Preparation, Assay, and Partial Characterization of a Neutral Endopeptidase from Rabbit Brain†

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ABSTRACT: In a study of the proteolytic enzymes in brain tissue which act on pharmacologically active peptides a neutral endopeptidase has been prepared that inactivates bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg by hydrolysis of the Phe[5]–Ser[6] peptide bond. The enzyme was isolated from the supernatant fraction of a 0.25 M sucrose homogenate of rabbit brain by gel filtration and chromatography on DEAE-cellulose. Enzyme activity was inhibited by *p*-chloromercuribenzoate and enhanced by dithiothreitol indicating the requirement of free sulfhydryl groups for activity. A chromatographic system employing the amino acid analyzer was used to identify the site of hydrolysis in bradykinin and to estimate quantitatively the products of the reaction Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg for kinetic studies of the endopeptidase acting on the nonapeptide substrate. The K_m for the reaction is 1.8×10^{-4} M at pH 7.5, 37°. The endopeptidase did not hydrolyze denatured hemoglobin. A second enzyme capable of inactivating bradykinin has been partially purified and tentatively identified as a carboxydipeptidase which releases the dipeptides Phe-Arg and Ser-Pro from the carboxyl terminus of the molecule.

Intracellular proteolytic enzymes participate in protein turnover, the regulation of enzyme levels and in the release and degradation of polypeptide hormones. These aspects of tissue protein and polypeptide biochemistry are particularly relevant to brain metabolism because of the extremely rapid turnover of brain protein and the limitations imposed by the blood brain barrier to amino acid transport (Davson, 1969; Schimke, 1970; Lajtha and Marks, 1971). The recent characterization of low molecular weight peptide hypothalamic regulatory hormones (*cf.* Gaul *et al.*, 1972; Guillemin and Burgus, 1972), substance P (Chang *et al.*, 1971; Zetler, 1970), and the demonstration of the action of peptide hormones such as bradykinin and angiotensin on the central nervous system, have focused attention on the proteases and peptidases present in brain tissue that are responsible for the release, interconversion, and inactivation of peptide hormones (Fischer-Ferraro *et al.*, 1971; Ganten *et al.*, 1971; Camargo *et al.*, 1972; Yang and Neff, 1972).

In an earlier study of the proteolytic enzymes in rabbit brain that are capable of hydrolyzing pharmacologically active peptides, we described a procedure for the preparation of a mixture of bradykinin activating enzymes (kininases) by gel filtration of an extract of the tissue (Camargo *et al.*, 1972). In the present work, the kininases in the mixture were separated into two fractions by DEAE-cellulose chromatography. One of the fractions consists mainly of an endopeptidase which hydrolyzes bradykinin at the Phe[5]–Ser[6] bond optimally at pH 7, and does not hydrolyze denatured hemoglobin. A chromatographic system employing the amino acid analyzer was used to identify the site of hydrolysis in bradykinin and for kinetic studies of the endopeptidase.

Materials

Frozen rabbit brain (type 3, mature animals) was purchased from Pel-Freeze Biologicals Inc., Rogers, Ark. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Aminex A-5 resin was purchased from Bio-Rad Laboratories, Richmond, Calif., and PA-28 resin from Beckman Instruments Inc., Spinco Division, Palo Alto, Calif. DEAE-cellulose (Whatman DE-52) was obtained from Reeve Angel Co., Clifton, N. J. Bradykinin was purchased from Schwarz Biochemicals Co., Orangeburg, N. Y. Hemoglobin substrate powder, trypsin, and chymotrypsin were products of Worthington Biochemicals, Inc., Freehold, N. J. *p*-Chloromercuribenzoate was a product of Aldrich Chemical Co., Milwaukee, Wis. Dithiothreitol was purchased from Calbiochem, Los Angeles, Calif.

Synthetic peptides corresponding to fragments of bradykinin were gifts from Dr. John M. Stewart (University of Colorado Medical School, Denver, Colo.) and Dr. Kenji Suzuki (Tohoku College of Pharmacy, Naukozawa, Seindai, Japan). The peptides had integral molar ratios of the constituent amino acids after acid hydrolysis and were homogeneous by high-voltage electrophoresis at pH 3.5 and 6.5.

Methods

Determination of Proteolytic Enzyme Activity Using Bradykinin as Substrate–Kininase Assay (Camargo et al., 1972). The isolated guinea pig ileum was used to monitor the loss of biological activity of bradykinin. Since all peptide fragments of bradykinin have less than 1% of the activity of the intact nonapeptide, the loss of biological activity reflects the hydrolysis of any one of eight peptide bonds in bradykinin.

Bradykinin (80 nmol/ml) was incubated with enzyme in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl for 10 and 20 min at 37°. The reaction was stopped by the addition of 0.010 ml of 12 N HCl/ml of incubation solution. Residual bradykinin activity was determined by a matching technique with the isolated guinea pig ileum bathed at 37° in 10 ml of Tyrodes buffer containing 3.5×10^{-7} M atropine and 1.7×10^{-6} M diphenylhydramine. Under these conditions, up to 50%

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inactivation of bradykinin was proportional to the amount of enzyme and the duration of incubation. This assay is the same as described previously except that the bradykinin concentration has been increased 80 times in order to facilitate analysis of the products with the amino acid analyzer. One unit of kininase activity is defined as 1 μmol of bradykinin inactivation/min measured with the isolated guinea pig ileum.

Determination of Proteolytic Activity Using Denatured Hemoglobin as Substrate. Barrett's modification of the Anson assay was employed using the Lowry procedure to detect trichloroacetic acid soluble peptides (Barrett, 1967, 1971). The incubation mixture containing enzyme in 1 ml of 0.05 M Tris-HCl buffer (pH 7.5), 0.1 M NaCl, and 0.25 ml of hemoglobin solution (8% w/v) was held at 45° for 1 hr. The reaction was stopped by the addition of 5 ml of 3% (w/v) trichloroacetic acid, incubated for 10 min, and then filtered. Aliquots of the filtrate (0.25 ml) were assayed by the Lowry method (Lowry *et al.*, 1951). Linear dose response curves were obtained with 0.5–8 μg of bovine chymotrypsin or trypsin under these conditions.

Determination of Arg-Pro-Pro-Gly-Phe, Ser-Pro-Phe-Arg, Ser-Pro, and Amino Acids Derived from Bradykinin. An automatic amino acid analyzer (Alonzo and Hirs, 1968) was operated by the method of Spackman *et al.* (1958) with Aminex A-5 and Pa-28 resins in the short (0.9 × 15 cm) and long (0.9 × 60 cm) columns, respectively. The columns were developed at 60 ml/hr and the ninhydrin reagent (Moore, 1968) was delivered at 30 ml/hr. The water bath was fitted with two thermostats to provide a gradual increase from 52 to 70° in the operating temperature of the columns. The sensitivity of the instrument was increased by using 1.2-cm flow cells in the colorimeter (MER Chromatographic, Mountain View, Calif.) and a 1-mV range card in the recorder (Hamilton, 1967).

The chromatographic system for the estimation of arginine, Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg on Aminex A-5 resin is illustrated with synthetic peptides in Figure 2 (top). The experimental conditions are given in the legend to the figure. The relative ninhydrin color yields (area/concentration) for arginine, the pentapeptide and the tetrapeptide were 1.00, 0.31, and 0.95, respectively.

The dipeptide Ser-Pro was eluted from the long column of the amino acid analyzer between leucine and phenylalanine. The column was developed in the usual manner with the 0.2 M sodium citrate buffer (pH 3.25). At 112 ml the eluting buffer was changed to 0.2 M sodium citrate buffer (pH 4.25). The temperature of the column was increased gradually from 52 to 70°, 28 min after the buffer change. The elution volumes and relative ninhydrin color yields (given in parentheses) for this region of the chromatogram were: isoleucine, 171 ml (0.98); leucine, 176 ml (1.00); Ser-Pro, 181 ml (0.64); and phenylalanine, 192 ml (0.98). The amino acids present in bradykinin, arginine, serine, proline, glycine, and phenylalanine, were eluted in their normal positions. Tris-HCl was eluted near lysine.

The rabbit brain extract and the supernatant fraction were dialyzed against the assay buffer at 4° before incubation with bradykinin to remove low molecular weight substances which interfere with the chromatographic analysis. Enzyme prepared by gel filtration or DEAE chromatography did not require dialysis. The enzyme assays were stopped by acidification as indicated in the legend to Figure 2, bottom, and applied directly to the amino acid analyzer without further treatment.

The elution positions of several other arginine containing bradykinin fragments have been determined for the chromatographic conditions used in Figure 2. Arg-Pro-Pro-Gly-Phe-Ser, Pro-Phe-Arg, and Phe-Arg did not interfere with the identification and quantitation of Arg-Pro-Pro-Gly-Phe or Ser-Pro-Phe-Arg. The heptapeptide Arg-Pro-Pro-Gly-Phe-Ser-Pro was eluted 6 ml before Arg-Pro-Pro-Gly-Phe thus interfering with the quantitation but not the identification of the pentapeptide. This is denoted "+" in Table III. Bradykinin, des-Arg^9-bradykinin, and Phe-Arg were not eluted from the column under the conditions employed.

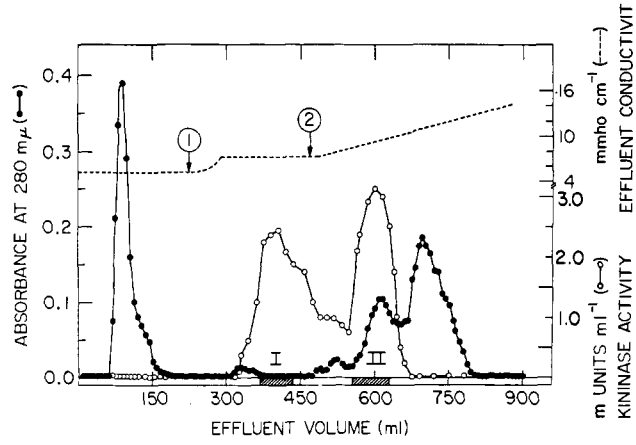

FIGURE 1: Chromatography on DEAE-cellulose of kininase activity present in the Sephadex G-100 fraction from rabbit brain. The column (1.8 × 40 cm) was equilibrated and developed with 0.05 M Tris-HCl buffer (pH 7.5)–0.08 M NaCl for 225 ml (arrow 1) when the eluting buffer was changed to 0.05 M Tris-HCl (pH 7.5)–0.1 M NaCl. At 465 ml (arrow 2) a linear gradient, prepared from 200 ml each of 0.05 M Tris-HCl buffer (pH 7.5)–0.1 M NaCl and 0.05 M Tris-HCl buffer (pH 7.5)–0.3 M NaCl, was applied to the column. The column was operated at 25 ml/hr, 4°, and fractions of 7.5 ml were collected. The sample contained 1.3 kininase units and 62 mg of protein in 25 ml of 0.05 M Tris-HCl (pH 7.5)–0.08 M NaCl. Kininase activity was determined with the isolated guinea pig ileum. (●) Absorbance at 280 mμ; (○) kininase activity; (- - -) effluent conductivity.

Preparation of Neutral Endopeptidase I from Rabbit Brain. The procedures used for the preparation of the supernatant fraction from the rabbit brain homogenate and the gel filtration of the fraction on Sephadex G-100 are similar to those described (Camargo *et al.*, 1972). The extract was prepared by homogenizing 90 g of thawed rabbit brain in 900 ml of 0.25 M sucrose, and centrifugation at 25,000*g* for 1 hr at 4°. After concentration from ~1 l. to 220 ml by lyophilization the supernatant fraction was filtered through Sephadex G-100 The column (7.6 × 175 cm) was equilibrated and developed with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl at 70 ml/hr, 4°. Fractions of 35 ml were collected. Kininase activity in the effluent was detected by assay with the isolated guinea pig ileum.

DEAE-cellulose Chromatography. Effluent from the G-100 column containing kininase activity was lyophilized and dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) containing 0.08 M NaCl at 4°. The DEAE-cellulose column was developed first by stepwise gradient followed by linear gradient elution as indicated in the legend to Figure 1.

Results

Chromatography of Rabbit Brain Supernatant Fraction. The supernatant fraction prepared from fresh or frozen rabbit brain in 0.25 M sucrose usually contained 60–70% of the

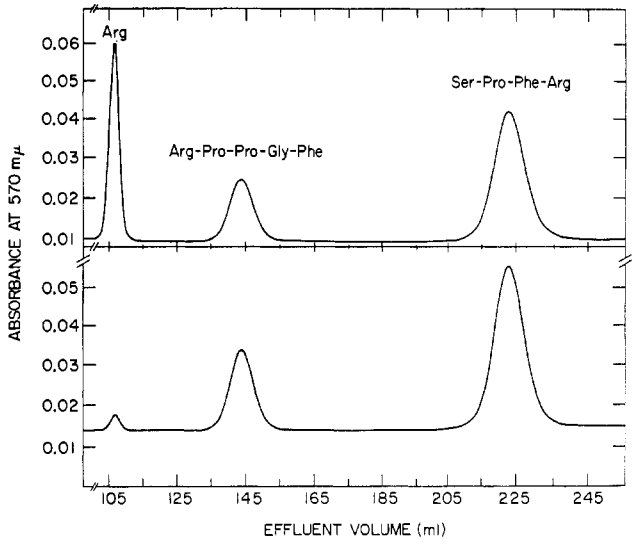

FIGURE 2: Chromatography of Arg-Pro-Pro-Gly-Phe, Ser-Pro-Phe-Arg, and products derived from bradykinin by hydrolysis with enzyme fraction DEAE-I. The chromatograms were obtained with an amino acid analyzer using Aminex A-5 resin (0.9 × 15 cm) equilibrated and developed with 0.35 M sodium citrate buffer (pH 5.28), to which sodium chloride (1.15 g/l.) had been added. The column was operated at 52° for 50 min after sample application and at 70° for the remainder the of analysis. Top: the sample contained 10 nmol of arginine and 29 nmol of each peptide. Bottom: bradykinin (80 nmol) and enzyme fraction DEAE-I (0.005 mg/protein) were incubated in 1.1 ml of 0.05 M Tris-HCl buffer (pH 7.5)–0.1 M NaCl for 40 min at 37°. The reaction was stopped by the addition of 1.0 ml of 0.2 M sodium citrate buffer. (pH 2.2)–0.12 M HCl containing 30% (v/v) polyethylene glycol. One milliliter of this solution, equivalent to 38 nmol of bradykinin, was applied to the column.

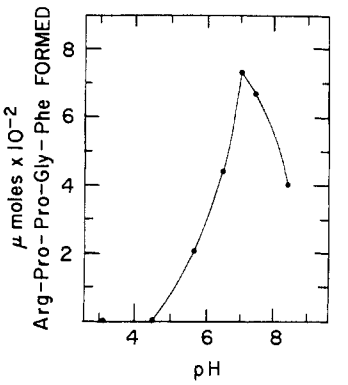

FIGURE 3: Effect of pH on the rate of hydrolysis of bradykinin by enzyme fraction DEAE-I. The pH of the enzyme solution in 0.05 M Tris-HCl buffer (pH 7.45)–0.1 M NaCl was adjusted to the appropriate pH with 0.1 M acetic acid or 0.1 N NaOH. The incubation mixture containing bradykinin (100 nmol/ml) and DEAE-I (0.0045 mg/ml of protein) was held at 37° for 30 min. The products of the reaction were determined with the amino acid analyzer as indicated in the legend to Figure 2.

kininase activity present in the homogenate. When the supernatant fraction was filtered on a 7.6 × 180 cm column of Sephadex G-100 the bulk of the protein was eluted at 2.8–3.3 l., arylamidase activity was eluted at 3.4–4.0 l., and all of the recovered kininase activity was eluted between 4.1 and 4.6 l.

TABLE I: Chromatographic Fractionation of Kininase Activity Present in Rabbit Brain. Prepared from 90 g of rabbit brain (4.3 g of Protein) as Described in the Text.

Fraction	Protein (mg)[a]	Act. Units[b]	Sp Act. (Units/mg)	Yield (%)
Supernatant	1386	6.90	0.005	(100)[c]
Sephadex G-100	62	1.31	0.021	19
DEAE-I	0.32	0.17	0.523	2.5
DEAE-II	4.34	0.25	0.058	3.6

[a] Protein determined by the Lowry method (Lowry *et al.*, 1951) using bovine trypsin as a standard except for DEAE-I which was determined by amino acid analysis after hydrolysis with 6 N HCl at 110° for 33 hr. No corrections have been made for the destruction of tryptophan, serine, or threonine. [b] The unit of kininase activity is defined as μmol min^{-1} bradykinin inactivation measured with the isolated guinea pig ileum (*cf.* Methods). [c] The supernatant fraction contained 60–70% of the kininase present in the tissue homogenate.

The elution diagram obtained was similar to that given in Figure 2 of Camargo *et al.* (1972) for a 1.8 × 85 cm column. The increased length of the column improved the separation of kininase and arylamidase activities.

The elution diagram obtained when effluent containing kininase activity from the Sephadex G-100 column was chromatographed on DEAE-cellulose is given in Figure 1. All of the kininase activity (o) was initially adsorbed to the anion-exchange resin and was subsequently eluted in two major fractions by step and gradient elution chromatography. The effluents indicated by the bars below the diagram were combined and are denoted DEAE-I and DEAE-II in the text and in the tables.

Data for the recovery of kininase activity and protein for each step of the isolation procedure are given in Table I. Only 19% of the kininase activity present in the supernatant fraction was recovered from the Sephadex G-100 column. Essentially all of the activity was located in the 4.1- to 4.6-l. region of the G-100 column effluent. During DEAE-cellulose chromatography 45% of the kininase activity was recovered from the column with DEAE-I and DEAE-II accounting for 13 and 19% of the recovered activity, respectively. The extreme lability of enzymatic activity found here for rabbit brain kininase has also been observed by others who have attempted to purify neutral endopeptidases from brain (*cf.* review of Marks and Lajtha, 1971).

The specific activity of DEAE-I, 0.5 μmol/min per mg of protein is 100 times that of the supernatant fraction from which it was derived. DEAE-II had a specific activity ten times that of the supernatant fraction. It should be pointed out, however, that these values do not accurately reflect the extent of purification of either fraction because the supernatant contains several enzymes which act on bradykinin and DEAE-II contains more than one kininase.

Enzymatic Properties of DEAE-I. Site of Peptide-Bond Hydrolysis in Bradykinin. The products of the enzymatic reaction were identified and estimated by chromatography on Aminex A-5 resin with an amino acid analyzer as shown in Figure 2. The top panel gives the elution profile obtained with 10 nmol of arginine and 29 nmol each of the synthetic peptides Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg. Brady-

TABLE II: Effect of Various Compounds on Kininase Activity of DEAE-I and DEAE-II.[a]

Addition	Concn (M × 10^{-4})	Rel Act. (%) DEAE-I	DEAE-II
$ZnCl_2$	1.0	−100	−100
$ZnCl_2$	0.1	−30	−80
$CaCl_2$	1.0	0	0
EDTA	10.0	0	0
p-Chloromercuribenzoate	10.0	−100	−100
Dithiothreitol	10.0	+35	+30

[a] Results are expressed as percentage changes from the activity without added compounds. Enzyme solutions were preincubated with and without additions in 0.05 M Tris-HCl buffer (pH 7.5), 0.1 N NaCl for 15 min at 22° before assay. The assay incubation conditions were as follows: DEAE-I (0.0045 mg/ml) or DEAE-II (0.041 mg/ml) were held at 37° for 30 min with bradykinin (80 nmol/ml) in the same buffer. Bradykinin activity was measured with the isolated guinea pig ileum. The incubation mixture was diluted 2000-fold in the ileum bathing solution precluding the direct action of the added compounds on the tissue.

kinin is not eluted from Aminex A-5 resin under these conditions.

Figure 2 (bottom) shows the elution profile obtained with 38 nmol of bradykinin which have been incubated with DEAE-I until all biological activity had been destroyed. Two peptides corresponding to 39 nmol of Arg-Pro-Pro-Gly-Phe, 35 nmol of Ser-Pro-Phe-Arg, and ~0.4 nmol of free arginine were detected. Analysis of a second aliquot of the reaction mixture on the long column of the amino acid analyzer indicated that the sample also contained ~0.4 mol of phenylalanine. No other peptides or free amino acids were detected. If the dipeptide Ser-Pro had been present at the level of Arg and Phe, its concentration would have been too low to have been detected. The identification of Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg was confirmed in separate experiments by collecting the Aminex A-5 effluent of the enzymatic hydrolysate before reaction with ninhydrin, and subjecting it to acid hydrolysis and amino acid analysis. The expected amino acid composition in essentially 1:1 molar ratios was obtained for each peptide.

In experiments where the hydrolysis of bradykinin was complete as shown in Figure 2 and in other experiments where the hydrolysis of bradykinin was incomplete (*cf.* Table III and Figure 4), both peptide fragments, Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg, were detected in 1:1 molar ratios. The data given in Table III for DEAE-I show that the extent of inactivation measured with the isolated guinea pig ileum was accounted for quantitatively in terms of the peptide fragments of bradykinin which had been recovered.

These experiments demonstrate the stoichiometric release of the peptide fragments from bradykinin as well as the positive identification of the fragments on the basis of amino acid composition. They show that the hydrolysis of bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, by DEAE-I takes place selectively at the Phe^5–Ser^6 peptide bond. The release of small equimolar amounts of Phe and Arg (~1% of the substrate hydrolyzed) is probably the result of contaminating peptidases present in DEAE-II which act on the carboxyl terminus of bradykinin and Ser-Pro-Phe-Arg (*cf.* Table III).

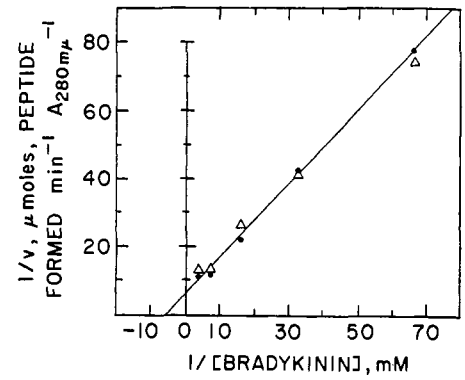

FIGURE 4: Double-reciprocal plot of the rate of hydrolysis of bradykinin by enzyme fraction DEAE-I as a function of bradykinin concentration. The bradykinin concentrations used were 0.0152 0.0304, 0.608, 0.1215 and, 0.243 mM. The enzyme (0.0045 mg/ml) was incubated with bradykinin in 0.05 M Tris-HCl buffer (pH 7.45) for 15 min at 37°. The enzymatic reaction was stopped and the products determined as indicated in the legend to Figure 2: (●) Arg-Pro-Pro-Gly-Phe; (△) Ser-Pro-Phe-Arg.

Determination of pH Optimum and K_m for the Hydrolysis of Bradykinin. The data given in Figure 3 show the effect of pH on the hydrolysis of bradykinin by DEAE-I. The amino acid analyzer system was used to determine the extent of hydrolysis and results for the release of Arg-Pro-Pro-Gly-Phe are given in the figure. The carboxyl-terminal peptide Ser-Pro-Phe-Arg was released in equimolar proportions over the entire pH range studied. No other amino acids or peptides in excess of 1% of the amount of substrate hydrolyzed were detected. These data show that the DEAE-I is an endopeptidase with pH optimum near pH 7. Specificity for the hydrolysis of the Phe^5–Ser^6 peptide bond was observed over the entire pH range studied.

The effect of substrate concentration on the rate of hydrolysis of the Phe^5–Ser^6 bond in bradykinin by DEAE-I is illustrated in the double-reciprocal plot given in Figure 4. The analytical results for both products of the reaction Arg-Pro-Pro-Gly-Phe (residues 1–5) and Ser-Pro-Phe-Arg (residues 6–9) are presented. The K_m for the reaction was found to be 1.8×10^{-4} M at pH 7.45, 37°.

Effect of Various Compounds on Kininase Activity of DEAE-I and DEAE-II. The data given in Table II show the effect of preincubation of the enzymes with metals, EDTA, and sulfhydryl reagents on the hydrolysis of bradykinin determined with the isolated guinea pig ileum. Both enzyme preparations were completely inhibited with 10^{-4} M $ZnCl_2$ and partially but differentially inhibited by 10^{-5} M $ZnCl_2$. The kininase activity of both fractions was not affected by 10^{-4} M $CaCl_2$ or 10^{-3} M EDTA. ClHgBz[1] completely inhibited both enzyme fractions whereas dithiothreitol caused 30 to 35% activation. The effects of ClHgBz and dithiothreitol upon the activity of DEAE-I and DEAE-II indicate a requirement for free SH group(s) for enzymatic activity.

Proteolytic Activity of DEAE-I and DEAE-II on Denatured Hemoglobin. When either 50 μg of DEAE-I or 100 μg of DEAE-II was incubated with denatured hemoglobin at pH 7.5 for 1 hr at 45°, no CCl_3COOH-soluble peptides in excess of

[1] Abbreviation used is: ClHgBz, *p*-chloromercuribenzoate.

TABLE III: Hydrolysis of Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Bradykinin) and Ser-Pro-Phe-Arg by Fractions Derived from Rabbit Brain.[a]

Fraction	Substrate	Extent of Hydrolysis (%)	Peptides (mol/mol of Peptide Hydrolyzed)			Amino Acids (mol/mol of Peptide Hydrolyzed)			
			$Arg^1 \rightarrow Phe^5$	$Ser^6 \rightarrow Arg^9$	$Ser^6 \rightarrow Pro^7$	Ser	Pro	Phe	Arg
Supernatant	Bradykinin	47	+	0.16	0.13	0.27	0.21	0.74	0.74
	Ser-Pro-Phe-Arg	67			0.20	0.77	0.77	0.74	0.74
DEAE-I	Bradykinin	65	1.01	0.98	0	0	0	0.004	0.004
DEAE-II	Bradykinin	85	+	0.13	0.26	0	0	0.68	0.61
	Ser-Pro-Phe-Arg	12			0.50	0	0	0.91	0.91

[a] The values are reported as mole peptide or amino acid recovered per mole substrate hydrolyzed. The extent of hydrolysis of bradykinin was determined by bioassay whereby the cleavage of any one peptide bond is detected. The extent of hydrolysis of Ser-Pro-Phe-Arg is based on the amount of the tetrapeptide recovered from the enzymatic hydrolysate. The enzymatic hydrolysates were prepared in the following manner: bradykinin (80 nmol) and Ser-Pro-Phe-Arg (94 nmol) were incubated in 1.0 ml of 0.05 M Tris-HCl buffer (pH 7.5)–0.1 M NaCl at 37° with 0.38 mg of supernatant fraction protein for 20 min; 0.0035 mg of DEAE-I protein for 30 min; or 0.041 mg of DEAE-II protein for 30 min. The reaction was stopped by the addition of 0.010 ml of 12 N HCl. Aliquots of 0.010 ml were removed from the bradykinin solutions for bioassay and the remainder subjected to chromatographic analysis (*cf.* Methods and legend to Figure 2). The symbol "+" indicates that both $Arg^1 \rightarrow Phe^5$ and $Arg^1 \rightarrow Pro^7$ were detected in the hydrolysate.

reagent controls were detected by the Lowry method. In the absence of demonstrable enzyme activity on denatured hemoglobin, an estimate of the upper limit of the specific activities of the enzyme preparations can be made in terms of the specific activity of chymotrypsin or trypsin which can be detected at the 0.3-μg level. On this basis, DEAE-I and DEAE-II have less than 0.6 and 0.3%, respectively, of the specific activity of chymotrypsin and trypsin for the hydrolysis of denatured hemoglobin at pH 7.5.

In an attempt to demonstrate interaction between DEAE-I and hemoglobin we examined the effect of added hemoglobin on the hydrolysis of bradykinin measured with the isolated guinea pig ileum assay. The incubation conditions were the same as given in the Methods section except that the solution contained 16 mg/ml of hemoglobin substrate. The 12-fold molar excess of hemoglobin (1.0 μmol of monomer/ml) had no demonstrable effect on the rate of hydrolysis of bradykinin (80 nmol/ml).

Hydrolysis of Bradykinin and Ser-Pro-Phe-Arg by DEAE-II. Some of the peptides and amino acids produced by the action of DEAE-II on bradykinin and the tetrapeptide are given in Table III. The data are reported as mole of peptide or amino acid released per mole of substrate hydrolyzed. The quantitative recovery of all of the amino acids in the hydrolyzed substrate was not achieved because some of the products of the reaction were not detected by the chromatographic systems employed.

The identifiable products of the cation of DEAE-II on bradykinin were equimolar quantities of phenylalanine and arginine and the following peptides: Arg-Pro-Pro-Gly-Phe-Ser-Pro (residues 1–7), Arg-Pro-Pro-Gly-Phe (residues 1–5), Ser-Pro-Phe-Arg (residues 6–9), and Ser-Pro (residues 6–7). In separate studies not documented here it was found that the heptapeptide (residues 1–7) was initially released more rapidly than the pentapeptide (residues 1–5) and that as the reaction proceeded the concentration of the pentapeptide increased as the heptapeptide decreased.

DEAE-II contains a mixture of enzymes. These data suggest, but do not prove, the following enzyme activities are present: a carboxydipeptidase which sequentially releases the dipeptides Phe-Arg and Ser-Pro; a dipeptidase which rapidly hydrolyzes Phe-Arg but which does not act on Ser-Pro; and the endopeptidase present in DEAE-I which hydrolyzes the Phe^5–Ser^6 bond of bradykinin. We interpret the release of Arg-Pro-Pro-Gly-Phe-Ser-Pro before Arg-Pro-Pro-Gly-Phe to indicate that the carboxydipeptidase is the first enzyme in the mixture to act on bradykinin. On this basis, the sensitivity to various compounds reported for the kininase activity of DEAE-II in Table II reflects the properties of the carboxydipeptidase.

The peptides and amino acids released from bradykinin and Ser-Pro-Phe-Arg by the action of the supernatant fraction are similar to those produced by the DEAE-II fraction with the exception that the supernatant fraction also released serine and proline (*cf.* Table III). Comparison of the ratios of the extent of hydrolysis of Ser-Pro-Phe-Arg and bradykinin by the supernatant fraction, 67:47, and DEAE-II, 12:85, shows that the supernatant fraction contains additional enzymes which act preferentially on Ser-Pro-Phe-Arg.

These conclusions concerning the properties of the enzymes present in DEAE-II shall be tested directly when the components of the mixture have been separated. The chromatographic system which utilizes the amino acid analyzer provides a useful method to monitor the steps in the fractionation procedure and to characterize the individual components of the mixture.

Discussion

The trivial name kininase has been applied to proteolytic enzymes which destroy the biological activity of bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, or related kinins, lysyl-bradykinin and methionyl-lysyl-bradykinin. Since hydrolysis of any peptide bond in the bradykinin portion of the molecule inactivates the hormone, many enzymes with different substrate specificities can act as kininases, *i.e.*, pan-

creatic chymotrypsin, pancreatic carboxypeptidase B, plasma carboxypeptidase N, and angiotensin-converting enzyme, *cf.* reviews by Erdös and Yang (1970) and Bakhle (1973).

The kininase activity of brain has been studied by several laboratories (Hooper, 1963; Hori, 1968; Iwata *et al.*, 1969; Camargo and Graeff, 1969; Shikimi and Iwata, 1970; Marks and Pirotta, 1971; Camargo *et al.*, 1972) who have shown that the pH optimum for activity is pH 7 to 8 and that more than 50% of the kininase activity can be recovered in the supernatant fraction from homogenates of brain tissue. Shikimi and Iwata (1970) demonstrated qualitatively that arginine and phenylalanine were released from bradykinin by a purified kininase from rabbit brain. The other products of the reaction were not identified. Since arginine and phenylalanine occur twice in each molecule of bradykinin, no conclusion could be drawn concerning the site of peptide-bond hydrolysis. Indirect evidence that hydrolysis occurs at the carboxyl terminus of bradykinin was presented by Camargo (1971) and Camargo *et al.* (1972) who reported similar rates of release of arginine and phenylalanine from bradykinin, lysyl-bradykinin, and methionyl-lysyl-bradykinin with a much slower rate of release of lysine and methionine from the higher homologues of bradykinin. Marks and Pirotta (1971) showed that partially purified neutral proteinase could degrade bradykinin, lysyl-bradykinin, and methionyl-lysyl-bradykinin to free amino acids upon prolonged treatment. Thus the information available in the literature does not provide data about the number of different enzymes present in brain tissue which have kininase activity or the sites of peptide bond hydrolysis in the hormone. This report shows that the rabbit brain supernatant fraction contains at least two enzymes capable of inactivating bradykinin, and provides an analytical system for the identification of some peptide fragments of bradykinin which can be used to assay the enzymes.

DEAE-I is an endopeptidase which hydrolyzes bradykinin at the Phe^5–Ser^6 peptide bond with a pH optimum near pH 7. Denatured hemoglobin is not hydrolyzed by the enzyme nor did the presence of this protein substrate reduce the rate of hydrolysis of bradykinin by the enzyme. Activity was inhibited by *p*-chloromercuribenzoate and enhanced by dithiothreitol indicating a requirement of free sulfhydryl groups for catalytic activity. EDTA and $CaCl_2$ had no effect on activity suggesting that the enzyme has no pronounced requirement for metal ions. The absence of a $CaCl_2$ requirement and the inability to hydrolyze denatured hemoglobin serve to distinguish DEAE-I from the neutral and alkaline proteinases isolated from brain tissue by Guroff (1964), Marks and Lajtha (1965), and Riekinnen and Rinne (1968) (*cf.* Marks, 1970; Marks and Lajtha, 1971, for recent reviews). DEAE-I also differs from the partially purified kininase from rat brain (Shikimi and Iwata, 1970; Shikimi *et al.*, 1970) which released free amino acids from bradykinin, did not act on denatured hemoglobin and was inhibited by EDTA and activated by ClHgBz. DEAE-I appears to be a highly purified preparation of an endopeptidase present in brain tissue which has not been previously described in the literature. The chromatographic system described here is a convenient method to assay the action of the enzyme on the nonpeptide bradykinin, which provides a method to detect and estimate both products of the reaction. A detailed study of physicochemical properties and activity toward substrates other than bradykinin is required before additional comparisons can be made between DEAE-I and other proteinases or peptidases described in the literature.

The presence of a carboxydipeptidase activity in extracts of rabbit brain confirms earlier reports of angiotensin-converting enzyme or carboxydipeptidase activity in brain tissue (Huggins and Thampi, 1968; Roth *et al.*, 1969; Cushman and Cheung, 1971; Yang and Neff, 1972). Yang and Neff reported that their angiotensin converting enzyme activity was inactivated by EDTA and not inhibited by ClHgBz. However, they assayed the enzyme preparation in the presence of ClHgBz thus precluding the possibility of detecting the carboxydipeptidase reported here which is inactivated by ClHgBz and insensitive to EDTA. Most of the preparations of angiotensin converting enzyme described in the literature are inactivated by EDTA and are insensitive to ClHgBz (*cf.* review by Bakhle, 1973). However, Huggins *et al.* (1970) have described a ClHgBz-sensitive angiotensin-converting enzyme from horse plasma and dog lung, and Boucher *et al.* (1972) and Fitz and Overturf (1972) have isolated EDTA-insensitive converting enzymes from rat submaxillary glands and from human lung, respectively.

The use of the biologically active peptide bradykinin as a substrate provides a sensitive method which should serve to complement other substrates now used for the assay of peptidase activity. A major problem in the study of intracellular proteinases and peptidases has been in the selection of the substrate used to detect enzyme activity in tissue homogenates and to monitor the steps in a purification procedure. The substrates most often employed are denatured proteins such as casein and hemoglobin; cellular proteins; low molecular weight pepsin-, trypsin-, and chymotrypsin-specific substrates; or simple di- or tripeptides and their amide derivatives (*cf.* Bohley *et al.*, 1971; Marks and Lajtha, 1971). These substrates have been extremely effective for the detection of the cathepsin proteinases and peptidases which have maximum activity in the acidic pH range but may not be as effective for the detection of intracellular proteinases and peptidases which act maximally in the pH range 6–8. The guinea pig ileum assay, when applied with a dose matching technique, provides an extremely sensitive, quantitative and reproducible method for detecting bradykinin destruction (*cf.* Camargo *et al.*, 1972). Since it is possible to detect and quantitate 10–100 pmol of bradykinin, this approach provides a method which could be sensitive to rates of hydrolysis of $\sim$1 pmol/min. The present chromatographic system for the identification of peptide fragments of bradykinin utilizes the ninhydrin reaction and requires at least 5 nmol of peptide for reliable quantitation. It should be possible to increase the sensitivity of this system by reducing the diameter of the column and by amplification of the photometer signal output (*cf.* Hamilton, 1967) or, alternately, to use other reagents for peptide detection (Udenfriend *et al.*, 1972).

The direct participation of bradykinin and angiotensin in the physiology and pathology of the nervous system has not yet been demonstrated. The search for brain proteolytic enzymes which act on peptide hormones, detailed information about their specificity, physicochemical properties, and sensitivity to inhibitors, should provide insight into the physiological role of protein and peptide metabolism in nervous tissue.

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