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CATABOLISM OF NEUROPEPTIDES BY A BRAIN PROLINE ENDOPEPTIDASE

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Summary. The substrate specificity of a rat brain proline endopeptidase has been examined with a variety of peptides having as few as 3 and as many as 30 amino acids. Each peptide is selectively cleaved on the carboxyl side of proline. The products of the reaction were separated by thin layer electrophoresis and identified by their amino acid compositions. Proteins such as reduced and alkylated serum albumin and denatured myoglobin were not substrates for the enzyme. The time course for hydrolysis of luteinizing hormone-releasing hormone and human angiotensin II demonstrated that both peptides were selectively and quantitatively cleaved at the expected site.

Enzymes which cleave on the carboxyl side of proline are few in number; however, recently, this laboratory purified to apparent homogeneity an enzyme from rat brain which deamidates thyrotropin-releasing hormone (TRH), releasing ammonia from the C-terminal proline residue (1). A fluorescent assay was also developed for the enzyme in which the amide residue of TRH was substituted by β-naphthylamide (1). Because the cleavage site was adjacent to a proline residue, it was of considerable interest to further examine the substrate specificity of the enzyme. Our results demonstrate that numerous polypeptides containing proline were cleaved by the enzyme; however, proteins such as hemoglobin or serum albumin having potential sites for cleavage, appear to be inert. The rat brain enzyme purified by this laboratory would thus appear to be a proline endopeptidase.

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From the substate specificity and number of peptides cleaved by the proline endopeptidase the possible use of this enzyme by protein chemists should not be overlooked. In addition, the fact that the enzyme is capable of proteolytically processing a number of neuropeptides by selective cleavage on the carboxyl side of proline warrant careful consideration of this enzyme in the catabolic events associated with postribosomal modifications of neuropeptides.

MATERIALS AND METHODS

Peptide hormones were obtained from the following companies: luteinizing hormone-releasing hormone (Beckman); angiotension II (human), oxytocin, neurotension (Bachem Fine Chemical Co.); and pGlu·His·Pro·Gly·NH_2 (Pennisula). Horse myoglobin (Sigma) was denatured and the heme removed by the method outlined by Teal (2), insulin (Elanco) and bovine serum albumin (Sigma) were reduced and alkylated by the method of Hirs (3). The β-chain of insulin was purified as described by Carmichael et al. (4).

The following β-naphthylamides were purchased from Bachem Chem. Co.: L-pGlu, CBZ-L-Arg, L-Arg, L-Pro, while L-Met was obtained from Becton-Dickerson; L-Ser, L-Trp, L-Leu and L-Tyr β-naphthylamides were purchased from Nutritional Biochemical Co.; L-Thr and L-Val were obtained from Mann Res. Lab.

Enzyme Purification. Purification of the rat brain proline endopeptidase and storage condition are given by Rupnow et al. (1). One unit of enzyme activity is defined as the amount of catalyst required to convert 1 nanomoles of substrate to product in one min.

Cleavage of some proline-containing peptides by rat brain proline endopeptidase. 1 mg of each peptide was dissolved in 0.25 ml of 0.01 M sodium phosphate pH 7.5 containing 1 mM EDTA and 1 mM 2-mercaptoethanol and incubated with 30 units of the proline endopeptidase at 37°. After 6 hours the samples were lyophilized and the residue suspended in 0.1 ml of 66% v/v methanol and spotted on plastic backed cellulose plates. The plates were then electrophoresed for 40 min. at 400 V at pH 6.5 in a buffer containing pyridine: acetic acid: water (100:3:879). After electrophoresis, one sample was visualized with fluorescamine and the corresponding sample was scraped from the plate. The peptide products were eluted from the cellulose by sequential washing with methanol, 1 M acetic acid and 0.5 M NH_4OH. The eluted products were dried over N_2 then subjected to amino acid analysis after hydrolysis in 6.0 N HCl for 24 hours at 110°.

Rate of cleavage of luteining hormone-releasing hormone and angiotensin II. Luteinizing hormone-releasing hormone (LH-RH) and angiotensin II (480 nanomoles) were dissolved in 0.09 ml of 0.10 M sodium phosphate pH 7.5 containing 1 mM EDTA and 1 mM 2-mercaptoethanol and incubated with 30 units of the proline endopeptidase (added in 0.01 ml) at 37°. At the indicated times 0.01 ml aliquots of the reaction mixtures were withdrawn and diluted into 1.74 ml of 0.2 M sodium borate, pH 9.0. After all aliquots were collected, 0.25 ml of a 0.30 mg/ml solution of fluorescamine in acetone was added and the fluorescense

read at an excitation wavelength of 390 nm and an emission wavelength of 490 nm. The fluorescence was then compared to known standards of the products of the reaction, glycineamide and phenylalanine.

Cleavage of proteins by the proline endopeptidase. 2 mg of reduced and alkylated bovine serum albumin and denatured myoglobin which had the heme removed was incubated with 0.2 ml 0.01 M sodium phosphate pH 7.5 containing 1 mM EDTA and 1 mM 2-mercaptoethanol at 37° with 30 units of proline endopeptidase added in 0.01 ml of enzyme buffer. After 12 hours the incubation mixtures were lyophilized. These samples were then subjected to analysis on SDS polyacrylamide (10%) gel electrophoresis according to the method of Weber, et al. (5). Protein was visualized by staining the gels with Coomassie brilliant blue R-250 and destaining in 10% acetic acid and 20% isopropanol.

RESULTS

Substrate Specificity of the Rat Brain Proline Endopeptidase. The rat brain enzyme was isolated using an analogue of TRH which formed a fluorescent product upon hydrolysis (1). Cleavage on the carboxyl side of proline resulted in formation of β-naphthylamine when pGlu-His-Pro-βNA or pGlu-(benzyl-imidazolyl)-His-Pro-βNA were used as substrates and ammonia when TRH was used as the substrate. The observations that both of the TRH analogues were better substrates for the enzyme than TRH itself and that the proline endopeptidase activity was found in many tissues outside the brain[1] suggested that the enzyme might have a function broader than the degradation of TRH. Because the three substrates named above were the only ones examined previously, it was of interest to determine how broad the substrate specificity of the enzyme might be. Table I summarizes the polypeptides and proteins examined as substrates with the proline endopeptidase. Cleavage of each polypeptide was noted to occur on the carboxyl side of proline. The cleavage products were separated by thin layer electrophoresis and the products identified by amino acid analysis (procedure described in Methods). When luteinizing hormone-releasing hormone, human angiotensin II and pGlu-His-Pro-Gly-NH_2 were used as substrates the products of the hydrolysis were shown to have R_f's identical to authentic glycineamide, phenylalanine and glycineamide, respectively. Amino acid composition of the peptide fragments resulting from cleavage of oxytocin, neurotensin, substance

[1] P. C. Andrews and J. E. Dixon (paper submitted for publication).

TABLE I
CLEAVAGE OF PROLINE CONTAINING PEPTIDES BY THE RAT BRAIN PROLINE ENDOPEPTIDASE

	Product identified on amino acid analysis	Number of amino acid residues	Position of Proline(s)
1. LH-RH pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	Gly	10	9
2. Angiotensin II (Human) Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	Phe	8	7
3. Oxytocin Cys-Tyr-Ile-Glu-Asn-Cys-Pro-Leu-Gly-NH_2 S-S	Leu Gly (0.93, 1.07)[a]	9	7
4. Neurotensin pGlu-Leu-Tyr-Glu-Asp-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	(Arg Arg Pro) (1.92, 1.08)[a]	13	7, 10
5. Insulin β chain[b] NH_2- - - - - - Tyr-Thr-Pro-Lys-Ala	Lys-Ala (0.86, 1.14)[a]	30	28
6. pGlu-His-Pro-Gly-NH_2	Gly	4	3
7. Albumin[b]	No Hydrolysis		
8. Myoglobin[c]	No Hydrolysis		

[a]Amino acid composition of isolated peptide.
[b]Reduced and carboxymethylated.
[c]The heme was removed by the procedure of Teal (2).

P, and the β-chain of insulin are also shown in Table I. In all cases the only products observed resulted from cleavage on the carboxyl side of proline and only the expected number of products were formed. The rat brain proline endopeptidase did not cleave reduced and alkylated bovine serum albumin or denatured myoglobin, as determined from analysis of the reaction products by SDS gel electrophoresis (data not shown). It should be noted that both proteins contain several potential sites for cleavage.

In addition to the peptides and proteins shown in Table I a number of L-amino acid β-naphthylamides were also examined as substrates. These included L-Pro, L-pGlu, L-Met, L-Ser, L-Trp, L-Leu, L-Arg, L-Thr, L-Val and L-Tyr β-naphthylamides. None of these compounds were hydrolyzed.

Rates of Cleavage of Proline containing Peptides. To establish the time course for cleavage of proline containing peptides, luteinizing hormone-releasing hormone and human angiotensin II were incubated with the proline endopeptidase for various times. The assay with each substrates takes advantage of the fact that cleavage of a peptide bond results in the appearance of one mole equivalent of glycineamide or phenylalamine which can be monitored using fluorescamine. Figure 1 shows the time course of hydrolysis

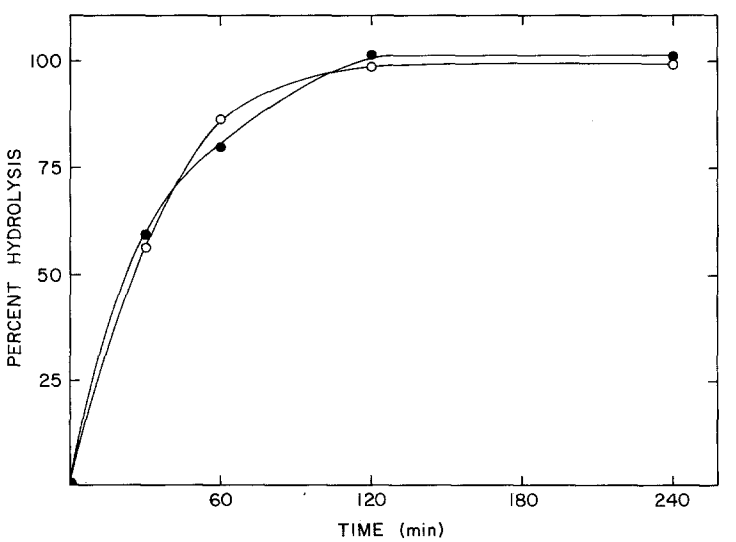

Figure 1. Time course for the hydrolysis of LH-RH(●) and angiotensin II (human) (o). Aliquots of the reaction mixture were monitored for peptide bond cleavage using fluorescamine (see Methods for details).

as determined by an increase in primary amino groups detected by fluorescamine. The hydrolysis of both peptides was complete within approximately 2 hrs. The increase in fluorescence for both compounds corresponds to the cleavage of a single bond in each substrate. The weight ratio of enzyme to substrate was 1:240 which is similar to that used in the digestion of numerous proteins with trypsin (6).

DISCUSSION

The results described herein demonstrate that the homogeneous rat brain proline endopeptidase is capable of cleaving a wide variety of peptides with a variety in amino acid compositions which contain as few as 3 and as many as 30 amino acid residues. The enzyme shows selective bond cleavage on the carboxyl side of proline in each peptide examined. It does not cleave denatured protein such horse myoglobin under the conditions examined. Thus the proline endopeptidase appears to be selective in the size of peptide, as well as the residue after which it will cleave. In addition to these experiments, we have also examined the cleavage of β-endophrin. However, difficulty in quantitatively removing the peptide from the thin layer chromatography plate precluded positive identification of the two peptides formed as a result of cleavage by the enzyme. In addition, a peptide from the active site of bovine intestinal alkaline phosphatase, having the tentative sequence of pGlu-Val-Pro-Asp-Ser-Ala-Gly-Thr-Ala-Thr, was also quantitatively cleaved after proline by this enzyme[2].

The time course shown in Figure 1 for cleavage of the two polypeptides clearly demonstrates peptide bond cleavage is both rapid and quantitative. These results suggest the possible utility of such an enzyme in formation of peptide fragments to aid in protein sequence determinations, however, the use of this enzyme would appear to be restricted to small polypeptides, because even denatured proteins of approximately 16,000 m.w. were not cleaved.

[2] J. Brotherton and L. G. Butler, personal communication.

Although enzymes which cleave on the carboxyl side of proline have been described previously (7,8), only the post-proline cleaving enzyme (9) and the post-proline dipeptidyl amino peptidase (10) have been purified to apparent homogenity and subjected to physical, chemical and kinetic analysis. A comparison of the properties of these enzymes clearly demonstrates that the rat brain proline endopeptidase is distinct from the above mentioned enzymes although all appear to be serine proteases[1](9). Evidence from this laboratory suggests that an enzyme similar to the brain proline endopeptidase is located in other tissues and may represent the major catabolic enzyme for cleavage of proline containing polypeptides[1]. Recently, Orlowski's laboratory has also described the purification of a similar enzyme from rabbit brain (11).

The specific function of this enzyme is at present unknown; however, we have obtained specific antibodies to the proline endopeptidase, which should afford an opportunity to localize this enzyme in brain as well as other tissues.

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