THE SPLITTING OF SUBSTANCE P BY THE POST-PROLINE CLEAVING ENZYME FROM LAMB KIDNEYS

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Received August 8th, 1979

The degradation of substance P (an undecapeptide with the following structure: Arg-Pro-Lys--Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) by the post-proline cleaving enzyme (EC 3.4.21.–) was investigated. The peptide bond between proline and glutamine in positions 4 and 5 of substance P was hydrolyzed in the presence of the enzyme. The tetrapeptide Z-Gly-Pro-Leu-Gly-NH_2 was synthetized and used for determining the enzyme activity during the purification procedure.

The post-proline cleaving enzyme (PPCE) (EC 3.4.21.–) is a serine endopeptidase which catalyzes the cleavage of the Pro-X peptide bond with remarkable specificity. The enzyme was first discovered in the human uterus[1]. Further studies[2] showed that enzymes with this specificity are present in many vertebrates. The enzyme has been recently isolated from lamb kidneys, purified and characterized as a serine protease having the asparagine, histidine, serine triad in its active site[3–6].

A great number of proline-containing peptides were studied[3,4] with the aim of finding possible substrates for the post-proline cleaving enzyme. In all substrates tested, the peptide bond at the carboxyl side of proline was cleaved irrespective of the nature of the following amino acid residue, provided it was not proline. The Pro-X peptide bond was not cleaved when a free proline occupied the N-terminal position, but was cleaved when the proline residue was within the peptide chain.

Substance P is a biologically active undecapeptide present in many vertebrates[7]. Its primary structure (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) enables various types of enzyme-catalyzed inactivation in tissues and organs. Substance P was found to be inactivated by an acid proteinase from the bovine hypothalamus[8], cathepsin D from the calf brain[9], cathepsin A from rat liver lysosomes[10], chymotrypsin[11,12], pepsin[9,11], dipeptidyl aminopeptidase IV from rat liver[13] and X-Pro dipeptidyl aminopeptidase from the human submaxillary gland[14]. Trypsin did not split substance P[11,12].

The presence of two proline residues in the molecule of substance P makes it susceptible to cleavage by proline-specific peptidases. In this paper, we describe the splitting of substance P by the post-proline cleaving enzyme isolated from lamb kidneys.

EXPERIMENTAL

Material

Gly-NH_2 and L-Leu-Gly-NH_2 were synthetized at the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences.

Benzyloxycarbonylglycyl-L-prolyl-L-leucyl-glycine Amide

To a solution of prolyl-leucyl-glycine amide (2·9 g) in dimethylformamide (30 ml), benzyloxycarbonyl-glycine *p*-nitrophenyl ester (3 g) was added and after 20 h of stirring, another portion of active ester (1 g) was added. 20 h later, the reaction mixture was evaporated, and the residue was triturated with ether until crystals were formed. The crystalline portion was filtered off and washed with ether, water, 1M-HCl, water, a 3% solution of ammonium hydroxide and again with water. Crystallization from ethanol yielded 2·5 g (51%) of product with a m.p. of 191–192°C. A further 0·26 g of product with the same m.p. was obtained from the mother liquor. R_F 0·52 (2-butanol–98% formic acid–water, 75 : 13·5 : 11·5), 0·48 (2-butanol–25% ammonia–water, 85 : 7·5 : 7·5), 0·59 (1-butanol–acetic acid–water, 4 : 1 : 1) and 0·68 (1-butanol–pyridine–acetic acid–water, 15 : 10 : 3 : 6). Amino acid analysis (after 20 h of hydrolysis with 6M-HCl at 105°C and 150 Pa): Pro 0·99, Gly 1·88, Leu 1·01. $[\alpha]_D$ + 73·6° (*c* 0·5, dimethylformamide). For $C_{23}H_{33}N_5O_6$ (475·5) calculated: 58·09% C, 6·99% H, 14·72% N; found: 58·09% C, 6·90% H, 14·58% N.

Substance P and its fragments (Arg-Pro-Lys-Pro, Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) were synthetized[15,16] at the Institute for Drug Research, Academy of Sciences, German Democratic Republic. Dansyl chloride was purchased from Fluka AG, Switzerland. Thin-layer sheets (Silufol) were a commercial product of Kavalier, Votice, Czechoslovakia. Paper Whatman No 3 MM was used for paper electrophoresis. Sephadex G-25, G-100 and DEAE-Sephadex A-50 were purchased from Pharmacia, Sweden. Lamb kidneys were obtained at the slaughter-house.

Methods

Isolation and purification of the post-proline cleaving enzyme. A purification procedure described earlier[3,4] was modified in the following way: the extract from lamb kidneys was subjected to fractionation with ammonium sulfate. The fraction which precipitated at 50–70% saturation with ammonium sulfate was desalted by dialysis and purified by ion-exchange chromatography on DEAE-Sephadex A-50, using elution with a 0-0·25M-NaCl gradient and by gel chromatography on Sephadex G-100. The preparation was precipitated by dialysis against ammonium sulfate solution (80% saturation), the precipitate was dissolved in water and desalted on a column of Sephadex G-25. During the purification procedure, 50 mM Na-phosphate buffer, pH 6·8, containing 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetate (EDTA), was used, if not stated otherwise. The resulting enzyme preparation had 30 times higher specific activity than the crude extract.* Its activity (determined as stated below) was 0.074 U/ml, and specific activity 0·18 U/mg of protein.

* The substrate used was hydrolyzed not only by the post-proline cleaving enzyme, but also by other enzymes present in the homogenate. The analytical method, based on the determination of free α-amino groups of amino acids or peptides by the ninhydrin method, did not make it possible to distinguish which portion of the cleavage products resulted from the action of the post-proline cleaving enzyme. The degree of purification was therefore greatly underestimated.

Determination of enzyme activity. The activity was estimated by incubating the enzyme with Z-Gly-Pro-Leu-Gly-NH_2 and determining the amount of liberated Leu-Gly-NH_2 by using the ninhydrin method[17]. The reaction mixture (1 ml) contained 1 mM substrate, 20 mM Na-phosphate buffer, pH 7·7, 1 mM EDTA, 1 mM DTT and 0·5–5 mU of enzymic preparation. The reaction was run for 30 min at 37°C and stopped by adding the ninhydrin solution. The enzymic activity was expressed in μmols of Leu-Gly-NH_2 liberated in 1 min at 37°C and pH 7·7 (= 1 U).

Incubation of the enzyme with substance P. The incubation mixture (200 μl) contained 2 mM substance P, 25 mM Na-phosphate buffer, pH 6·8, 0.5 mM DTT, 1 mM EDTA and 7·4 mU of enzyme. The mixture was incubated at 37°C; 20 μl aliquots were taken at 1 h intervals and subjected to thin-layer chromatography. After 3 h of incubation, the N-terminal amino acids present in the incubation mixture were determined by the dansylation method.

Thin-layer chromatography. The peptide fragments resulting from the cleavage of Z-Gly-Pro--Leu-Gly-NH_2 and substance P were analyzed by thin-layer chromatography in the following systems: 1-butanol : pyridine : acetic acid : water (30 : 20 : 6 : 12) in the case of Z-Gly-Pro-Leu--Gly-NH_2 and 1-butanol : pyridine : acetic acid : water (10·5 : 6 : 1 : 7·5) in the case of substance P. The individual compounds were identified by means of the ninhydrin reaction and in the case of substance P also by chlorination. The following compounds were used as standards: Gly-NH_2, Leu-Gly-NH_2, Arg-Pro-Lys-Pro, Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, Lys-Pro-Gln-Gln-Phe--Phe-Gly-Leu-Met-NH_2 and substance P.

Determination of the N-terminal amino acids by the dansylation method. The peptide bond which had been split in substance P was identified by determining the N-terminal amino acids of the cleavage products formed during the incubation of substance P with the enzyme. The peptides were dansylated[18], the dansyl derivatives of amino acids were separated by thin-layer chromatography in the following systems[19]: 1-butanol : toluene : 25% ammonium (8 : 1 : 1) (S1), chloroform : toluene : 1-butanol : acetic acid (7 : 1 : 1 : 1) (S2), ethanol : toluene : 25% ammonia (7 : 2 : 1) (S3).

Paper electrophoresis. The dansyl derivatives of amino acids were also separated by electrophoresis on paper in 6% acetic acid, pH 2·4, at a potential drop of 25 V/cm.

Determination of protein concentration in the enzyme preparation was performed according to the method of Lowry and coworkers[20].

RESULTS AND DISCUSSION

During the incubation of the enzyme with the protected tetrapeptide Z-Gly-Pro--Leu-Gly-NH_2, a ninhydrin-positive product was formed which had the same mobility as the dipeptide Leu-Gly-NH_2. Glycinamide was not detected; this indicated that, under the given conditions, the bond between proline and leucine was split specifically and that the preparation was not contaminated with carboxyamidopeptidase activity.

When the incubation mixture of the enzyme and substance P was analyzed by thin layer chromatography, a cleavage product was observed which had the same mobility as the C-terminal heptapeptide of substance P. Thin-layer chromatography of the dansylated incubation mixture revealed the presence of dansyl derivatives of arginine, glutamic acid and lysine. The same result was obtained by performing paper electrophoresis of the dansyl derivatives of amino acids.

The above-mentioned results suggest that the enzyme splits the peptide bond between proline and glutamine in positions 4 and 5. This is documented by the appearance of the corresponding heptapeptide and the free α-amino group of glutamine in the incubation mixture. We have no proof that our enzyme preparation is not contaminated by X-Pro dipeptidyl aminopeptidase (EC 3.4.14.–). It is therefore possible that, under the given conditions, the bond between proline and lysine in positions 2 and 3 is also split. The absence of the nonapeptide Lys-Pro-Gln-Gln-Phe-Phe-Gly--Leu-Met-NH_2 in the incubation mixture is not relevant, because the final product of dipeptidase cleavage, *i.e.* heptapeptide Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, is identical with the product of the reaction catalyzed by the post-proline cleaving enzyme. It has been found[6] that the post-proline cleaving enzyme does not have dipeptidyl aminopeptidase activity.

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Translated by L. Servitová.