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DEGRADATION OF BRADYKININ BY ISOLATED NEUTRAL ENDOPEPTIDASES OF BRAIN AND PITUITARY

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SUMMARY

The degradation of bradykinin by a highly purified preparation of rabbit brain prolyl endopeptidase and by an apparently homogeneous preparation of a bovine pituitary cation-sensitive neutral endopeptidase was studied. Peptide fragments were separated and isolated by high performance liquid chromatography and identified by amino acid analysis. Prolyl endopeptidase rapidly cleaves bradykinin at the Pro^{7}-Phe^{8} bond. A slower cleavage also occurs at the Pro^{3}-Gly^{4} bond. Cation-sensitive neutral endopeptidase splits bradykinin at the Phe^{5}-Ser^{6} bond. These enzymes may participate in the regulation of brain concentrations of bradykinin.

INTRODUCTION

Immunoreactive bradykinin has recently been localized to neuronal cells and fibers in rat brain (1). Although in the past few years considerable progress has been made in our knowledge of the nature, function and distribution of biologically active peptides in brain and pituitary (2), little is known about the enzymology of their formation and degradation (3). Bradykinin can be degraded by a number of tissue and plasma exo- and endopeptidases (4). Bradykinin-degrading enzymes have also been detected in brain (5-7), however the responsible enzymes have not been sufficiently purified or characterized.

We have recently reported on the purification of two neutral endopeptidases of brain and pituitary. A prolyl endopeptidase has been purified from rabbit brain (8) and a cation-sensitive high molecular weight neutral endopeptidase was isolated from bovine pituitaries (9). We report here the action of these enzymes on bradykinin and the use of HPLC coupled with amino acid analysis to separate and identify the products of enzymatic reaction.

Abbreviations: HPLC - high performance liquid chromatography

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MATERIALS AND METHODS

Bradykinin triacetate was obtained from Bachem Inc. Torrance, CA. Methanol Chrom AR grade was a product of Mallinkrodt Inc. St. Louis, MO. AcA-34 Ultrogel was purchased from LKB Inc. Rockville, MD. Materials used in the purification and assay of the enzymes were obtained from sources previously described (8,9).

Prolyl endopeptidase was purified about 1000 fold from rabbit brain. Enzymatic activity was determined with the chromogenic substrate α-N-benzyloxycarbonylglycyl-L-prolyl sulfamethoxazole as previously described (8). A unit of enzyme activity is defined as the amount of enzyme causing the release of 1μmole sulfamethoxazole/hr. The preparation used had a specific activity of 700 units/mg protein.

Cation-sensitive neutral endopeptidase was prepared from bovine pituitaries by a modification of the published procedure (9). Enzymatic activity was determined using the chromogenic substrate α-N-benzyloxycarbonylglycylglycyl-L-leucyl-p-nitroanilide (9). After step 4 of the purification procedure the enzyme was rechromatographed on a 1.5 x 17 cm column of diethylaminoethylcellulose (DE-52) equilibrated at pH 8.3 with 0.01 M Tris-EDTA. The enzyme was eluted with a gradient established between 200 ml of the 0.01 M buffer and 200 ml of 0.4 M Tris-EDTA buffer at the same pH. Fractions containing enzymatic activity were pooled, concentrated by ultrafiltration, and subjected to gel filtration on a 2.5 x 45 cm column packed with AcA-34 ultrogel buffered at pH 7.5 with 0.01 M Tris-EDTA. The enzyme emerged as a single peak coincident with a protein peak. Polyacrylamide gel electrophoresis (5% gels) in a 0.05 M Tris-HCl buffer, pH 8.3 (10) revealed a single protein band. A unit of activity was defined as the amount of enzyme catalyzing the release of 1μmole p-nitroaniline per hour from the chromogenic substrate. The specific activity of the purified enzyme used in these studies was 8.4 units/mg protein representing an 1100 fold purification in this preparation.

HPLC was carried out using a Perkin-Elmer series 2 liquid chromatograph coupled to an LC-55 variable wavelength UV detector. Samples were injected onto a 0.46 x 25 cm reverse phase column (ODS-SIL-X-1, Perkin-Elmer). A linear gradient was established between 15% methanol in 0.05M KH_2PO_4 adjusted to pH 2.0 with phosphoric acid and pure methanol (11). The gradient was programmed for an increase of 2%/min of the methanol concentration. The total flow was 2 ml/min and the effluent was monitored continuously at 210 nm. Fractions corresponding to discrete peaks were collected, evaporated under a stream of nitrogen, and the residue hydrolyzed in vacuo in 6N HCl for 18 hr at 105°. The hydrolyzate was evaporated under nitrogen and the residue subjected to amino acid analysis using a Technicon amino acid autoanalyzer.

Cation-sensitive neutral endopeptidase was incubated with bradykinin at 37° in a reaction mixture (final volume 0.2 ml) containing 1.1μmole peptide, Tris-EDTA buffer (0.01 M; pH 7.5) and enzyme (0.7 units). The progress of the reaction was monitored by injection of 5μl aliquots onto the HPLC column at varying time intervals. After 19 hr the reaction was terminated by chromatographing the remaining sample in three equal portions. Fractions were collected and pooled for amino acid analysis as described.

The effect of prolyl endopeptidase on bradykinin was studied in a similar manner. The reaction mixture contained 1.1μmole peptide, 50μl prolyl endopeptidase (1.35 units in 0.01 M potassium phosphate buffer,

pH 8.0), 10µl 0.01 M dithiothreitol and 150µl 0.01 M potassium phosphate buffer pH 8.0. The progress of the reaction was monitored as above and terminated after 5 hr by chromatography of the mixture as described above.

RESULTS

HPLC analysis of the incubation mixture of bradykinin with prolyl endopeptidase revealed two major and two minor components (Fig. 1). Amino acid analysis of the resolved major components was consistent with the structures Arg-Pro-Pro-Gly-Phe-Ser-Pro and Phe-Arg establishing a major point of cleavage at the Pro^7-Phe^8 bond. Amino acid analysis of the resolved minor components was consistent with the structures Arg-Pro-Pro and Gly-Phe-Ser-Pro establishing a minor cleavage point at the Pro^3-Gly^4 bond.

HPLC analysis of the incubation mixture of bradykinin with cation-sensitive neutral endopeptidase revealed two peptide products (Fig. 2). Amino acid analysis of the resolved products was consistent with the structures Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg establishing a point of cleavage at the Phe^5-Ser^6 bond by this enzyme.

HPLC on a reverse phase column was capable of resolving all peptide products from each other and from unreacted bradykinin. The retention times of the peptides increased with increasing peptide chain length (Table 1).

DISCUSSION

Our studies have illustrated the value of HPLC coupled with amino acid analysis for the separation and characterization of peptides. This method is therefore highly suitable in studies on the specificity of proteolytic enzymes and their action on biologically active peptides.

The cleavage of bradykinin by prolyl endopeptidase as studied by HPLC confirms our earlier conclusions based on a microdansylation technique to identify the points of cleavage (8). The major cleavage of the Pro^7-Phe^8 bond and minor cleavage of the Pro^3-Gly^4 bond is consis-

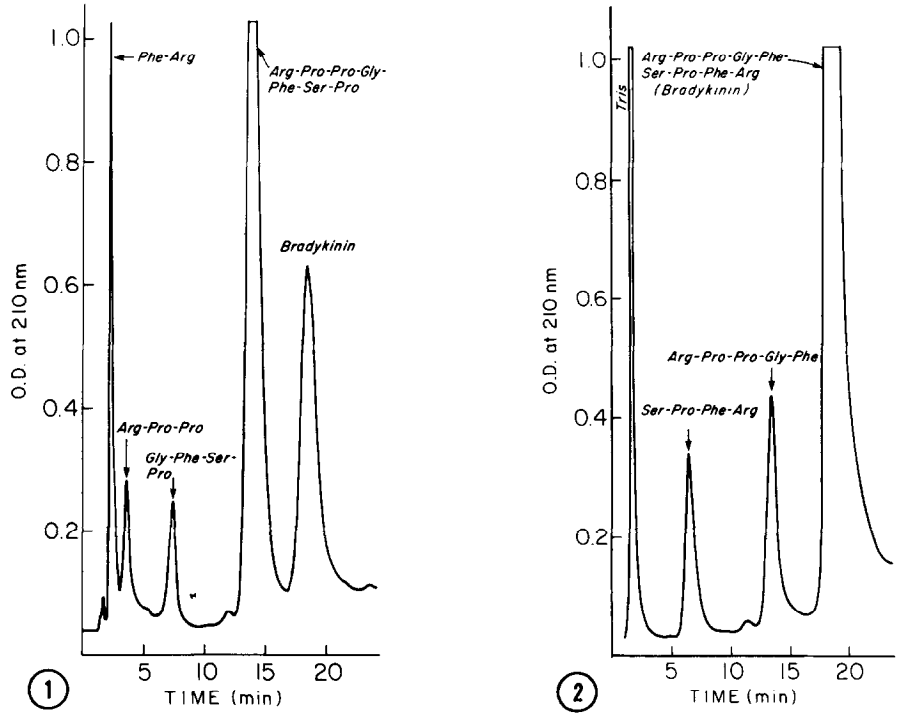

Fig. 1 Reverse phase HPLC separation of peptide products formed by the action of prolyl endopeptidase on bradykinin. Peptides were eluted with a linear gradient established between 15% methanol in 0.05 M KH_2PO_4 adjusted to pH 2.0 with H_3PO_4 and pure methanol. The gradient was programmed for an increase of 2% min of the methanol concentration and the total flow was 2 ml/min. Peptide products were identified by amino acid analysis as described in the text.

Fig. 2 Reverse phase HPLC separation of peptide products formed by the action of cation-sensitive neutral endopeptidase on bradykinin. Chromatographic conditions as in Fig. 1.

TABLE 1

HPLC SEPARATION OF PEPTIDES DERIVED FROM ENZYMATIC DEGRADATION OF BRADYKININ

Peptide[a]	Retention Time (min.)[b]
Phe-Arg	3
Arg-Pro-Pro	4.8
Ser-Pro-Phe-Arg	7.5
Gly-Phe-Ser-Pro	9
Arg-Pro-Pro-Gly-Phe	14
Arg-Pro-Pro-Gly-Phe-Ser-Pro	16.3
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	19.5

a)Amino acid composition determined by amino acid analysis
b)Chromatographic conditions given in the text

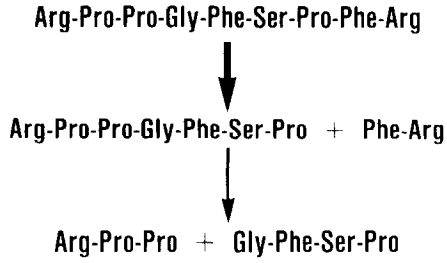

Fig. 3 Degradation of bradykinin by prolyl endopeptidase. Heavy arrow represents fast reaction and light arrow represents slow reaction.

tent with the scheme for degradation shown in Fig. 3. The peptide Gly-Phe-Ser-Pro-Phe-Arg which could be formed by direct cleavage of the Pro^3-Gly^4 bond was not isolated because it was apparently rapidly degraded to Gly-Phe-Ser-Pro and Phe-Arg.

Prolyl endopeptidase, isolated by us from rabbit brain (8) has a specificity similar to the "post-proline cleaving enzyme" isolated from lamb kidney by Walter and co-workers (12,13). This enzyme however differs from the kidney enzyme by its high sensitivity to sulfhydryl blocking agents and by a molecular weight which is about half of that of the kidney enzyme (8). It is highly unlikely that brain prolyl endopeptidase is a monomeric form of "post-proline cleaving enzyme", since dimerization of the enzyme was never observed. This enzyme also clearly differs from "peptidase P" which also splits the Pro^7-Phe^8 bond of bradykinin (4) and was later shown to be apparently identical with angiotensin converting enzyme (14).

Cation-sensitive neutral endopeptidase purified from bovine pituitaries to a single band on polyacrylamide gel electrophoresis cleaves the Phe^5-Ser^6 bond of bradykinin. Other investigators have detected cleavage of this bond in crude or partially purified brain preparations (6,15), however the responsible enzymes have not been isolated or sufficiently characterized. The pituitary enzyme used in our studies has a molecular weight well in excess of 200,000 and a similar enzyme has

recently been purified in our laboratory from rabbit brain (unpublished data). Both enzymes are characterized by a great sensitivity to inhibition by monovalent cations. The enzyme is clearly distinguishable from other neutral endopeptidases detected in brain (15). Although cleavage of the Phe^5-Ser^6 bond is a chymotrypsin-like split, it should be noted that chymotrypsin preferentially cleaves the Phe^8-Arg^9 bond of bradykinin with only minor cleavage of the Phe^5-Ser^6 bond (16).

The high concentration in brain of the neutral endopeptidases described makes it likely that these enzymes play an important role in controlling the levels of bradykinin and other biologically active peptides in brain. This function acquires special significance in view of the known central effects of bradykinin and the recent finding of bradykinin-like immunoreactive neuronal systems in rat brain (1).

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