Inhibition of rabbit tissue kininase by anti-(endo-oligopeptidase A) antibodies

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Highly purified rabbit brain endo-oligopeptidase A injected into goats produced, after 60 days of immunization, antisera that specifically inhibit purified rabbit brain endo-oligopeptidase A. An immunoreactive kininase having the same specificity as rabbit brain endo-oligopeptidase A for bradykinin was detected in several rabbit tissues. The highest amount of this immunoreactive kininase was found in the 25 000g supernatant fraction (S fraction) of heart, liver, skeletal muscle, ovary, brain and testis homogenates, corresponding to 89, 86, 78, 59, 56 and 53% respectively of the whole kininase activity found in the S fraction.

A growing number of peptides are being reported in the literature as chemical modulators or transmitters of physiological functions (Hökfelt *et al.*, 1980). These discoveries have stimulated the development of new areas of investigation on the chemistry and biology of peptides, which present one of the most prominent problems of neuroendocrinology. Among the variety of aspects that have been pursued by several investigators, the least studied is the mechanism involved in turning on and off these biological messengers. It is obvious that tissue peptidases must be involved in forming and destroying biologically active polypeptides. However, very little is known about the number, characteristics and distribution of tissue peptidases (Barrett, 1977; Marks, 1977).

In a previous paper we described the presence of two brain endopeptidases that hydrolyse bradykinin (Arg₍₁₎-Pro₍₂₎-Pro₍₃₎-Gly₍₄₎-Phe₍₅₎-Ser₍₆₎-Pro₍₇₎-Phe₍₈₎-Arg₍₉₎) and various oligopeptides derived from bradykinin (Oliveira *et al.*, 1976), as well as angiotensins I and II, luliberin, substance P and neurotensin (Martins *et al.*, 1980). Endopeptidase A (Camargo *et al.*, 1973) and endopeptidase B (Martins *et al.*, 1980) are neutral thiol-activated peptides that hydrolyse bradykinin at the Phe₍₅₎-Ser₍₆₎ and Pro₍₇₎-Phe₍₈₎ peptide bonds respectively.

One property that distinguishes these neuro-peptide-metabolizing brain endopeptidases from most proteinases (Barrett, 1977) is the fact that their activity is apparently restricted towards oligopeptides (Camargo *et al.*, 1979a). The existence in other tissues of enzymes exhibiting properties similar to those of brain endo-oligopeptidases was indicated by

Cicilini *et al.* (1977) and Camargo *et al.* (1979b) on the basis of sites of cleavage in the bradykinin molecule as well as of the resistance of peptides attached to agarose to hydrolysis by tissue kininases. In the present paper we describe the development of monospecific antibodies against brain endo-oligopeptidase A and the use of antibodies to study the distribution in rabbit tissues of an immunoreactive endopeptidase that hydrolyses bradykinin at the same peptide bond as does brain endo-oligopeptidase A.

Materials and methods

Materials

Bradykinin and related peptides were synthesized by the solid-phase method (Stewart & Young, 1969) and purified by countercurrent distribution and ion-exchange chromatography by Professor A. C. M. Paiva and Professor L. Juliano, Escola Paulista de Medicina, São Paulo, Brazil. Peptides were homogeneous by the criterion of amino acid composition and by high-voltage electrophoresis at pH 3.5. The chemicals used for the amino acid analyser were products of Pierce Chemical Co. (Rockford, IL, U.S.A.).

Bacto-agar and Freund's adjuvant were products of Difco (Detroit, MI, U.S.A.). Agarose was purchased from BDH Chemicals (Poole, Dorset, U.K.) and DEAE-cellulose (Whatman DE-52) was from Reeve Angel (London E.C.4, U.K.). Ethyl chloroformate was a product of Merck Schuchardt (Munich, Germany). Rabbit brains were purchased from Granja Seleta (Itú, São Paulo, Brazil).

Enzyme preparation

Brain endo-oligopeptidases A and B were purified from the pH5 supernatant fraction of 195 rabbit brains homogenized in 0.25 M-sucrose as described by Oliveira *et al.* (1976), with modifications in chromatography on DEAE-cellulose described by Martins *et al.* (1980). An analytical system based on the amino acid analyser was used to show that these preparations of brain endo-oligopeptidases A and B hydrolyse bradykinin ($\text{Arg}_{(1)}\text{-Pro}_{(2)}\text{-Pro}_{(3)}\text{-Gly}_{(4)}\text{-Phe}_{(5)}\text{-Ser}_{(6)}\text{-Pro}_{(7)}\text{-Phe}_{(8)}\text{-Arg}_{(9)}$) at the $\text{Phe}_{(5)}\text{-Ser}_{(6)}$ and $\text{Pro}_{(7)}\text{-Phe}_{(8)}$ peptide bonds respectively, releasing stoichiometric amounts of products (Oliveira *et al.*, 1976). The amount of brain endo-oligopeptidase A used as antigen was 2.5 mg, with a specific activity of 96 munits/mg of protein. One unit of kininase activity is defined as the amount of enzyme that hydrolyses 1 μmol of bradykinin/min at 37°C in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl.

Portions (0.5 ml) of purified brain endo-oligopeptidase A in 50 mM-Tris/HCl buffer, pH 7.5, containing 12 munits of enzyme activity and 125 μg of protein, were stored at -20°C. No loss of activity was observed after 6 months of storage.

Raising and testing antisera

Antienzyme sera were produced with 0.5 ml of purified brain endopeptidase A (stored as described above) emulsified with equal volumes of complete Freund's adjuvant injected intradermally at multiple sites on the abdomen of goats every 2 weeks. The sera, heat-treated at 56°C for 30 min, were tested for anticatalytic activity at regular intervals. Antisera with optimum anticatalytic activity were obtained by bleeding the animals after 60 days of immunization. The heat-treated sera had no kininase activity. The animals were bled before immunization to provide control serum. Control serum and antisera were all stored at -20°C with 0.02% NaN_3 . Although several goats had been submitted to the same treatment, resulting in the production of antisera with high anticatalytic activity, in the present study we used the antiserum obtained from one single animal.

Immunopurification of antibodies

The immunoadsorbent was prepared with 500 mg of protein from the 25 000 g supernatant fraction of the 0.25 M-sucrose homogenate of rabbit brain mixed with proteins from 11 ml of rabbit serum dissolved in 34 ml of 0.1 M-sodium acetate buffer, pH 5. The mixture was incubated at 56°C for 30 min, which completely inactivated brain kininase. The protein was made insoluble by the addition of ethyl chloroformate as described by Avrameas & Ternynck (1967). Less than 2.5% of the original protein was found in the supernatant after centrifugation.

Immunoadsorption was performed by mixing 10 ml of goat antiserum with immunoadsorbent. The mixture was incubated for 15 min at 37°C and kept at 0°C for 2 h. The insolubilized immunoadsorbent was removed by centrifugation, and immunoadsorption was repeated at least three times until monospecific antiserum was obtained in the supernatant. The immunoglobulin of the supernatant fraction was purified by chromatography on DEAE-cellulose as described by Das & Soffer (1976).

Enzyme assay

Bradykinin (58 μM) was incubated with enzyme in 1.0 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl at 37°C. The reaction was stopped by the addition of 1.2 ml of 0.2 M-sodium citrate buffer, pH 2.2, containing 15% (v/v) polyethylene glycol. The hydrolysis of bradykinin was measured by bioassay with the isolated guinea-pig ileum, which measures inactivation of bradykinin, and/or by direct quantification of peptide products by using the amino acid analyser (see below).

The bioassay with the isolated guinea-pig ileum, described in detail by Camargo *et al.* (1972), is based on the fact that when any peptide bond in bradykinin is hydrolysed the peptide loses 95% or more of its biological activity. The residual activity of bradykinin after incubation with enzyme was determined by a matching technique in which the response of the ileum to enzyme incubation mixture is matched with that of a standard solution of bradykinin, whose composition was determined by amino acid analysis. The assay is linear in the range of up to 60% hydrolysis under the conditions used. All measurements were performed in duplicate, and the deviation from the average was $\pm 15\%$. When the amino acid analyser was used to monitor the reaction, the rate of inactivation was also linear to 60%, and the deviation from the average of duplicate analysis was $\pm 10\%$.

In separate experiments, not documented here, the concentration of bradykinin was varied between 5 and 58 μM for all tissue preparations, and the latter concentration was shown to give maximum rates of inactivation.

Assay of anticatalytic activity

A typical reaction mixture (1 ml) contained 50 mM-Tris/HCl buffer, pH 7.5, 0.1 M-NaCl, 1.5 munits of kininase activity and antibody. After preincubation for 5 min at 37°C, 58 nmol of bradykinin was added to the reaction mixture and incubated at 37°C. Samples (10–50 μl) of incubation mixture were removed every 5 min for enzyme assay by the isolated-guinea-pig-ileum method. A parallel observation indicated that the reaction of the enzyme with the antibody is instantaneous, and there is no dissociation of the

enzyme-antibody complex under the conditions of enzyme assay. Enzyme inactivation with increasing concentrations of antibody was linear up to about 70% inhibition. We define 1 unit of anticatalytic activity as the amount of antibody required to inhibit completely the activity of 1 unit of purified brain endopeptidase A under the conditions described above.

Immunochemical methods

The microimmunoelectrophoretic method of Scheidegger (1955) was conducted by using 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. The plates were stained with 1% Thiazin Red for 90 min and washed with 0.9% NaCl for 48 h.

Preparation of supernatant and particulate fractions from rabbit tissues

Crude enzyme preparations from rabbit tissue homogenates were prepared by the method described by Cicilini *et al.* (1977). Tissue homogenates were centrifuged at 25000 *g* for 1 h. We used as enzyme preparations the corresponding particulate (P) and supernatant (S) fractions. The P fractions were resuspended up to the initial volume of the homogenate in 0.25 M-sucrose in 50 mM-Tris/HCl

buffer, pH 7.5. These enzyme preparations are stable for at least 3 weeks when stored at -20°C.

Protein determination

The method of Lowry *et al.* (1951) was employed for the enzyme preparations, with crystalline albumin as the standard. Immunoglobulin G concentration was estimated by using $A_{280}^{1\%} = 13.1$.

Determination of peptides and amino acids derived from bradykinin

Analysis of the peptides derived from bradykinin was performed with an amino acid analyser (Alonzo & Hirs, 1968) operated as previously described (Oliveira *et al.*, 1976). The following modifications were introduced into the system: (i) the size of the Aminex A-6 column was 0.63 cm × 15 cm; (ii) the flow rate of the buffer was 36 ml/h; (iii) SnCl₂ was replaced by NaBH₄ in the ninhydrin solution recommended by Takahashi (1978); (iv) the 0.388 M-sodium citrate buffer, pH 7.5 (buffer 2), was introduced into the column immediately after elution of peptide Arg₍₁₎→Gly₍₄₎.

As a result of these modifications, the detection and quantification of the peptides was significantly improved. Fig. 1 shows the elution diagram for lysine and arginine (3.75 nmol), Arg₍₁₎→Gly₍₄₎ (41.5 nmol), Arg₍₁₎→Pro₍₇₎ (15.5 nmol), Arg₍₁₎→Phe₍₅₎ (24.7 nmol), Ser₍₆₎→Arg₍₉₎ (4.2 nmol), Phe-Arg (4.0 nmol), Phe₍₅₎→Arg₍₉₎ (5.6 nmol) and

Fig. 1. Chromatography of Lys, Arg, Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe-Ser-Pro, Arg-Pro-Pro-Gly-Phe, Ser-Pro-Phe-Arg, Phe-Arg, Phe-Ser-Pro-Phe-Arg and Gly-Phe-Ser-Pro-Phe-Arg

The chromatogram was obtained with an Aminex A-5 resin column (0.63 cm × 15 cm) equilibrated and developed at 80°C with 0.65 M-sodium citrate buffer, pH 4.2, and 0.388 M-sodium citrate buffer, pH 7.5, after 84 min. The sample contained 3.75 nmol of Lys and Arg, 41.5 nmol of Arg₍₁₎→Gly₍₄₎, 15.5 nmol of Arg₍₁₎→Pro₍₇₎, 24.7 nmol of Arg₍₁₎→Phe₍₅₎, 4.2 nmol of Ser₍₆₎→Arg₍₉₎, 4.06 nmol of Phe-Arg, 5.6 nmol of Phe₍₅₎→Arg₍₉₎ and 8.2 nmol of Gly₍₄₎→Arg₍₉₎.

Gly₍₄₎→Arg₍₉₎ (8.2 nmol). Bradykinin, des-Arg₍₁₎-bradykinin, des-Arg-Pro-bradykinin and Pro-Phe-Arg are not detectable by this method, whereas Arg-Pro, Arg-Pro-Pro and des-Arg₍₉₎-bradykinin can be detected if we extend the use of buffer 1 for an additional 15 min. These modifications permitted us to obtain narrower peaks for peptides Arg₍₁₎→Ser₍₆₎, Arg₍₁₎→Pro₍₇₎ and Arg₍₁₎→Phe₍₅₎, and increased 2.4-fold the sensitivity of peptides and amino acids. The samples were also analysed for

free amino acids with the two-column system of Spackman *et al.* (1958).

Results

Preparation of specific anticatalytic antiserum

The antiserum yielded a single precipitin band by immunoelectrophoresis against the purified enzyme, and three bands with the S fraction of rabbit brain homogenate (Fig. 2). The pre-immune serum did not

Fig. 2. Immunoelectrophoresis of purified brain endo-oligopeptidase A and of the S fraction of rabbit brain against anti-(endo-oligopeptidase A) antibodies

(a) The upper well contains 5 μ l (0.02 mg of protein) and the bottom well 10 μ l (0.04 mg of protein) of purified brain endo-oligopeptidase A. After the run, 74 μ l of antiserum (70 mg of protein) was added to the longitudinal basin. (b) The well contains 10 μ l (0.3 mg of protein) of the S fraction of rabbit brain homogenate, concentrated 10-fold. The bottom basin contains 75 μ l of total antiserum (70 mg of protein) and the upper basin has 75 μ l of immuno-adsorbed anti-(endo-oligopeptidase A) immunoglobulin (0.5 mg).

Table 1. Purification of anti-(endo-oligopeptidase A) immunoglobulin from goat antiserum

A 10 ml portion of goat antiserum was immuno-adsorbed as described in the Materials and methods section. The insolubilized immuno-adsorbent was removed by centrifugation, and the supernatant was submitted to chromatography on DEAE-cellulose, performed on a 2.8 cm $\times$ 38 cm column at 4°C, eluted with 15 mM-potassium phosphate buffer, pH 8.0, at a flow rate of 30 ml/h. The immunoglobulin fraction appears in the effluent region corresponding to 66–129 ml.

	Volume (ml)	Protein (mg/ml)	Anticatalytic activity (munits/ml)	Specific activity (munits/mg of protein)	Recovery (%)
Antiserum	10	35	10.2	0.28	100
Immuno-adsorbed antiserum	7.5	12	9.7	0.8	73
Immunoglobulin fraction of immuno-adsorbed antiserum	5.6	2.8	6.9	2.5	38

show any of these characteristics. When purified brain endo-oligopeptidase A and the S fraction were previously incubated for 30 min at 56°C, kinase activity disappeared from both enzyme preparations. The immunoelectrophoresis obtained with the heat-treated purified brain endo-oligopeptidase A or S fraction against antiserum showed disappearance of the only precipitin band yielded by the purified enzyme and the disappearance of one of the precipitin bands yielded by the S fraction. The missing precipitation band in the immunoelectrophoresis of the S fraction incubated at 56°C for 30 min corresponds to the band obtained with active purified brain endo-oligopeptidase A. This result indicates that the inactivation of brain endo-oligopeptidase A affects the ability of this enzyme to interact with antiserum, whereas the other proteins that contaminate the purified rabbit brain endo-oligopeptidase A retain the ability to interact with antibodies. This property was used to purify the antibodies in order to obtain monospecific anti-(endo-oligopeptidase A) antibodies.

In a separate experiment, we identified one of the contaminating bands obtained by immunoelectrophoresis as the interaction between rabbit serum protein and antibodies formed against purified brain endo-oligopeptidase A. Thus, when antiserum was adsorbed with insolubilized proteins from rabbit serum and rabbit brain that had been treated at 56°C for 30 min, the immunoelectrophoretic pattern obtained with this antiserum gave only one precipitin band for both purified brain endo-oligopeptidase A and for the S fraction (Fig. 2b). This result led us to the conclusion that adsorption of anti-(endo-oligopeptidase A) serum yields a serum that is monospecific for this enzyme.

The adsorbed goat antisera were applied to a column (25 cm × 3.8 cm) of DEAE-cellulose equilibrated and developed with 15 mM-potassium phosphate buffer, pH 8.0. Anticatalytic activity for brain endo-oligopeptidase A was found in the immunoglobulin fraction, which appears in the effluent region corresponding to 66–129 ml. The results presented in Table 1 indicate that the specific anticatalytic activity of purified immunoglobulin was 8.9-fold higher than that of native antiserum. About 40% of this anticatalytic activity was recovered after immunoadsorption followed by chromatography on DEAE-cellulose.

Effect of anti-(endo-oligopeptidase A) immunoglobulins against rabbit brain endo-oligopeptidases A and B

When purified brain endo-oligopeptidase A was incubated at 37°C with anti-(endo-oligopeptidase A) immunoglobulins, total inhibition of kinase activity was observed within 2 min and remained unchanged for at least 60 min.

Fig. 3 shows that anti-(endo-oligopeptidase A) immunoglobulins completely inhibit the activity of purified brain endo-oligopeptidase A but have no activity on purified brain endo-oligopeptidase B. Preimmune serum had no anticatalytic effect on either purified brain endo-oligopeptidase.

Determination of brain endo-oligopeptidase A in rabbit brain homogenate

Anti-(endo-oligopeptidase A) immunoglobulins inhibit about 65% of the kinase activity in the S fraction (Fig. 4a), whereas the antibody diminishes the kinase activity of the P fraction of rabbit brain homogenate by only 45% (Fig. 4b). Measurements of bradykinin products generated by incubation of

Fig. 3. Effect of anti-(endo-oligopeptidase A) antibodies on the activities of purified brain endo-oligopeptidases A and B

Purified brain endo-oligopeptidase A (1.5 munits) or endo-oligopeptidase B (1.2 munits) was preincubated with 0.4 mg of immunoreadsorbed anti-(endo-oligopeptidase A) antibodies or 0.6 mg of immunoglobulins from pre-immune goat serum at 37°C, in 1 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl. After 5 min of preincubation, bradykinin (58 nmol) was added to the incubation media. Samples (5–10 μl) were removed every 10 min and bioassayed by the isolated-guinea-pig-ileum method. Δ, Endo-oligopeptidase A preincubated with anti-(endo-oligopeptidase A) antibodies; ▲, endo-oligopeptidase A preincubated with pre-immune goat immunoglobulins; ○, endo-oligopeptidase A preincubated with anti-(endo-oligopeptidase A) antibodies; ●, endo-oligopeptidase B preincubated with pre-immune goat immunoglobulins; □, endo-oligopeptidase A without immunoglobulins.

Fig. 4. Effect of anti-(endo-oligopeptidase A) antibodies on bradykinin hydrolysis by the S and P fractions of rabbit brain homogenate

Samples containing approx. 1.3 munits of kinase activity from the S fraction (a) or the P fraction (b) of rabbit brain homogenate were preincubated at 37°C with 0.04, 0.08, 0.4 and 1.6 mg/ml of immunoabsorbed anti-(endo-oligopeptidase A) immunoglobulins in 1 ml of 60 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl. After 5 min of preincubation, bradykinin (58 nmol) was added to the incubation medium. After 15 min of incubation, a 150 µl sample was transferred to a tube containing 340 µl of 5 mM-HCl for bioassay (○—○). Then 1 ml of 30% (v/v) polyethylene glycol in 0.32 M-sodium citrate buffer, pH 3.2, containing 50 mM-HCl was added to the remaining incubation medium and the products of bradykinin hydrolysis (□, Arg₍₁₎→Phe₍₅₎; ■, Arg₍₁₎→Pro₍₇₎) were determined by using the amino acid analyser.

the S fraction of rabbit brain homogenate with bradykinin indicate a decrease in the concentrations of products Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg that is proportional to the concentration of antibody. The products of bradykinin hydrolysis by brain endo-oligopeptidase A were undetectable at an antibody concentration of about 0.8 mg/ml (Fig. 4a). On the other hand, the rate of bradykinin products generated by the activity of brain endo-oligopeptidase A (Arg-Pro-Pro-Gly-Phe-Ser and Phe-Arg)

present in the S fraction was not significantly altered. For the P fraction, however, the decrease in the amount of products of brain endo-oligopeptidase A acting on bradykinin is more than 70% even with large amounts of antibody.

Distribution of an immunoreactive kinase in rabbit tissues

The property of the anti-(endo-oligopeptidase A) immunoglobulin of inhibiting the kinase activity of brain homogenates, with the corresponding decrease in or abolition of the formation of bradykinin products generated by hydrolysis of the Phe₍₅₎-Ser₍₆₎ bond of bradykinin, was applied in an investigation of other rabbit tissue homogenates in an attempt to identify and quantify the presence of an immunoreactive endopeptidase similar to brain endo-oligopeptidase A. In order to compare the effect of antibodies on the kinase activities of the different tissues we studied the effect of substrate concentration on the rate of bradykinin inactivation by the S and P fractions of tissue homogenates. Results not reported in detail in the present paper indicate that the concentration of bradykinin used in the immunoinhibition experiments was at an apparent saturation value.

Fig. 5 shows the effect of anti-(endo-oligopeptidase A) antibody on the rate of bradykinin hydrolysis by tissue kinase. The upper part of the Figure gives results of total kinase activity measured by bioassay. The lower part of the Figure shows the effect of antibody on the liberation of two fragments, Arg-Phe and Ser-Arg. The recovery of all arginine peptides was close to 100% for these experiments. The highest inhibition activity of anti-(endo-oligopeptidase A) immunoglobulin was found in the S fraction of heart (89%), followed by liver (86%), skeletal muscle (78%), brain (65%), ovary (59%) and testis (53%), being low in smooth muscle (23%) and undetectable in the lung (S), kidney (S and P) and spleen (S and P). In the particulate fractions, the inhibitory effect of the antibodies was always less than 50%, except for the heart homogenate (61%). Fig. 5 also presents the products Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg obtained by hydrolysis of bradykinin by peptidases of the S and P fractions of tissue in the absence and in the presence of twice the saturating concentration of purified anti-(endo-oligopeptidase A) immunoglobulin. [We consider a saturating concentration of anti-(endo-oligopeptidase A) immunoglobulin to be the amount of antibody that would inhibit completely the kinase activity present in the incubation medium, assuming only brain endo-oligopeptidase A to be present in the enzyme preparation.] As shown in Fig. 5, anti-(endo-oligopeptidase A) immunoglobulin completely abolished the formation of Arg-Pro-Pro-Gly-Phe and Ser-

Fig. 5. Effect of anti-(endo-oligopeptidase A) antibodies on the rate of bradykinin hydrolysis by tissue kinases. Samples containing 1.0–1.5 munits of kinase activity from the S fraction (▨) or the P fraction (□) of various rabbit tissue homogenates (A, heart; B, liver; C, skeletal muscle; D, brain; E, ovary; F, testis; G, smooth muscle; H, lung; I, kidney; J, spleen) were preincubated at 37°C with 1.6 mg of immunoadsorbed anti-(endo-oligopeptidase A) immunoglobulins/ml or 1.6 mg of immunoglobulins from preimmune goat serum/ml, in 1 ml of 50 mM-Tris/HCl, pH 7.5, containing 0.1 M-NaCl. After 5 min of preincubation, 58 nmol of bradykinin was added to the mixture, which was submitted to incubation at 37°C until about 50% of the bradykinin had been inactivated. The reaction was stopped by addition of 1.2 ml of 30% (v/v) polyethylene glycol in 0.32 M-sodium citrate buffer, pH 3.2, containing 50 mM-HCl. The rate of bradykinin inactivation was followed by bioassay on the isolated guinea-pig ileum, and the products of bradykinin hydrolysis ($\text{Arg}_{(1)} \rightarrow \text{Phe}_{(5)}$, upper half of circles; $\text{Ser}_{(6)} \rightarrow \text{Arg}_{(9)}$, lower half of circles) were determined by chromatographic analysis.

Pro-Phe-Arg in the incubation medium of the S fractions of heart, liver, skeletal muscle and ovary, a finding similar to that obtained with the S fraction of brain, indicating that the antibodies inhibit an endopeptidase with the same specificity for the $\text{Phe}_{(5)}\text{--Ser}_{(6)}$ bond of bradykinin as has brain endo-oligopeptidase A. Pre-immune serum had no effect either on the kinase activity or on the formation of bradykinin fragments. For the S fraction of testis and smooth muscle, the amount of the Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg fragments formed was decreased by only 50–60% by the action of the antibodies. The amounts of Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg formed from bradykinin when the P fraction of the tissues was incubated in the presence of antibodies decreased by 50–90% when compared with the respective controls, except for the heart fraction, where the concentration of Arg-Pro-Pro-Gly-Phe was higher in the presence than in the absence of antibodies.

Discussion

Endo-oligopeptidase A isolated from the S fraction of rabbit brain homogenate is a highly purified preparation of brain endo-oligopeptidase A, since antiserum prepared against this enzyme preparation develops only one line of precipitation with the

antigen as shown by immunoelectrophoresis. Two minor contaminants present in the purified preparation of this enzyme should explain the appearance of two other lines of precipitation when antiserum was tested against the S fraction of rabbit brain homogenate concentrated 10-fold.

On the other hand, rabbit brain endo-oligopeptidase A has proved to be a good immunogen in goats, since potent antisera were produced in response to injection of small amounts of enzyme. The antisera raised against brain endo-oligopeptidase A produced complete inhibition in quantities of 30–50 μl /munit of brain endo-oligopeptidase A. However, these antisera do not form a line of precipitation against the inactive enzyme, as shown by immunoelectrophoresis.

It is known that antibodies to native proteins, particularly globular proteins, are made primarily to conformational determinants (Cinader, 1963). Alterations that affect the conformation of a determinant in lysozyme (Atassi *et al.*, 1976) and in penicillinase (Pollock, 1963) affect the antigen–antibody reactions. This must explain why brain endo-oligopeptidase A lost its ability to react with specific antibodies when inactivated by heat (50°C for 30 min). The loss of reactivity to the specific antibody shown by inactive brain endo-oligopeptidase A is an important characteristic of the

interaction between many proteolytic enzymes and high-molecular-weight inhibitors such as immunoglobulins and α_2 -macroglobulin, since only the active form of the enzyme reacts with the inhibitor (Shapira & Arnon, 1967; Barrett & Starkey, 1973). This property was used to purify by immunoadsorption the antiserum obtained with the purified preparation of brain endo-oligopeptidase A. The immunoadsorbed goat antiserum gave only one line of precipitation by immunoelectrophoresis against the S fraction, and kept its property of binding purified endo-oligopeptidase A, as indicated by the inhibition of kininase activity and by the formation of a precipitation line by immunoelectrophoresis against purified brain endo-oligopeptidase A. The possibility of non-specific inhibition of endopeptidase by α_2 -macroglobulin can be ruled out, because pre-immune serum had no effect on kininase activity, and because the purification of immunoglobulin by chromatography on DEAE-cellulose completely eliminated α_2 -macroglobulin (Das & Soffer, 1976).

The high degree of selectivity of immunoreaction with antibodies, combined with the specificity of brain endo-oligopeptidase A for the $\text{Phe}_{(5)}\text{-Ser}_{(6)}$ bond of bradykinin, permitted us to use the immunoinhibition of kininase activity, complemented by the quantification of bradykinin products, to study the distribution of an isofunctional and immunoreactive endopeptidase in crude enzyme preparations of rabbit tissues.

The expression of endo-oligopeptidase A activity in crude preparations of tissue peptidases was evaluated both by the degree of antibody inhibition of kininase activity and by the disappearance of products resulting from hydrolysis of the $\text{Phe}_{(5)}\text{-Ser}_{(6)}$ bond of bradykinin. Application of these methods to the S fraction of rabbit brain confirmed the result obtained by Oliveira *et al.* (1976), who showed that brain endo-oligopeptidase A contributes more than 60% of the total kininase activity present in this tissue preparation. The presence of antibodies in the incubation media does not seem to affect other peptidases present in the S fraction of rabbit brain homogenate that act on bradykinin or on its products.

Contrary to what happens in the incubation performed with the S fraction of most rabbit tissue homogenates, where the anti-(endo-oligopeptidase A) antibodies abolish the generation of fragments resulting from hydrolysis of the $\text{Phe}_{(5)}\text{-Ser}_{(6)}$ bond of bradykinin, in the incubation with the P and S fractions of smooth muscle and testis $\text{Arg}_{(1)}\text{-Phe}_{(5)}$ formation is decreased but not abolished in the incubation mixture. These results may be explained by the presence of another brain endo-oligopeptidase A-like enzyme or by the inaccessibility of the antibodies to the antigenic determinants of the

particulate enzyme (Cinader, 1963, 1977), or even by the presence of other peptidases, such as angiotensin-converting enzyme (EC 3.4.15.1), which form the same product $\text{Arg}_{(1)}\rightarrow\text{Phe}_{(5)}$ by sequential cleavage of the $\text{Pro}_{(7)}\text{-Phe}_{(8)}$ and $\text{Phe}_{(5)}\text{-Ser}_{(6)}$ peptide bonds of bradykinin (Dorer *et al.*, 1974).

The results of immunoinhibition by anti-(endo-oligopeptidase A) immunoglobulins of tissue kininases that specifically hydrolyse the $\text{Phe}_{(5)}\text{-Ser}_{(6)}$ bond of bradykinin suggest that virtually all of it is antigenically related to brain endo-oligopeptidase A. A question to be raised is whether these immunoreactive tissue kininases exhibiting the same specificity as brain endo-oligopeptidase A for bradykinin are the same enzyme or multiple molecular forms of brain endo-oligopeptidase A.

Multiple forms of enzymes with similar immunological reactivity, but exhibiting differences in molecular weight or kinetics and inhibition properties, have been described for several enzymes (Atassi *et al.*, 1976). Although it is not yet possible to state for sure that tissue kininases that are inhibited by anti-(endo-oligopeptidase A) immunoglobulins constitute multiple forms of brain endo-oligopeptidase A, results obtained by M. A. Cicilini, H. L. L. Coelho & A. C. M. Camargo (unpublished work) support the idea that, at least for brain and heart endopeptidases, this hypothesis seems likely.

We have previously shown that endo-oligopeptidases of nervous tissue have specificity for certain peptide bonds of bradykinin (Oliveira *et al.*, 1976), angiotensins I and II and luliberin (Martins *et al.*, 1980). In the present study we have shown that the isofunctional and immunoreactive form of brain endo-oligopeptidase A is widely distributed in peripheral tissue. Thus, if endo-oligopeptidase A-like enzymes are part of the system responsible for the physiological termination of the effect of biologically active peptides, we should presume that this system may be selective as to substrate size and as to specificity of the susceptible peptide bond, but not restricted to a specific polypeptide or to a particular tissue.

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