# Purification of Rabbit Brain Endooligopeptidases and Preparation of Anti-Enzyme Antibodies†

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ABSTRACT: Endooligopeptidase A was purified ~3000-fold and endooligopeptidase B ~1200-fold from the 25000*g* supernatant fraction of rabbit brain homogenate. The purified enzymes were homogeneous on the basis of acrylamide gel electrophoresis under denaturing and nondenaturing conditions, isoelectric focusing, immunochemical criteria, and specific activities of the elution profile of gel filtration on Sephadex G-100. The only peptide bond cleaved by endooligopeptidase A in bradykinin, Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Phe[8]-Arg[9], is Phe[5]–Ser[6], whereas endooligopeptidase B hydrolyzes the Pro[7]–Phe[8] peptide bond of bradykinin and the Pro[3]–Gly[4] bond of des-Phe[8]-Arg[9]-bradykinin. The specific activity of the homogeneous enzymes using bradykinin as substrate was 1087 nmol min$^{-1}$ mg$^{-1}$ for endooligopeptidase A and 292 nmol min$^{-1}$ mg$^{-1}$ for endooligopeptidase B. Gel filtration suggested molecular weights of 75 000 and 68 000 for endooligopeptidases A and B, respectively. Sodium dodecyl sulfate gel electrophoresis suggested that each endooligopeptidase consisted of a single polypeptide chain with molecular weights of 74 000 and 69 000 for the A and B enzymes, respectively. Purified endooligopeptidase A or B injected into goats produces monospecific antisera directed against each enzyme. The antibody prepared against each endooligopeptidase did not react with or inhibit the activity of the other enzyme.

The growing number of biologically active peptides recently demonstrated in the central nervous system (Hökfelt et al., 1980) has stimulated the search for peptidases that could participate in the modulation of peptide activity in nervous tissue (see Discussion). Since most biologically active polypeptides are small molecules consisting of 3–15 amino acid residues per molecule (Fujino & Shioiri, 1980), enzymes such as endooligopeptidases A and B that selectively hydrolyze oligopeptides (Camargo et al., 1979a) are reasonable candidates for the modulation of the action of peptide hormones in the central nervous system. Endooligopeptidases A and B are neutral thiol-activated endopeptidases isolated from the supernatant fraction of rabbit brain homogenate (Camargo et al., 1973; Oliveira et al., 1976). Brain endooligopeptidase A hydrolyzes the carboxyl side of the aromatic residues of peptides derived from bradykinin, whereas brain endooligopeptidase B hydrolyzes the carboxyl side of proline in peptides structurally related to bradykinin (BK), the Pro–Phe bond of angiotensins I and II, and the Pro–Gly-$NH_2$ bond of LH-RH[1] (Oliveira et al., 1976; Camargo et al., 1979b; Greene et al., 1982). They are distinct from pancreatic proteolytic enzymes because they do not hydrolyze denatured hemoglobin (Camargo et al., 1973), the bradykinin moiety in the reduced and carboxymethylated kininogen (Oliveira et al., 1976), nor Gly-Gly-Gly-Arg-BK when this polypeptide is covalently bound to high molecular weight carriers through its terminal amino group (Camargo et al., 1979a). The susceptibility of small peptides to hydrolysis by these brain endopeptidases and the resistance of proteins to hydrolysis by the enzymes led us to suggest that brain endopeptidases A and B are endooligopeptidases (Camargo et al., 1979a) by analogy with the brush border aminooligopeptidases from intestinal mucosa cells that only attack the amino-terminal residue of oligopeptides (Gray & Santiago, 1977). This report describes the purification of brain endooligopeptidases A and B to apparent homogeneity and the production of monospecific goat antisera directed against these enzymes.

## Materials and Methods

Bradykinin and related peptides were synthesized by the solid-phase method and purified by countercurrent distribution and ion-exchange chromatography by Professors A. C. M. Paiva and L. Juliano, Escola Paulista de Medicina, São Paulo, Brazil. The peptides were homogeneous by the criteria of amino acid composition and high-voltage electrophoresis at pH 3.5. The chemicals used for the amino acid analyzer, ammonium sulfate and β-mercaptoethanol, were products of Pierce Chemical Co. (Rockford, IL). Acrylamide and *N*,*N*′-dimethylenebis(acrylamide) were from Fluka AG, Buchs SG, Switzerland. Dithiothreitol, amino acids, sodium dodecyl sulfate, blue dextran, cytochrome *c*, bovine serum albumin, and ovalbumin were obtained from Sigma Chemical Co. Sephadex G-100 fine and superfine were from Pharmacia, and ampholines for isoelectric focusing were from LKB-Produkter. Chymotrypsinogen was obtained from Worthington. DEAE-cellulose (Whatman DE-52) was obtained from Reeve Angel (London E.C.Y., United Kingdom). Aminex A-5 and A-6 resins were purchased from Bio-Rad Laboratories, Richmond, CA. Freund's adjuvant was a product of Difco (Detroit, MI). Agarose was purchased from BDH Chemicals (Poole, Dorset, United Kingdom). Rabbit brains were purchased from Granja Seleta (Itú, São Paulo, Brazil).

*Enzyme Extraction.* The enzymes were prepared from 550 rabbit brains (2.62 kg) homogenized in 9800 mL of 0.25 M sucrose at 4 °C for 1 min at top speed in a 2-L Waring blender. The homogenate was centrifuged at 25000*g* for 30 min at 4 °C and the supernatant retained (fraction St).

*Acid Precipitation.* The pH of fraction St was adjusted to 5.0 by the dropwise addition of 0.5 M acetic acid and held at 4 °C for 90 min. The suspension was centrifuged at 8000*g* for 30 min, and the precipitate was discarded. The pH of the

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[1] Abbreviations used: LH-RH, luteinizing hormone-releasing hormone; BK, bradykinin; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; TRH, thyrotropin-releasing hormone; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

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supernatant was adjusted to 7.5 by addition of 0.5 N NaOH (fraction St').

*$(NH_4)_2SO_4$ Fractionation.* Solid ammonium sulfate (1820 g) was added slowly to 7.5 L of St' held in an ice bath to achieve 40% saturation. The pH of the solution was maintained at 7.5 by adding $NH_4OH$ (2.5 M). After 1 h at 4 °C, without stirring, the sediment was collected by centrifugation and discarded. The supernatant was rapidly brought to 80% $(NH_4)_2SO_4$ saturation by addition of 1995 g of $(NH_4)_2SO_4$ to 7.0 L of supernatant. The suspension was allowed to stand for 2 h without stirring and then centrifuged. The sediment that contained the enzyme activity was resuspended in 900 mL of 0.05 M Tris-HCl, pH 7.5, containing 0.03 M NaCl and dialyzed against 5 L of the same buffer at 4 °C. Dialysis was repeated 4 times at 8-h intervals with fresh buffer. This fraction is denoted $(NH_4)_2SO_4$ fraction in Table I.

*Preparative Polyacrylamide Gel Electrophoresis.* Preparative gel electrophoresis was performed with 6% acrylamide gel in a 4.3 × 10 cm column with an apparatus described by Morris & Morris (1976). The collection chamber, 4.3 × 2.5 cm, located on the bottom of an acrylamide gel column was filled with Sephadex G-25 supported by a polyacetate membrane separating the resin from the anode. Tris–glycine, pH 8.2, was used as the cathode and anode buffer. The elution buffer (Tris–glycine, pH 8.2) was introduced into the collection chamber at 4 mL/h from the two sides of the interface between the acrylamide gel and the Sephadex layer and exited through a hole in the center of the polyacetate membrane to which a Teflon filter was connected. Fractions of 1 mL were collected. The experimental conditions are given in the legend to Figure 4.

*Measurement of Enzyme Activity.* (*a*) *Kininase Assay* (*Camargo et al., 1972*). A bioassay with the isolated guinea pig ileum was used to measure bradykinin activity. The assay consisted of the determination of the loss of bradykinin activity, due to enzymatic inactivation, by a matching technique. The isolated guinea pig ileum was bathed at 37 °C in 10 mL of Tyrode buffer containing $3.5 \times 10^{-6}$ M atropine and $1.7 \times 10^{-6}$ M diphenylhydramine. Bradykinin, 10 nmol, was incubated with the enzyme in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and $5 \times 10^{-4}$ M dithiothreitol for 10–20 min at 37 °C. The enzyme concentration was selected to give 40–60% inactivation in 15 min. A unit of kininase activity corresponds to the inactivation of 1 μmol of bradykinin/min. This assay was used to monitor the kininase activity present in the column effluent. The biological assay detected the hydrolysis of any peptide bond in bradykinin (Erdös & Yang, 1970).

(*b*) *Determination of Products Derived from Bradykinin.* The enzyme was incubated with 58 nmol of bradykinin in 1 mL of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and $5 \times 10^{-4}$ M dithiothreitol. The reaction was stopped by acidification with 1 mL of 0.05 N HCl and 0.2 M sodium citrate containing 15% (w/v) poly(ethylene glycol) and applied directly to the amino acid analyzer without further treatment. An automatic amino acid analyzer (Alonzo & Hirs, 1968) was used to measure Arg-Pro-Pro, Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe, Arg-Pro-Pro-Gly-Phe-Ser-Pro, des-Arg$^9$-BK, Gly-Phe-Ser-Pro-Phe-Arg, Phe-Ser-Pro-Phe-Arg, Ser-Pro-Phe-Arg, and Phe-Arg as described by Oliveira et al. (1976) with the modifications indicated in the legend to Figure 1. Free amino acids were determined by the method of Spackman et al. (1958).

*Raising and Testing Antisera for Endooligopeptidases A and B.* Antienzyme antisera were produced with purified brain

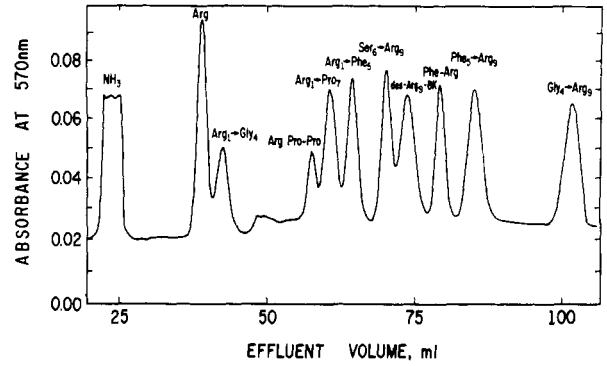


FIGURE 1: Chromatography of Arg, Arg-Pro-Pro, Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe, Arg-Pro-Pro-Gly-Phe-Ser-Pro, des-Arg$^9$-BK, Gly-Phe-Ser-Pro-Phe-Arg, Phe-Ser-Pro-Phe-Arg, Ser-Pro-Phe-Arg, and Phe-Arg. The chromatogram was obtained with Aminex A-5 resin (0.6 × 25 cm) equilibrated and developed at 80 °C with 0.65 M sodium citrate buffer (pH 4.2) and 0.388 M sodium citrate, pH 7.5, after 38 min. The flow rate of buffers was 60 mL/h, and ninhydrin was delivered at a flow rate of 30 mL/h. The sample contained 6.7 nmol of arginine, 24.7 nmol of Arg-Pro-Pro, 65.3 nmol of Arg-Pro-Pro-Gly, 32.0 nmol of Arg-Pro-Pro-Gly-Phe, 25.6 nmol of Arg-Pro-Pro-Gly-Phe-Ser-Pro, 18.6 nmol of des-Arg$^9$-BK, 12.0 nmol of Gly-Phe-Ser-Pro-Phe-Arg, 9.3 nmol of Phe-Ser-Pro-Phe-Arg, 7.0 nmol of Ser-Pro-Phe-Arg, and 6.7 nmol of Phe-Arg.

endooligopeptidase A (125 μg in 0.5 mL) or B (207 μg in 0.5 mL) emulsified with 0.5 mL of complete Freund's adjuvant by intradermal injection at multiple sites into the abdomen of goats every 10 days. During immunization, the serum, heat treated at 56 °C for 30 min, was tested for anticatalytic activity at regular intervals. A high level of anticatalytic activity was obtained after 60 days of immunization. The heat-treated serum had no kininase activity. The animals were bled before immunization to provide control serum. Both the control serum and antisera were stored at −20 °C in the presence of 0.02% sodium azide. The reaction mixture for assay of antibody anticatalytic activity consisted of 1 mL of 0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1.5 milliunits of kininase activity, and antiserum. After preincubation for 5 min at 37 °C, 58 nmol of bradykinin was added to the reaction mixture, which was held at 37 °C. Aliquots of 10–50 μL of the incubation mixture were removed every 5 min for measurement of kininase activity with the isolated guinea pig ileum. In separate experiments not documented here, it was shown that the reaction of the enzyme with anti-endooligopeptidase A or B antibody was instantaneous and that no dissociation of the enzyme–antibody complex occurred under the conditions of the kininase assay.

*Polyacrylamide Gel Electrophoresis.* The purity of the enzyme preparations was determined by disc gel electrophoresis (Maizel, 1969). A 6% separation gel in 0.44 M Tris-HCl (pH 8.2) was prepared with ammonium persulfate as the catalyst. The enzyme preparation was dissolved in 50 μL of Tris–glycine, pH 8.2, containing 20% glycerol (v/v). Electrophoresis was carried out at 4 °C with Tris–glycine buffer, pH 8.2, with a constant current of 2 mA/tube for 2.5 h. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed by the method of Weber & Osborn (1969). Samples were run at a constant current of 5.0 mA/tube in 10% gels for 90 min. All gels were stained for protein with 0.2% Coomassie Brilliant Blue.

*Isoelectric Focusing.* Isoelectric focusing was used to determine the purity of the enzyme preparation in 6% acrylamide gels containing 2% (w/v) ampholine, pH 4–6, in 0.6 × 5 cm tubes by the method of Wrigley (1968). Focusing was started at 1 mA/gel. After approximately 60 min, the voltage was gradually increased to 350 V while a current of 1 mA/gel was

Table I: Purification of Endooligopeptidases A and B[a]

| purification step | total protein (mg) | | sp act. (milliunits/mg) | | purification | | yield (%) | |
|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B |
| homogenate | 272.736 | | 0.34 | 0.22 | 1 | 1 | 100 | 100 |
| sucrose supernatant fraction St | 43.730 | | 1.16 | 0.52 | 3.41 | 2.36 | 54.9 | 37.6 |
| pH 5 supernatant fraction St' | 15.655 | | 2.98 | 1.36 | 8.76 | 6.18 | 50.6 | 34 |
| ammonium sulfate fraction $(NH_4)_2SO_4$ | 6.719 | | 5.29 | 2.41 | 15.56 | 10.95 | 38.8 | 26 |
| DEAE-cellulose | | | | | | | | |
| fractions A-I and A'I | 391 | –[b] | 34.85 | | 102.5 | – | 14.8 | – |
| fraction B-I | – | 190.8 | – | 54.51 | – | 247.77 | – | 16.9 |
| Sephadex G-100 fraction A-II | 71.9 | – | 106.9 | – | 314.5 | – | 8.3 | – |
| gel electrophoresis | | | | | | | | |
| fraction A-III | 9.14 | – | 1.087 | – | 3.021 | – | 5.0 | – |
| fraction B-II | – | 31.5 | – | 293 | – | 1.272 | – | 10.6 |

[a] The experimental procedure is described in the legend to Table II. [b] (–) indicates <0.05.

maintained. After 3.5 h at 350 V, the gels were stained with 0.2% Coomassie Brilliant Blue.

The isoelectric points of endooligopeptidases A and B were determined in 6% acrylamide gels containing 2% (w/v) ampholine, pH 4–6, in 0.6 × 16 cm tubes by the method of Righetti & Drysdale (1971). Experimental details are given in the legend to Figure 10.

*Determination of Molecular Weight.* The molecular weight of the native enzyme was determined by gel filtration on a Sephadex G-100 (superfine) column (1.3 × 120 cm) according to the method of Andrews (1970). The experimental details are given in the legend to Figure 2. Molecular weight was also determined by acrylamide gel electrophoresis under dissociating conditions by the method of Weber & Osborn (1969).

*Immunochemical Methods.* The microimmunoelectrophoretic method of Scheidegger (1955) was carried out with a 1-mm layer of agarose (2% in 50 mM sodium veronal buffer, pH 8.4) on a microscope slide (27 mm × 75 mm). Double radial immunodiffusion (Ouchterlony, 1958) was performed at 22 °C in a humid atmosphere for 48 h. Photographs of the gel-diffusion reactions were taken in a dark field with an apparatus similar to the one described by Chase (1971).

*Protein Determination.* Protein was determined by the method of Bensadoun & Weinstein (1976).

## Results

*Purification.* A summary of the purification procedure is presented in Table I. The procedure results in 3021- and 1270-fold purification with a 5% and 10.6% yield for brain endooligopeptidases A and B, respectively. The activity of each enzyme was calculated on the basis of the rate of appearance of $Arg^1 \rightarrow Phe^5$ (endooligopeptidase A) and $Arg^1 \rightarrow Pro^7$ (endooligopeptidase B) from bradykinin, since these fragments are the major peptide products of the enzyme (Oliveira et al., 1976). The three initial steps of enzyme purification [up to $(NH_4)_2SO_4$] produced about 15- and 11-fold purification of brain endooligopeptidases A and B, respectively.

When the $(NH_4)_2SO_4$ fraction was applied to a column of DEAE-cellulose equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.03 M NaCl, the enzymes were bound to the column while the bulk of contaminating proteins passed through. The profile of total kininase activity shown in Figure 2 was obtained by bioassay with the isolated guinea pig ileum. The solid bars at the bottom of Figure 2 (A-I, A'I, B-I, C, and D) indicate the fractions that were pooled. The presence of endooligopeptidases A and B in each pool was determined on the basis of fragments released from BK (Table II). Endo-

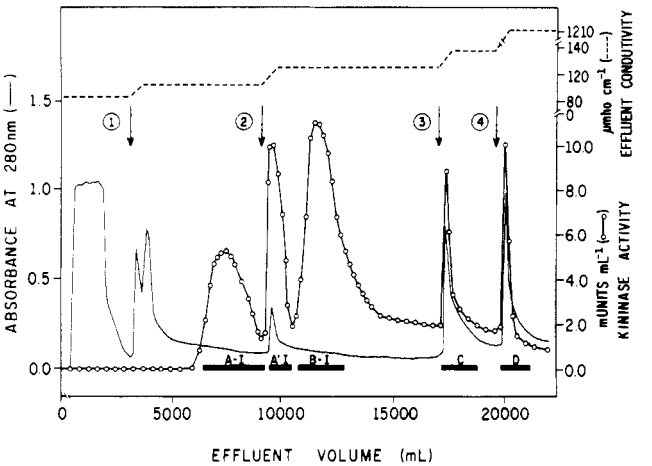


FIGURE 2: Chromatography on DEAE-cellulose of the $(NH_4)_2SO_4$ fraction. The column (5 × 40 cm) was equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.03 M NaCl. The sample contained 75.1 kininase units and 6.71 g of protein in 1290 mL of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.03 M NaCl. After sample application, the column was developed by step gradients of NaCl with 6 L of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl (arrow 1), 7.5 L of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.07 M NaCl (arrow 2), 3 L of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl (arrow 3), and 3 L of 0.05 M Tris-HCl buffer, pH 7.5, containing 1.0 M NaCl (arrow 4). The column was operated at 250 mL/h, 4 °C, and fractions of 21 mL were collected. Kininase activity was determined by bioassay with the isolated guinea pig ileum. (—) Absorbance at 280 nm; (O) kininase activity. The bars indicate the fractions that were pooled.

oligopeptidase A was eluted as two peaks and separated from endooligopeptidase B. The first peak (A-I), corresponding to 28% of the total endooligopeptidase A activity applied to the column, was eluted under equilibrium conditions by 50 mM NaCl buffer (arrow 1). The second peak (A'I), corresponding to 16% of the total endooligopeptidase A applied to the column, was eluted in the breakthrough peak of the second step of 70 mM NaCl (arrow 2). Fractions A-I and A'I contained endooligopeptidase A activity but no endooligopeptidase B activity.

Fraction B-I, 65% of the endooligopeptidase B applied to the column, was eluted well after the breakthrough of the 70 mM NaCl step (arrow 2). This fraction still contained some endooligopeptidase A and other peptidase activity (cf. Table II).

Fractions A-I, A'I, and B-I accounted for more than 80% of the endooligopeptidase A and B activity present in the effluent of the DEAE-cellulose column. The other two peaks

Table II: Analysis of Products Derived from Incubation of Bradykinin with Fractions Obtained during the Purification of Endooligopeptidases A and B from Rabbit Brain Homogenate[a]

| fractionation step | recovery (%) | bradykinin products (nmol/nmol of bradykinin hydrolyzed) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Arg[1] → Phe[5] | Ser[6] → Arg[9] | Arg[1] → Pro[7] | Phe-Arg | Arg[1] → Pro[3] | Gly[4] → Arg[9] | Arg[1] → Phe[8] | Arg |
| homogenate | 86 | 0.47 | 0.12 | 0.19 | 0.08 | –[b] | – | – | 0.86 |
| sucrose supernatant fraction St | 89 | 0.68 | 0.29 | 0.32 | 0.17 | – | – | – | 0.33 |
| ammonium sulfate fraction $(NH_4)_2SO_4$ | 92 | 0.78 | 0.22 | 0.28 | 0.19 | – | – | – | 0.38 |
| DEAE-cellulose | | | | | | | | | |
| fraction A-I | 94 | 0.95 | 0.87 | – | – | – | – | – | 0.06 |
| fraction A'I | 102 | 1.05 | 0.88 | – | – | – | – | – | 0.11 |
| fraction B-I | 107 | 0.25 | 0.12 | 0.72 | 0.27 | – | – | – | 0.78 |
| fraction C | 89 | 0.27 | 0.08 | 0.62 | – | – | – | – | 0.81 |
| fraction D | 73 | 0.11 | – | 0.23 | – | – | – | – | 1.12 |
| Sephadex fraction A-II | 102 | 1.07 | 0.93 | – | – | – | – | – | 0.05 |
| gel electrophoresis | | | | | | | | | |
| fraction A-III | 94 | 0.97 | 0.92 | – | – | – | – | – | – |
| fraction B-II[c] | 94 | – | – | 0.95 | 0.93 | – | – | – | – |
| fraction B-II[d] | 92 | – | – | 0.68 | 0.94 | 0.23 | – | – | – |
| fraction B-III | 98 | 0.17 | 0.10 | 0.81 | 0.88 | – | – | – | – |

[a] Bradykinin (58 nmol) was incubated at 37 °C with 1.5–1.8 milliunits of kininase activity of enzyme preparations for 15 min in 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl and $5 \times 10^{-4}$ M DTT. The extent of bradykinin hydrolyzed was measured by the isolated guinea pig ileum method. The reaction was stopped when 45–55% of bradykinin was inactivated. The amino acid analyzer was used to quantitate bradykinin fragments. [b] (–) indicates <0.05. [c] 58 nmol of bradykinin incubated at 37 °C with fraction B-II for 15 min (45% inactivation). [d] 58 nmol of bradykinin incubated at 37 °C with fraction B-II for 50 min.

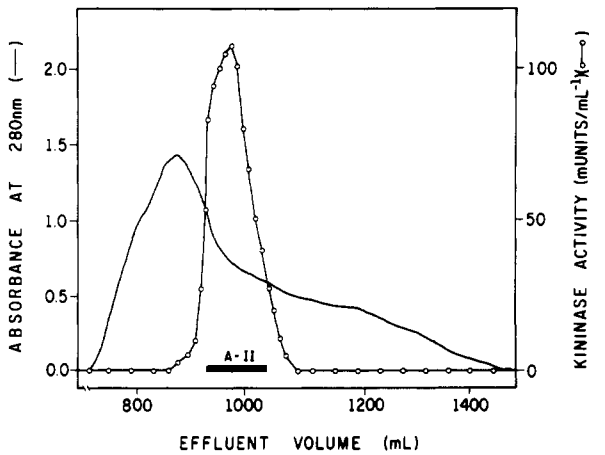


FIGURE 3: Gel filtration on Sephadex G-100 of a mixture of fractions A-I and A'I prepared by chromatography on DEAE-cellulose (Figure 2). The sample was concentrated to 60.2 mL under reduced pressure at 4 °C in an 8/32 Nojax visking casing (Berggård, 1961). The column (5 × 90 cm) was equilibrated and developed at 4 °C with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl at a flow rate of 22 mL/h, and 5.5-mL fractions were collected. Kininase activity was determined by bioassay with the isolated guinea pig ileum. The sample contained 13.63 units of kininase activity and 391 mg of protein in 81.5 mL of 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl. (—) Absorbance at 280 nm; (O) kininase activity. The bar indicates the fraction that was pooled.

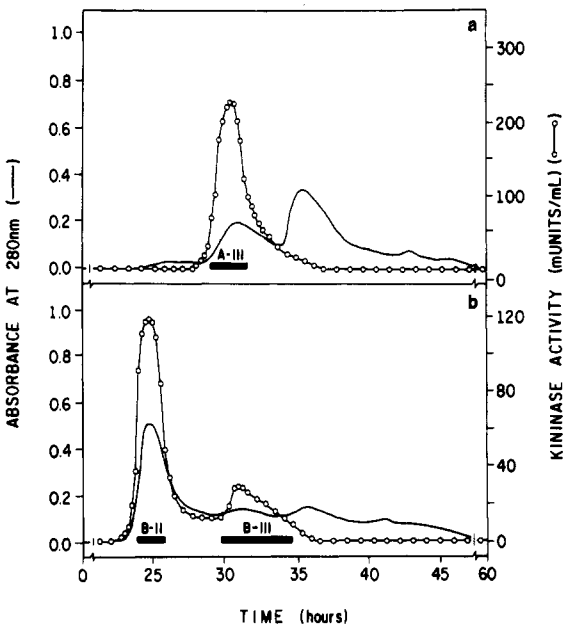


FIGURE 4: Preparative polyacrylamide gel electrophoresis. Electrophoresis in a 6% acrylamide gel column (4.3 × 10 cm) with Tris–glycine buffer, pH 8.2, in the cathode and anode chambers was run at 60 mA for 60 min as a pretreatment. (a) 4.3 mL of fraction A-II (Figure 3) containing 2.5 units of kininase activity and 24 mg of protein in 20% (v/v) glycerol and (b) 1.9 mL of fraction B-I (Figure 2) containing 1.1 units of kininase activity and 21.2 mg of protein in 20% (v/v) glycerol were applied to the column. Electrophoresis was run at 60 mA for 48 h. The elution buffer was run at 4 mL/h and 1-mL fractions were collected. (—) Absorbance at 280 nm; (O) kininase activity. The bars represent the fractions that were pooled.

of kininase activity (fractions C and D) contained a mixture of exo- and endopeptidases (Table II) and were not studied further.

Gel filtration of pooled fractions A-I and A'I on Sephadex G-100 increased the specific activity 3-fold and yielded fraction A-II (Figure 3). When fraction A-II was subjected to polyacrylamide gel electrophoresis, a single peak of kininase was obtained (A-III), which coincided with the second peak of material absorbing at 280 nm (Figure 4a).

Peak B-I from the DEAE-cellulose column was further purified. Gel filtration on Sephadex G-100 of fraction B-I was not used for purification because it neither improved the specific activity of endooligopeptidase B nor separated it from contamination by endooligopeptidase A activity. Instead, fraction B-I was separated into two peaks of kininase activity by preparative gel electrophoresis (Figure 4b). The first peak (B-II) accounted for 62% of the recovered kininase activity and contained only endooligopeptidase B activity (Table II). The second peak of kininase activity (B-III) was a mixture of endooligopeptidase B and endooligopeptidase A. The entire purification procedure provided an overall purification of 3021-

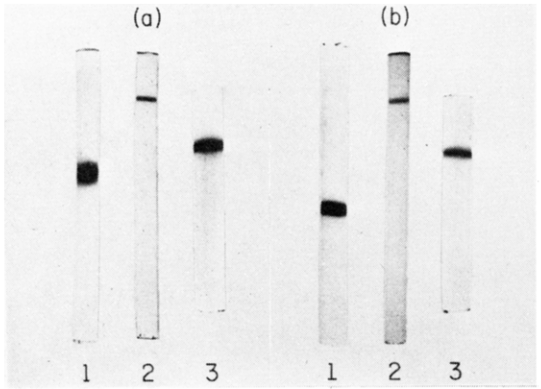


FIGURE 5: Electrophoretic patterns of endooligopeptidases A and B in acrylamide gels: (a) 1, 2, and 3 represent the electrophoresis of fraction A-II under regular disc, $NaDodSO_4$, and isoelectric focusing gel electrophoresis, respectively; (b) 1, 2, and 3 represent the electrophoresis of fraction B-II under regular disc, $NaDodSO_4$, and isoelectric focusing gel electrophoresis, respectively. Enzyme samples contained 50–100 μg of protein/gel. Experimental details are given under Materials and Methods.

and 1272-fold for endooligopeptidases A and B, respectively.

*Bradykinin Hydrolysis by Endooligopeptidases A and B.* Table II shows that the only peptide bond cleaved by endooligopeptidase A (fraction A-III) was $Phe^5$–$Ser^6$ and that both fragments were recovered quantitatively. Endooligopeptidase B hydrolyzed BK at the $Pro^7$–$Phe^8$ bond as shown by the data for 15-min incubation (45% hydrolysis), entry B-II[c] in Table II. Upon more extensive hydrolysis (entry B-II[d]), the amount of $Arg^1 \rightarrow Pro^7$ was less than the amount of Phe-Arg and Arg-Pro-Pro that appeared. These data, taken together with the absence of $Gly^4 \rightarrow Arg^9$, in both short and long incubations, suggest that Arg-Pro-Pro is a hydrolysis product of $Arg^1 \rightarrow Pro^7$ rather than BK.

*Purity of Endooligopeptidases A and B.* Analytical acrylamide gel electrophoresis of fractions A-III and B-II resulted in a single band of protein each under dissociating and nondissociating conditions and by isoelectric focusing (Figure 5). Additional support for homogeneous proteins was obtained by immunochemical methods and by gel filtration on Sephadex G-100, which will be described in the following sections.

*Anti-Endooligopeptidase A and B Antisera.* Anti-enzyme antisera produced by injecting fractions A-III and B-II into goats indicated that both enzymes are good antigens, since small amounts of each antigen produced antiserum with high anticatalytic activity after 60 days of immunization. The specificity and the level of anticatalytic activity of the antibodies can be evaluated on the basis of the data in Figure 6. The activity of 1.5 milliunits of endooligopeptidase A or B was completely abolished by 250 and 50 μL of anti-endooligopeptidase A and B antisera, respectively. No inhibition was obtained when endooligopeptidase A was preincubated with anti-endooligopeptidase B antiserum or vice versa. The monospecificity of the two antisera was determined by double radial immunodiffusion (Figure 7) and by immunoelectrophoresis (Figure 8). Only one line of precipitin was obtained when the antibodies were run against their respective antigens. Additional support for the monospecificity of both antisera was obtained when fraction St, concentrated 10 times, was used in place of the purified antigen (wells 1 and 4, Figure 7). Under these circumstances, the only line of precipitin coincided with that of the purified antigen. Immunodiffusion also showed that anti-endooligopeptidase A antiserum does not cross-react with endooligopeptidase B and vice versa (Figure 7).

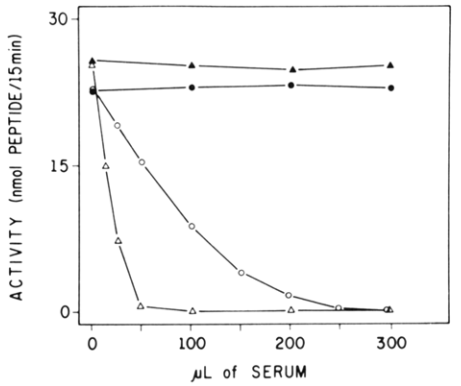


FIGURE 6: Inactivation of endooligopeptidase A and endooligopeptidase B by anti-endooligopeptidase A and anti-endooligopeptidase B antisera, respectively. Endooligopeptidase A (1.6 milliunits) and endooligopeptidase B (1.8 milliunits) in 1 mL of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and $5 \times 10^{-4}$ M DTT were incubated with 58 nmol of bradykinin in the presence of increasing amounts of antiserum. (▲) Endooligopeptidase A incubated with anti-endooligopeptidase B antiserum; (△) endooligopeptidase A incubated with anti-endooligopeptidase A antiserum; (●) endooligopeptidase B incubated with anti-endooligopeptidase A antiserum; (○) endooligopeptidase B incubated with anti-endooligopeptidase B antiserum. The enzyme activity is expressed as the amount of $Arg^1 \rightarrow Phe^5$ or $Arg^1 \rightarrow Pro^7$ formed when bradykinin was incubated at 37 °C for 15 min in the presence of the appropriate antiserum.

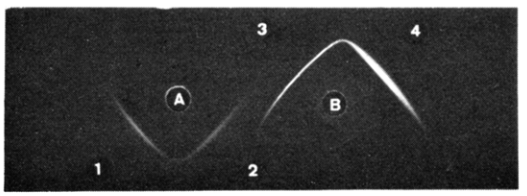


FIGURE 7: Ouchterlony immunodiffusion of purified endooligopeptidases A and B. Immunodiffusion experiments were carried out as described under Materials and Methods. Wells A and B: 5 μL of anti-endooligopeptidase A and B antisera, respectively. Wells 2 and 3: 5 μL of fraction A-III and fraction B-II, respectively. Wells 1 and 4: 5 μL of the 25000*g* supernatant fraction of rabbit brain homogenate concentrated 10-fold.

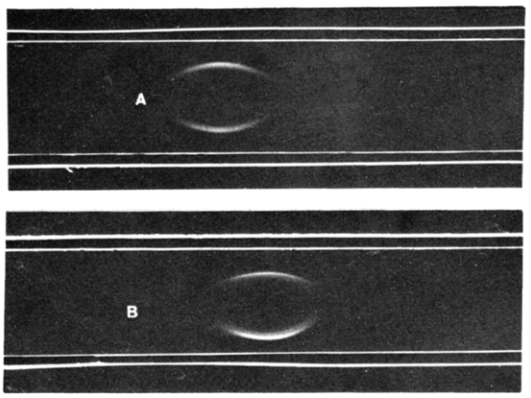


FIGURE 8: Immunoelectrophoresis of purified brain endooligopeptidases A and B against anti-endooligopeptidase A and B antibodies. (A) The well contains 5 μL (5.7 μg of protein) of fraction A-III. After the run, 75 μL of antiserum (15 mg of protein) was added to the longitudinal basin. (B) The well contains 5 μL (25.6 μg of protein) of fraction B-II. After the run, 60 μL of antiserum (12 mg of protein) was added to the longitudinal basins.

*Molecular Weight.* Two methods were employed to estimate the molecular weights of the enzymes. Fractions A-III and B-II were submitted separately to gel filtration on a 1.3 × 120 cm column of Sephadex G-100, and in both experiments all the material absorbing at 230 nm was eluted in a single peak, which was completely superimposable to the kininase

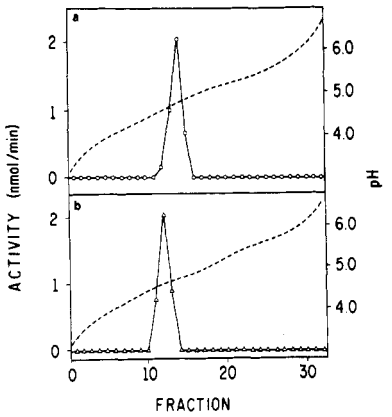


FIGURE 9: Isoelectric focusing of endooligopeptidases A and B. A 50-$\mu$g sample of fraction A-III (panel a) and a 60-$\mu$g sample of fraction B-II (panel b) were subjected to isoelectric focusing in 6% acrylamide gel containing 2% amopholine, pH 4–6. The current was kept at 1 mA/tube until the voltage had risen to 400 V. Voltage was thereafter maintained at 400 V for 7 h with total electrophoresis time limited to 10 h. Gels 16 cm in length were cut into 5-mm sections for determination of pH gradient and kininase activity. The gradient was measured at room temperature with a digital pH meter after gel sections had been macerated in 0.5 mL of distilled water. Samples (200 $\mu$L) of the gel section extract were incubated with 10 nmol of bradykinin in 200 $\mu$L of 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl and $5 \times 10^{-4}$ M DTT for kininase assay. (○) Kininase activity of fraction A-III; (△) kininase activity of fraction B-II; (---) pH gradient.

activity. The elution volume of each enzyme was treated by the method of Andrews (1970) (data not shown), and an apparent molecular weight of 75 000 was found for endooligopeptidase A and of 68 000 for endooligopeptidase B. The protein standards used were ribonuclease, chymotrypsinogen, ovalbumin, and bovine serum albumin. The molecular weight of both enzymes was also determined by gel electrophoresis in the presence of $NaDodSO_4$ according to the procedure of Weber & Osborn (1969). A plot of the relative mobilities of ribonuclease, chymotrypsinogen, ovalbumin, bovine serum albumin, and $\beta$-galactosidase vs. the log of their molecular weights was linear (data not shown), and the relative mobilities of brain endooligopeptidases A and B on 7.5% gels were compatible with proteins having a molecular weight of 74 000 and 69 000, respectively. The molecular weight determinations suggest that endooligopeptidases A and B consist of a single polypeptide chain each.

*Isoelectric Focusing.* Fractions A-III and B-II were submitted to isoelectric focusing in a 0.5 × 16 cm tube in 6% acrylamide gels as described in the legend to Figure 9. Both endooligopeptidases A and B appeared as a single peak of kininase activity each, having isoelectric points of 4.75 and 4.55, respectively (Figure 9).

## Discussion

Endooligopeptidases A and B were isolated from the supernatant fraction of rabbit brain homogenate in apparently homogeneous form. The enzymes, with specific activities of 1087 and 293 milliunits/mg for endooligopeptidases A and B, respectively, were isolated from 2.62 kg of rabbit brain in yields of 5% for endooligopeptidase A and 10.6% for endooligopeptidase B. The purity of the enzymes was demonstrated by a single band in acrylamide gel electrophoresis, in $NaDodSO_4$–polyacrylamide gel electrophoresis, and in acrylamide gel isoelectric focusing. Additional support for the homogeneity of endooligopeptidases A and B was obtained in double immunodiffusion and immunoelectrophoresis studies. The molecular weights and isoelectric points for endooligopeptidases A and B described in this study are very close to the values previously reported by Oliveira et al. (1976) for partially purified enzyme preparations. Both enzymes proved to be good immunogens in goats, since a potent antiserum was produced in response to small amounts of enzymes. The antisera against brain endooligopeptidase A or B produced complete inhibition in quantities of 170 $\mu$L/milliunit for endooligopeptidase A and 35 $\mu$L/milliunit for endooligopeptidase B. Immunoelectrophoresis and Ouchterlony immunodiffusion of endooligopeptidases A and B indicated that the antibodies are monospecific, since a single line of precipitin was formed when each enzyme was tested against its respective antigen. Furthermore, the antibody directed against endooligopeptidase A did not cross-react with endooligopeptidase B and vice versa. The antibody has proved to be a useful tool for determining the presence of endooligopeptidase A in peripheral tissues (Coelho et al., 1981).

The purification index and the recovery of enzymatic activity were based on the quantitative determination of bradykinin products formed when this peptide was incubated with enzymes at all stages of the purification. Previous reports from our laboratory indicated that, when bradykinin is incubated with purified brain endooligopeptidase A or B, the major peptides formed are $Arg^1 \rightarrow Phe^5$ and $Arg^1 \rightarrow Pro^7$, respectively (Oliveira et al., 1976). Since these fragments are slowly degraded by brain peptidases present in tissue homogenate (Cicilini et al., 1977; Coelho et al., 1981), the amount of $Arg^1 \rightarrow Phe^5$ and $Arg^1 \rightarrow Pro^7$ formed when bradykinin is incubated with enzyme preparations was used to evaluate the relative activity of brain endooligopeptidases A and B, respectively, present in the early stage of enzyme purification. The quantitative recovery of bradykinin products formed by the action of brain peptidases clearly shows that purified brain endooligopeptidases A and B are free from any other peptidases acting on bradykinin or on its products. This would not be possible if we used a chromogenic or fluorogenic substrate rather than a polypeptide.

Recently several laboratories have published descriptions of purified brain peptidases that hydrolyze oligopeptides such as bradykinin (Orlowski et al., 1979), angiotensin II (Knisatscheck & Bauer, 1979; Orlowski et al., 1979; Taylor & Dixon, 1980), substance P (Blumberg et al., 1980; Akopyan et al., 1978), LH-RH (Kock et al., 1974; Knisatscheck & Bauer, 1979; Orlowski et al., 1979; Hersch & McKelvy, 1979; Akopyan et al., 1978; Kuhl et al., 1979; Horsthemke & Bauer, 1980; Taylor & Dixon, 1980), and TRH (Rupnow et al., 1979). However, comparison of the properties of these enzymes with those of endooligopeptidases A and B indicates several similarities. The few differences could be attributable to differences in the methodology applied for enzyme detection, to impurities, or to the different sources of enzyme preparations. For example, the neutral endopeptidase from bovine anterior pituitary described by Horsthemke & Bauer (1980), which hydrolyzes LH-RH at the $Tyr^5$–$Gly^6$ and $His^2$–$Trp^3$ bonds, has a molecular weight of 83 000, is inhibited by SH-reactive agents, and is $Ca^{2+}$ independent. Except for the molecular weight, which is about 8000 higher than that of endooligopeptidase A, all other properties are similar to those previously described for brain endooligopeptidase A (Camargo et al., 1973; Oliveira et al., 1976). On the other hand, brain endooligopeptidase B has similar molecular weight, pH optimum, isoelectric point, and selectivity for peptide bonds involving the carboxyl group of proline (Oliveira et al., 1976) when compared to the proline endopeptidase described by Orlowski et al. (1979), Knisatscheck & Bauer (1979), and Rupnow et al. (1979). Even the differences pointed out by

Orlowski et al. (1979) related to the cleavage of $Pro^3$-$Gly^4$ in the bradykinin moiety by their enzymatic preparation cannot be used to distinguish it from endooligopeptidase B, since this enzyme hydrolyzes the $Pro^3$–$Gly^4$ peptide bond of des-$Phe^8$-$Arg^9$-BK after extensive incubation time (Table II).

Recently, proline-specific endopeptidases were subjected to a critical review by Walter et al. (1980). According to these authors, brain endooligopeptidase B is distinct from the kidney post-proline-cleaving enzyme because the latter does not cleave the peptide Ser-Pro-Phe-Arg, which is the best substrate for endooligopeptidase B (Oliveira et al., 1976).

On this basis, it seems likely that until clear distinctions can be documented in the literature, the neutral thiol-activated brain endopeptidases, which selectively hydrolyze small polypeptides on the carboxyl side of aromatic or proline residues, respectively, should be considered closely related to or identical with brain endooligopeptidases A and B previously described by Camargo et al. (1973) and Oliveira et al. (1976) and confirmed in this study.

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