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DETERMINATION OF SPECIFICITY OF ENDOPEPTIDASES BY COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND AMINO ACID ANALYSIS

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SUMMARY

The specificity of three neutral endopeptidases toward several biologically active peptides was determined by combined high-performance liquid chromatography and amino acid analysis of the degradation products. Incubation mixtures were chromatographed on a reversed-phase column equilibrated with a mixture of acetonitrile and potassium phosphate buffer (0 05 *M*; pH 2 0) Reaction products were eluted with a linear gradient of acetonitrile and the absorbance of the effluent monitored at 210 nm Fractions corresponding to discrete peaks were subjected to quantitative amino acid analysis The peptide bond undergoing cleavage is readily assigned from the knowledge of the primary structure of the peptide and the amino acid composition of the reaction products.

INTRODUCTION

Recognition of the important role of peptides in neural function and also in the regulation of function of peripheral tissues, has stimulated renewed interest in the isolation, characterization and determination of the specificty of endopeptidases from brain and other tissues. These enzymes are likely to function in the formation of active peptides from precursor molecules and also in the degradation of these peptides, thereby terminating their activity Determination of the substrate specificity of endopeptidases requires techniques for the separation and identification of the peptides formed by their action on peptide and protein substrates. The frequently tedious classical electrophoretic and chromatographic methods for peptide separation are now being replaced by more efficient, simple and rapid high-performance liquid chromatographic (HPLC) techniques. Reversed-phase liquid chromatography with ion suppression achieves a high efficiency resolution of peptides The separation process can be monitored by measuring the absorbance of the peptide bond at 210 nm and emerging peptides collected for definitive characterization by amino acid analysis.

We describe here the application of HPLC and amino acid analysis to the elucidation of the substrate specificity of three neutral endopeptidases that we have isolated from various tissues including brain and pituitary[1–4].

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MATERIALS AND METHODS

Prolyl endopeptidase was prepared from rabbit brain as described[1] The specific activity of the preparation used in these studies was 600 U/mg. Membrane-bound metalloendopeptidase was isolated from rabbit kidney cortex using the procedure described for isolation of the enzyme from bovine pituitaries[4]. The specific activity was 1000 U/mg. Cation sensitive neutral endopeptidase was isolated from bovine pituitaries as described[3]. The specific activity of the preparation used in these studies was 4 U/mg. Bradykinin triacetate and angiotensin II acetate were obtained from Bachem, Torrance, CA, U.S A. Leucine enkephalin and neurotensin were obtained from Sigma, St. Louis, MO, U.S.A. All other chemicals were of the highest analytical grade available. Z-D-Ala–Leu–Arg–2NA* was synthesized in this laboratory by Dr Stevens Pearce

HPLC was carried out on a Perkin-Elmer series 2 liquid chromatograph equipped with a variable-wavelength spectrophotometric detector (LC-55). Peptide mixtures were chromatographed on a 250 × 4 mm Bio-Sil ODS-10 (10 μm) reversed-phase column (Bio-Rad Labs.). The column was equilibrated with a mixture of acetonitrile and potassium phosphate buffer (0.05 *M*; pH 2 0)[5]. The starting acetonitrile concentration was 10%. A linear gradient was programmed for an increase in the acetonitrile concentration of 1%/min or 2%/min as required. The flow-rate was 1 ml/min. Peptide elution was monitored at 210 nm.

Incubations were carried out in a final volume of 250 μl. Details of individual incubations are given in the legends to the figures. The progress of the reaction was monitored by subjecting 10-μl aliquots of the incubation mixture to reversed-phase chromatography. After a sufficient amount of reaction product was formed, the remainder of the incubation mixture was injected on the column. Fractions corresponding to discrete peaks were manually collected and concentrated under a stream of nitrogen. The concentrated samples were transferred to 1-ml ampules and the contents evaporated to dryness *in vacuo*. To the dried residue was added 150 μl constant boiling hydrochloric acid (6 *N*) and 10 μl phenol to tyrosine-containing peptides in order to prevent oxidation of the phenolic ring. The ampules were evacuated, sealed, and the contents hydrolyzed at 105°C for 18 h. The contents of the ampules were then evaporated to dryness under nitrogen and the hydrolysate subjected to amino acid analysis on a Technicon TSM amino acid autoanalyzer.

RESULTS

Reversed-phase HPLC was found to be an excellent system for the rapid and efficient resolution of peptides. Moreover peptides could be readily collected as they emerged from the column for definitive characterization by amino acid analysis. The combination of HPLC and amino acid analysis was applied to the study of endopeptidase specificity. Sites of cleavage were assigned from a knowledge of the primary structure of the peptide substrate and the amino acid composition of the products. Representative examples are given below.

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* 2NA = 2-Naphthylamide.

Membrane-bound metalloendopeptidase

Incubation of this enzyme with leucine enkephalin gave two peptide products (Fig. 1). Analysis of the amino acid composition of the products revealed a split on the amino side of the phenylalanine residue (Table I) Incubation of the metalloendopeptidase with bradykinin gave four peptide products (Fig 2) Analysis of the amino acid composition of the products revealed two splits of the peptide chain, again each occurring on the amino side of a phenylalanine residue (Table I) The specificity of this enzyme has been studied in considerable detail both with model chromogenic substrates[4] as well as with neuropeptides[6] The results are consistent with a "thermolysin-like" specificity in which peptide bonds at the amino side of hydrophobic amino acids are preferentially cleaved.

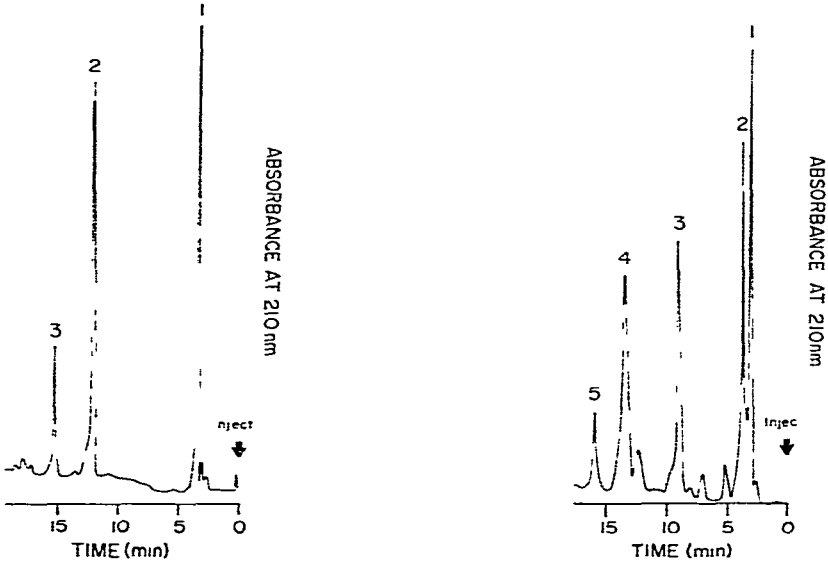

Fig 1 Action of metalloendopeptidase on leucine enkephalin The incubation was carried out at 37°C for 1 h in a Tris–HCl buffer (0 05 *M*, pH 7 3) The incubation mixture (total volume 0 25 ml) contained 330 nmoles leucine enkephalin and 1 2 units of enzyme A 10-μl aliquot was chromatographed on a 250 × 4 mm Bio-Sil ODS-10 column equilibrated with a mixture of acetonitrile and potassium phosphate buffer (0 05 *M*, pH 2 0) The starting concentration of acetonitrile was 10% and was linearly increased at a rate of 2% min The flow-rate was 1 ml/min Absorbance was monitored at 210 nm (0 2 a u f s)

Fig 2. Action of metalloendopeptidase on bradykinin Incubation conditions as in Fig 1 Chromatographic conditions as in Fig 1 except for a 1%/min increase in acetonitrile concentration 0 1 a u f s

Prolyl endopeptidase

Incubation of prolyl endopeptidase with angiotensin II gave two peptide products (Fig. 3) The larger fragment was readily identified as des-phenylalanyl angiotensin II, demonstrating cleavage at the carboxyl side of the single prolyl residue (Table II) Incubation of the enzyme with neurotensin for 3 h gave two peptide products consistent with a split of the Pro–Tyr bond (Fig. 4, Table II) An increase in the time of incubation led to a split of the Pro–Arg bond as well (data not shown).

TABLE I

ACTION OF MEMBRANE-BOUND METALLOENDOPEPTIDASE ON BRADYKININ AND LEUCINE ENKEPHALIN

*Peak**	*Amino acids***	*Assignment*
Bradykinin		
1	Gly (1 0), Pro (2.1), Arg (0 8)	Arg–Pro–Pro–Gly
2	Arg (1.0), Phe (0 8)	Phe–Arg
3	Ser (1 0), Pro (1 1), Phe (1 2)	Ser–Pro–Phe
4	Gly (1 0), Pro (3 2), Ser (1.2), Phe (1 2), Arg (0 8)	Arg–Pro–Pro–Gly–Phe–Ser–Pro
5	—	Bradykinin***
	Arg–Pro–Pro–Gly↓Phe–Ser–Pro↓Phe–Arg	
Leucine enkephalin		
1	Tyr (1 0), Gly (2.0)	Tyr–Gly–Gly
2	Phe (1 0), Leu (0 9)	Phe–Leu
3	—	Leucine enkephalin***
	Tyr–Gly–Gly↓Phe–Leu	

* Peak numbers refer to Fig. 2 (bradykinin) and Fig. 1 (leucine enkephalin)
** Amino acid concentrations expressed relative to first amino acid listed
*** Assignment based on retention time

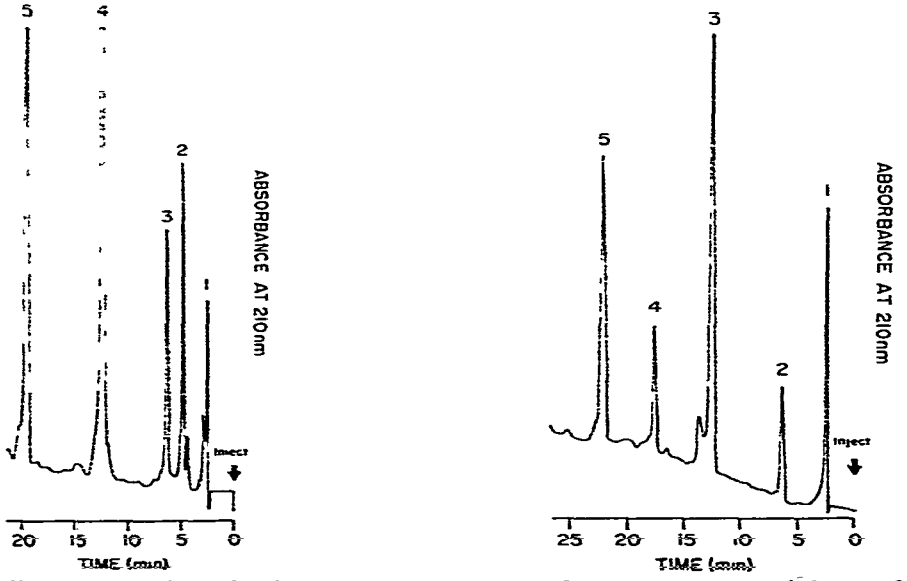

Fig. 3 Action of prolyl endopeptidase on angiotensin II. Incubation was carried out at 37°C for 5 h in a Tris–HCl buffer (0 1 *M*; pH 8.3). The incubation mixture (total volume 0.25 ml) contained 190 nmoles angiotensin II, 250 nmoles dithiothreitol and 0 6 units of enzyme. Chromatographic conditions as in Fig. 2.

Fig. 4. Action of prolyl endopeptidase on neurotensin. Incubation was carried out for 3 h at 37°C in a Tris–HCl buffer (0.1 *M*; pH 8.3). The incubation mixture (total volume 0.25 ml) contained 60 nmoles neurotensin, 250 nmoles dithiothreitol and 0 6 units enzyme. Chromatographic conditions as in Fig. 2.

TABLE II

ACTION OF PROLYL ENDOPEPTIDASE ON ANGIOTENSIN II AND NEUROTENSIN

*Peak**	*Amino acids***	*Assignment*
Angiotensin II		
1	No amino acids	‡
2	Phe (1 0)	Phe
3	No amino acids	‡
4	His (1 0), Arg (1 0) Asp (0 8) Pro (1 0), Val (0 8), Ile (0 8) Tyr (1 0)	Asp–Arg–Val–Tyr–Ile–His–Pro
5	– Asp–Arg–Val–Tyr–Ile–His–Pro↓Phe	Angiotensin II***
Neurotensin		
1	No amino acids	‡
2	No amino acids	‡
3	Asp (1 0) Lys (1 4) Arg (2.3) Glu (2 2), Pro (1 9), Leu (0 7) Tyr (1 1)	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro
4	Ile (1 0), Leu (0 9) Tyr (1 3)	Tyr–Ile–Leu
5	– pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro↓ –Tyr–Ile–Leu	Neurotensin***

* Peak numbers refer to Fig 3 (angiotensin II) and Fig 4 (neurotensin)
** Amino acid concentrations expressed relative to first amino acid listed
*** Assignment based on retention time
‡ Present in unincubated control

Cation-sensitive neutral endopeptidase

Incubation of this enzyme with neurotensin produced three primary cleavages (Fig. 5). These were assigned on the basis of the amino acid composition of the products to a split of the Glu–Asn, Asn–Lys and Ile–Leu bonds (Table III). Incubation of cation-sensitive neutral endopeptidase with the model chromogenic substrate *Z*-D-Ala–Leu–Arg–2NA revealed a trypsin-like activity with cleavage of the Arg–2NA bond (Fig. 6, Table III).

DISCUSSION

HPLC and amino acid analysis was used for the separation and characterization of peptide products formed by the action of endopeptidases on neuropeptide substrates. The specificity of three neutral endopeptidases isolated from brain, pituitary and other tissues was readily determined using this technique Its speed, simplicity and reliability should make it the method of choice for such studies. The

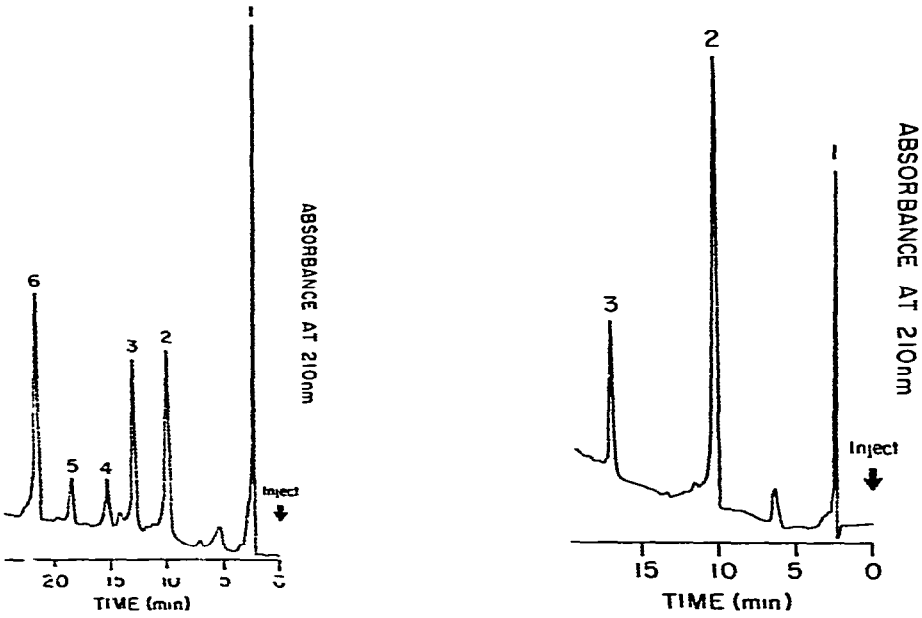

Fig. 5. Action of cation-sensitive neutral endopeptidase on neurotensin. Incubation was carried out for 16 h at 37°C in a Tris–HCl buffer (0 05 *M*; pH 7.5). The incubation mixture (total volume 0.25 ml) contained 60 nmoles neurotensin and 0.1 units of enzyme. Chromatographic conditions as in Fig. 2.

Fig. 6 Action of cation-sensitive neutral endopeptidase on *Z*-D-Ala–Leu–Arg–2NA. Incubation was carried out for 20 h at 37°C in a Tris–HCl buffer (0 05 *M*; pH 7 5). The incubation mixture (total volume 0 25 ml) contained 200 nmoles *Z*-D-Ala–Leu–Arg–2NA, 250 nmoles dithiothreitol and 0.1 units of enzyme. Chromatographic conditions as in Fig. 1.

classification of endopeptidases on the basis of substrate specificity is illustrated with the three enzymes used in this study.

Membrane-bound metalloendopeptidase can be viewed as a thermolysin-like enzyme on the basis of its resemblance to the specificity of the bacterial enzyme[7]. Both enzymes cleave peptide bonds on the amino side of a hydrophobic amino acid. The action of the metalloendopeptidase is illustrated here with bradykinin and with leucine enkephalin. Although leucine enkephalin is cleaved to release a C-terminal dipeptide, the enzyme is not a dipeptidyl carboxypeptidase. It will cleave a C-terminal dipeptide if a hydrophobic amino acid is present in the penultimate position, but its action on bradykinin and on C-terminal blocked peptides[4] shows that a free carboxyl group is not a requirement for activity. Table IV summarizes the action of this enzyme on other peptide substrates[6].

Prolyl endopeptidase cleaves peptidyl prolyl peptide and peptidyl prolyl amino acid bonds. The enzyme has a preference for bonds in which the amino function is provided by a hydrophobic amino acid. This is seen with neurotensin as substrate in which the Pro–Tyr bond is readily cleaved (Fig. 4, Table II) and in which the Pro–Arg bond undergoes cleavage only after prolonged incubation[1]. Although the enzyme releases the terminal phenylalanine from angiotensin II (Fig. 3, Table II) it is not a carboxypeptidase. A C-terminal amino acid will be split if a proline residue is present

TABLE III

ACTION OF CATION-SENSITIVE NEUTRAL ENDOPEPTIDASE ON NEUROTENSIN AND *Z*-DAla–Leu–Arg–2NA

*Peak**	*Amino acids***	*Assignment*
Neurotensin		
1	Asp (1 0)	Asp
2	Tyr (1 0), Ile (0 9) Pro (2 2), Arg (2 0), Lys (1 3)	Lys–Pro–Arg–Arg–Pro–Tyr–Ile
3	Tyr (1 0), Leu (0 8) Glu (2.0)	pGlu–Leu–Tyr–Glu
4	Traces	–
5	Traces	–
6	–	Neurotensin***
	pGlu–Leu–Tyr–Glu↓Asn↓Lys–Pro–Arg–Arg–Pro–Tyr–Ile↓Leu	
Z-D-Ala–Leu–Arg–2NA		
1	None	–[§]
2	None	2-naphthylamine***
3	Arg (1 0) Ala (0 9), Leu (0 8)	*Z*-D-Ala–Leu–Arg
	Z-D-Ala–Leu–Arg↓2NA	

* Peak numbers refer to Fig 5 (neurotensin) and Fig 6 (*Z*-D-Ala–Leu–Arg–2NA)

** Amino acid concentrations expressed relative to first amino acid listed.

*** Assignment based on retention time

§ Present in unincubated control

TABLE IV

CLEAVAGE OF BIOLOGICALLY ACTIVE PEPTIDES BY MEMBRANE-BOUND METALLOENDOPEPTIDASE

Peptide	*Site of cleavage*
Substance P	Arg–Pro–Lys–Pro–Gln–Gln↓Phe↓Phe–Gly↓Leu–MetNH_2
Met-Enkephalin	Tyr–Gly–Gly↓Phe–Met
Oxytocin	Cys–Tyr–Ile–Gln–Asn–Cys–Pro↓Leu–GlyNH_2
Neurotensin	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro↓Tyr↓Ile–Leu

in the penultimate position. However, the action of this enzyme on the C-terminal blocked peptide LHRH[8] and on other neuropeptide substrates[1] shows that a free carboxyl group is not a requirement for activity. It is now clear that prolyl endopeptidase is identical to enzymes designated as "post-proline cleaving enzyme"[9,10], "TRH deamidase"[11–13] and "bradykinin kininase B"[14]. Table V summarizes the action of this enzyme on other peptide substrates[1,8,15]

TABLE V

CLEAVAGE OF BIOLOGICALLY ACTIVE PEPTIDES BY PROLYL ENDOPEPTIDASE

Peptide	*Site of cleavage*
Bradykinin	Arg–Pro–Pro↓Gly–Phe–Ser–Pro↓Phe–Arg*
TRH	pGlu–His–Pro↓NH_2
LHRH	pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro↓Gly–NH_2

* The Pro–Gly bond of bradykinin is cleaved at an appreciably slower rate than the Pro–Phe bond

Cation-sensitive neutral endopeptidase, a high-molecular-weight enzyme first isolated from bovine pituitaries[2,3] has recently been shown to be a multi-catalytic protease complex[16]. It exhibits chymotrypsinlike, trypsinlike and peptidylglutamyl-peptide bond hydrolyzing activities. Experiments with selective inhibitors and activators have shown that the activities are catalyzed by distinct active sites The chymotrypsinlike and peptidylglutamyl-peptide bond hydrolyzing activities are observed in the action of this enzyme complex on neurotensin (Fig. 5, Table III) The trypsinlike activity is observed in the cleavage of the model chromogenic substrate Z-D-Ala–Leu–Arg–2NA. With the latter substrate, two major products are observed on the HPLC chromatogram (Fig. 6). One peak containing no amino acids is attributed on the basis of its retention time to 2NA The amino acid composition of the other

TABLE VI

CLEAVAGE OF BIOLOGICALLY ACTIVE PEPTIDES BY CATION-SENSITIVE NEUTRAL ENDOPEPTIDASE

Peptide	*Site of cleavage**
Angiotensin II	Asp—Arg—Val↓Tyr↓Ile↓His—Pro↓Phe
Bradykinin	Arg—Pro—Pro—Gly—Phe↓Ser—Pro—Phe—Arg
LHRH	pGlu—His—Trp↓Ser—Tyr↓Gly—Leu—Arg—Pro—Gly—NH_2
α-MSH	Ac—Ser—Tyr↓Ser—Met—Gly—His↓ Phe↓Arg—Trp↓Gly—Lys—Pro—Val—NH_2
Substance P	Arg—Pro—Lys—Pro—Gln—Gln—Phe↓Phe↓Gly—Leu—Met—NH_2

* Heavy arrows indicate rapid cleavage and dashed arrows indicate slow cleavage

product is similar to that of the unchanged substrate but is identified as Z-D-Ala–Leu–Arg because it is well separated from the substrate Thus a major cleavage between Arg and 2NA is assigned (Table III) Table VI summarizes the action of this enzyme on other peptide substrates[3 16 17]

In summary, the bonds cleaved by the action of an endopeptidase on a peptide substrate can be readily deduced from a knowledge of the primary structure of the substrate and the amino acid composition of the products HPLC provides a simple and rapid means for peptide separation The non-destructive UV detector allows for the collection of the products for further characterization by amino acid analysis

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