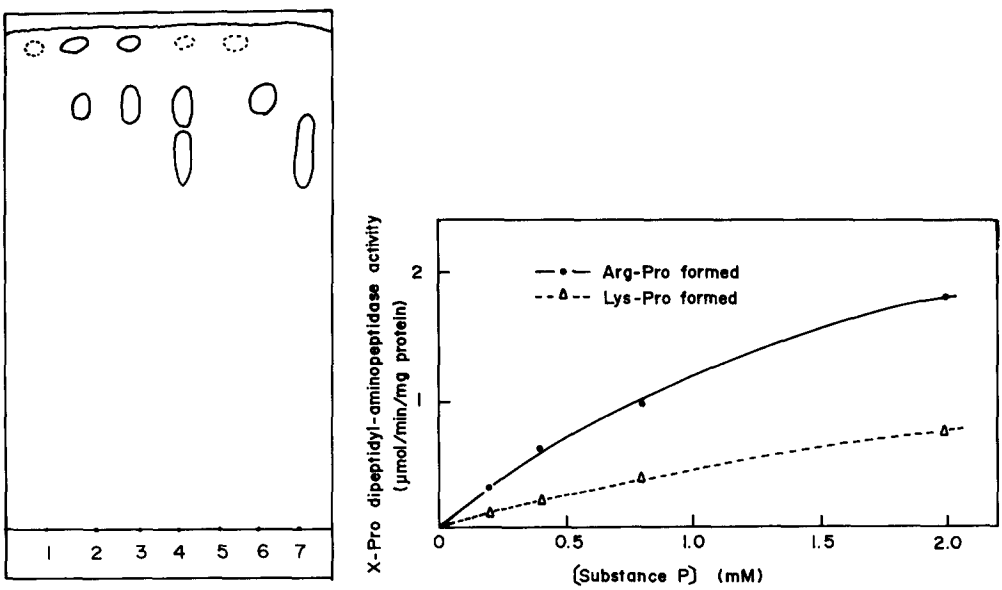

Fig. 1. Hydrolysis of substance P by X-Pro dipeptidyl-aminopeptidase. Substance P was incubated with homogeneous X-Pro dipeptidyl-aminopeptidase purified from human submaxillary gland, and the reaction products were analyzed by paper chromatography, as described in Materials and Methods. *n*-Butanol/acetic acid/water (2 : 1 : 1, v/v) was used as solvent for paper chromatography. A one-way ascending paper chromatogram was prepared for 22 h at room temperature in a solvent flow of 30 cm. 1, substance P, zero-time; 2, substance P, 5 min; 3, substance P, 20 min; 4, substance P, 120 min; 5, substance P, boiled enzyme, 120 min; 6, Arg-Pro *p*-nitroanilide, 20 min; 7, Lys-Pro *p*-nitroanilide, 20 min.

Fig. 2. Michaelis-Menten plots illustrating the effect of the concentrations of substance P on the rates of release of N-terminal dipeptides, Arg^1-Pro^2 and Lys^3-Pro^4. The incubation mixture and the assay procedure were described in the text. The dipeptides released were assayed by an amino acid analyzer.

Arg[1]-Pro[2] and Lys[3]-Pro[4] from substance P by X-Pro dipeptidyl-aminopeptidase.

In amino acid analysis for determining dipeptides by the amino acid analyzer, *p*-nitroanilides of the dipeptides did not appear in the chromatogram, and no ninhydrin-positive peak was observed in the blank incubation of either Arg-Pro *p*-nitroanilide or Lys-Pro *p*-nitroanilide without the enzyme or with the boiled enzyme. In the experimental incubation of *p*-nitroanilides of the dipeptides with the enzyme, a single peak from either Arg-Pro *p*-nitroanilide or Lys-Pro *p*-nitroanilide, which is expected to correspond to Arg-Pro or Lys-Pro, appeared at 125 min or 57 min, respectively. The retention time of Arg or Lys was 90 min or 43 min, respectively, but when *p*-nitroanilide of Arg-Pro or Lys-Pro was incubated with the enzyme, neither Arg nor Lys was observed.

For the identification of the split products of the reaction, Arg-Pro *p*-nitroanilide or Lys-Pro *p*-nitroanilide was incubated with the enzyme at 37°C for 120 min, and then subjected to paper chromatography as shown in Fig. 1. The part of the paper corresponding to Arg-Pro or Lys-Pro, was eluted with 2 M acetic acid, and the eluate was evaporated and dried under reduced pressure. When the sample was subjected to the amino acid analyzer, a single peak appeared at 125 min or 57 min from Arg-Pro *p*-nitroanilide or Lys-Pro *p*-nitroanilide, respectively. The retention time of the ninhydrin-positive material was identical to that by the direct analysis with the amino acid analyzer described above. On the other hand, the material eluted from the paper chromatogram was hydrolyzed in 6 M HCl for 20 h, then analyzed with the amino acid analyzer, and Arg and Pro, or Lys and Pro were detected in the sample from Arg-Pro *p*-nintroanilide or Lys-Pro *p*-nitroanilide, respectively. Based on these results we establish in amino acid analysis that the ninhydrin-positive material assayed was identical with the dipeptide released (Arg-Pro or Lys-Pro) and not with amino acid released sequentially.

When substance P was incubated in the absence of the enzyme or with boiled enzyme, no peak corresponding to dipeptides (Arg-Pro and Lys-Pro) or to amino acids (Arg or Lys) appeared in the amino acid analysis. In contrast, in the experiment where substance P was incubated with the enzyme, two peaks appeared at 125 min and 57 min, which correspond to Arg-Pro and Lys-Pro, as

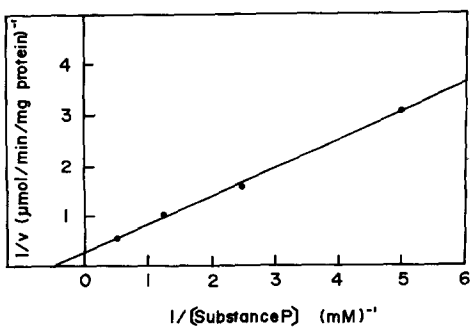

Fig. 3. Lineweaver-Burk plot illustrating the effect of the concentration of substance P on the rate of release of N-terminal dipeptide Arg[1]-Pro[2] calculated from the values in Fig. 1. The incubation mixture and the assay procedure were described in the text. Arg[1]-Pro[2] released from substance P were assayed by an amino acid analyzer. K_m and V values were calculated as 2.0 mM and 3.6 μmol/min per mg protein, respectively.

TABLE I

RATE OF HYDROLYSIS OF SUBSTANCE P, BRADYKININ AND X-PRO *p*-NITROANILIDES (X = ARG, LYS, AND GLY) WITH X-PRO DIPEPTIDYL-AMINOPEPTIDASE

The enzyme activity was assayed as described in Materials and Methods with each substrate at a concentration of 2 mM and at pH 7.0.

Substrate	Activity (μmol/min per mg protein)
Substance P (Arg-Pro formed)	1.78
(Lys-Pro formed)	0.73
Bradykinin (Arg-Pro formed)	0.00
Arg-Pro *p*-nitroanilide	4.04
Lys-Pro *p*-nitroanilide	4.33
Gly-Pro *p*-nitroanilide	3.58

identified in the above experiment. However, no peak was observed at the positions of Arg (90 min) and Lys (43 min).

Fig. 2. shows Michaelis-Menten plots illustrating the effect of substance P concentration on the rate of formation of Arg^1-Pro^2 and Lys^3-Pro^4. The Lineweaver-Burk reciprocal plot of the rate of Arg^1-Pro^2 release versus substance P concentrations (Fig. 3) gave a K_m value of 2.0 mM and a V value of 3.6 μmol/min per mg protein.

In contrast to substance P, bradykinin was not hydrolyzed at all with X-Pro dipeptidyl-aminopeptidase in the same incubation condition as substance P.

Table I shows the rates of hydrolysis of substance P at pH 7 based on the release of Arg^1-Pro^2 and Lys^3-Pro^4, and those of *p*-nitroanilides of Arg-Pro, Lys-Pro and Gly-Pro. Substance P was shown to be a substrate as good as *p*-nitroanilides of various X-Pro for X-Pro dipeptidyl-aminopeptidase.

Discussion

X-Pro dipeptidyl-aminopeptidase can hydrolyze various N-terminal X-Pro from a peptide. We reported that Gly-Pro *p*-nitroanilide gave the highest activity at an optimal pH of 8.7 among various X-Pro *p*-nitroanilides, but at pH 7.0 *p*-nitroanilides of X-Pro with N-terminal basic amino acid, i.e., either Arg-Pro or Lys-Pro, gave similar activities as Gly-Pro *p*-nitroanilide [7]. It is expected, therefore, that not only peptides with N-terminal Gly-Pro structure probably predominantly derived from collagen, but also any peptide with N-terminal X-Pro sequence can be hydrolyzed by X-Pro dipeptidyl-aminopeptidase.

Among various biologically active natural peptides, substance P is a unique molecule, since it has two successive N-terminal X-Pro structures, namely Arg^1-Pro^2-Lys^3-Pro^4 sequence [15]. In the present study, these two N-terminal dipeptides, Arg^1-Pro^2 and Lys^3-Pro^4, were shown to be released from substance P at pH 7.0 by homogeneous X-Pro dipeptidyl-aminopeptidase purified from the human submaxillary gland. Substance P is thought to be a neurotransmitter in the brain and spinal cord [13,14], and we have found X-Pro dipeptidyl-aminopeptidase activity in human and rat brains (Kato, T., Nagatsu, T., Nagatsu, I. and Sakakibara, S., to be published) and in human cerebrospinal fluid [9]. It is

conceivable, therefore, that substance P can be hydrolyzed by X-Pro dipeptidyl-aminopeptidase in the brain. Yajima et al. reported that the C-terminal heptapeptide amide without N-terminal Arg^1-Pro^2-Lys^3-Pro^4 of substance P exhibited much higher activity than substance P based on contractility on isolated guinea-pig ileum [18]. The change in the activity as a neurotransmitter due to the formation of the pentapeptide amide from substance P by X-Pro dipeptidyl-aminopeptidase should be considered in the future study.

In contrast to substance P, bradykinin, which also has N-terminal Arg^1-Pro^2 sequence [16], was proved not to be hydrolyzed by X-Pro dipeptidyl-aminopeptidase. This is expected, since we had shown that the enzyme splits the peptide which possesses the arrangement of Gly-Pro and any amino acid or peptide except Pro or Hyp at the position next to N-terminal Gly-Pro in a peptide [10].

The physiological significance of degradation of substance P and the formation of the heptapeptide amide from substance P by X-Pro dipeptidyl-aminopeptidase and purification and properties of the brain enzyme remain for further study.

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