CENTRAL ACTION OF BRADYKININ (II) SEPARATION OF BRADYKININ DEGRADING ENZYME FROM THE RAT BRAIN*

Kimio KARIYA, Hideo IWAKI, Aiko YAMAUCHI, Midori OKAMOTO, Yuko TSUDA** and Yoshio OKADA**
Department of Pharmacology and Chemistry**, Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Ikawadani-cho, Tarumi-ku, Kobe 673, Japan
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Abstract—Separation of kininases from the rat brain and identification of the products produced by these enzymes were examined using DEAE-cellulose column chromatography and thin-layer chromatography, respectively. Fraction A (F-A), which was developed with 70 mM NaCl, released Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg, fraction B (F-B), with 100 mM NaCl, cleaved the Pro7-Phe8 and Phe8-Arg9 peptide bonds of the bradykinin molecule and fraction C (F-C) with 150 mM NaCl hydrolyzed bradykinin to amino acids. The specific activity of each fraction determined by the bioassay system was 158.7, 51.0 and 14.8 nmole bradykinin/mg protein/min, respectively. The intensities of their fluorescence on the chromatogram showed visibly that o-phenanthroline effectively inhibited F-A, B and C at a concentration of 0.5 mM. These results suggest that heterogeneous enzyme systems are present in the rat brain and regulate the levels of bradykinin in the brain.

Peptide hormones circulating in peripheral tissues are not only directed to target organs, but also have certain effects on the central nervous system (1). Bradykinin (BK) is one such hormone. BK-like immunoreactive neurons are localized histochemically in the rat brain (2). Further, the enzymatic inactivating system of BK at neutral pH was found in tissues in the kidney, lung, heart, spleen, liver and brain (3). Endopeptidases of brain tissue have been isolated and characterized (4–9). However, few authors have discussed the peptides released from BK. We have already reported that the Ser-Pro containing peptide fragments of BK prolonged pentobarbital induced sleeping time in mice and the possible importance of Ser-Pro for the sedation phase of behavior induced by BK was suggested (10). It is interesting that not only the native hormone, but also the products of degradation have certain pharmacological effects. In the present work, the three kinds of enzymes were separated from the rat whole brain and determination made of the position of hydrolysis of BK by these enzymes.

MATERIALS AND METHODS

Enzyme preparation: Male Sprague-Dawley strain rats weighing 200 to 300 g were anesthetized with pentobarbital (50 mg/kg, i.p.) and both carotid arteries were cannulated for the elimination of blood, a rich source of plasma kininase I and II (11). After the blood was flushed from the jugular

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veins with cold saline, the whole brain was removed and homogenized with a Teflon pestle in five volumes of 0.25 M sucrose. The homogenate was centrifuged at 25,000×g for 60 min, the supernatant was adjusted to pH 5.0 by the addition of 0.5 M acetic acid and the preparation left standing at 4°C for 2 hr. The suspension was centrifuged at 900×g for 15 min and this supernatant was adjusted to pH 7.5 by adding 0.5 N NaOH. After dialysis against a large volume of 50 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl, the preparation was then applied to a DEAE-cellulose column previously equilibrated with the dialyzed buffer and active fractions were eluted by the stepwise increase of the concentrations of sodium chloride in buffer, as described by Oliveria et al. (12). The column (0.9×30 cm) was run at 17 ml/hr, at 4°C, and fractions of 5.0 ml were collected. Active fractions which were determined by the bioassay method were pooled and condensed to about one twentieth of their original volume by using collodion bags, under reduced pressure. Determinations for protein were made using the methods of Lowry et al. (13) using bovine serum albumin as a standard.

**Determination of kininase activity:** The Magnus method and thin-layer chromatography coupled with microdansylation were used to detect kininase activity. With this bioassay method, the reaction mixture contained 1 nmole BK and 0.8–1.0 μg enzyme protein in 50 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl in a total volume of 0.5 ml. Incubations were carried out for 20 min at 37°C. The reaction was stopped by the addition of 10 μl of 0.5 N HCl, and the residual BK was bioassayed using the ileum isolated from a guinea pig and which had been bathed in Tyrode’s solution containing 1.7 μM atropine sulphate at 28°C.

In the thin-layer chromatographic analysis, the reaction mixtures contained 40 nmole BK, 10–20 μg enzyme protein, with or without inhibitors, in a total of 100 μl of 50 mM sodium phosphate buffer (pH 7.5). Incubations were carried out for 60 min at 37°C with 20 μl of the reaction mixtures being transferred to a micro test tube containing 10 μl of 0.5 N HCl. Dansylation was accomplished according to the method of Gray (14). The so-obtained peptides were dissolved in 20 μl of ethanol and spotted on the precoated silica gel G plate (Silica gel 60/Kieselguhr F254, Merck A.G.). The plate was developed with the solvent (n-butanol: acetic acid : water=4 : 1 : 5 v/v). After the chromatography, the dansylated peptides were visually detected as fluorescent spots when irradiated with UV light (360 nm) and these spots were sketched.

**Reagents:** Bradykinin diacetate was obtained from the Protein Research Foundation (Osaka). The peptide fragments related to BK were synthesized by liquid phase synthesis, as described previously (10). Other chemicals used were of a special grade.

**RESULTS**

**Preparation of kininases from the rat brain:** Figure 1 shows protein concentrations and kininase activities on the elution diagram. Kininase activities appeared as a stepwise gradient in various sodium chloride concentrations. Kininase eluted by a concentration of 70 mM NaCl showed the highest activity and was termed F-A, by 100 mM NaCl was F-B and by 150 mM NaCl was F-C. Little activity was obtained when elution was with 50 mM NaCl as the first step.

Purification of kininases from the rat whole brain homogenate are summarized in Table 1. The 25,000×g supernatant fraction showed about half of the kininase activity present in the homogenate. When the pH of the supernatant was adjusted to 5.0, the specific
activity increased 10.8 fold over the homogenate. F-A was purified about 122.1 times from the homogenate and showed a specific activity of 158.7 nmole/mg protein/min.

**Table 1.** Purification of bradykinin degrading enzymes from rat brain

| Fraction            | Protein (mg) | Specific Activity (nmole/mg protein/min) | Degree of purification | Yield (%) |
|---------------------|--------------|------------------------------------------|------------------------|-----------|
| Homogenate          | 460          | 1.3                                      | 1.0                    | 100       |
| 25,000 g Supernatant| 120          | 2.4                                      | 1.8                    | 50        |
| pH 5.0 Supernatant  | 20.6         | 14.1                                     | 10.8                   | 50        |
| DEAE-Cellulose column |            |                                          |                        |           |
| A                   | 0.73         | 158.7                                    | 122.1                  | 20        |
| B                   | 0.32         | 51.0                                     | 39.2                   | 2.8       |
| C                   | 0.99         | 14.8                                     | 11.4                   | 2.5       |

Bradykinin degrading activity of each fraction was bioassayed as described in MATERIALS AND METHODS using an isolated guinea pig ileum.

**Fig. 1.** Chromatogram on DEAE-cellulose of pH 5.0 supernatant fraction from the rat brain. The pH 5.0 supernatant fraction was applied as described in MATERIALS AND METHODS and the column was developed by stepwise gradients of NaCl as shown in this figure. The sample was eluted at the rate of 17 ml/hr and fractions of 5.0 ml were collected. Kininase activity was determined using an isolated guinea pig ileum. (●) Absorbance at 280 nm, (○) Kininase activity.

**Fig. 2.** Thin-layer chromatogram of the dansylated reaction mixtures: Figure 2 illustrates thin-layer chromatogram of the dansylated au-
authentic peptides, amino acids and the hydrolyzed products of BK by F-A, B and C. Fluorescent spots corresponding to Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg appeared after 60 min incubation in the case of F-A. F-B released peptides and amino acids corresponding to Phe-Arg, arginine and phenylalanine from BK. And also another spot was observed at the second position from the bottom in the column of fraction B. All amino acids constituting BK appeared when BK was incubated with F-C for 60 min at 37°C.

Effects of some compounds on kininase activities of F-A, B and C: The intensities of the fluorescence on the chromatogram were observed visually under UV radiation. Such were classified as bright, moderately bright and faint. The results are shown in Table 2. Fluorescence of BK disappeared, but each product, as shown in Fig. 2, appeared on the plate after the incubation for 60 min without inhibitors. The enzymes of fraction A, B and C did not cleave any peptide bond of BK, in the presence of 0.5 mM o-phenanthroline. 8-Hydroxyquinol-
line did not influence the appearance of products, at this concentration. With the presence of diethylidithiocarbamate, all spots on the chromatogram showed moderately bright intensities, indicating that all of the enzymes, were to some extent inhibited.

**DISCUSSION**

With regard to the possible release of pharmacologically active peptides in the central nervous system, it was reported that the excited state was elicited by the authentic BK whereas its metabolites might cause a depression (15). Previously we observed that BK and some fragments containing Ser-Pro, such as Gly-Phe-Ser-Pro, Phe-Ser-Pro and Ser-Pro prolonged pentobarbital induced sleeping time in mice (10). These results suggest that not only native BK, but also its degraded fragments have a pharmacological effect on the central nervous system. Preliminary experiments showed that partially purified BK degrading enzymes from the rat brain released peptides which had phenylalanine, serine and proline as N-terminal amino acids (16).

In this report, this enzyme was further purified by DEAE-cellulose column chromatography. The most active F-A which was eluted by 70 mM NaCl, cleaved the BK molecule and released Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg. In respect to the cleavage point, this enzyme was similar to kininase A purified from rabbit cerebral cortex by Camargo et al. (8). Another enzyme which shows the same cleavage point was purified from bovine pituitaries by Wilk et al. (9). Although F-B released Phe-Arg, arginine and phenylalanine from the C-terminal position of BK, one spot corresponding to the second position from the bottom of the fraction B column in Fig. 2 was not identified with an authentic peptide. This spot may be Arg-Pro-Pro-Gly-Phe-Ser-Pro which divided Phe-Arg from BK.

As cleaving point of this enzyme Shikimi et al. (17) showed that arginine and phenylalanine were released by incubating BK for 24 hr with the enzyme from the whole brain homogenate which had been purified 32 fold. However, the residual peptides which released phenylalanine and arginine from BK were not clearly revealed on their chromatogram. Oliveira et al. (12) purified kininase B and which also releases Phe-Arg from the C-terminal position of BK. From such evidence, F-B is more likely to be the latter enzyme and it may be contaminated by a peptidylamino acid hydrolase such as carboxypeptidase B. Incubation mixtures of F-C contained all the amino acids derived from BK, indicating the similarity of F-C to a carboxypeptidase. The specific activity of F-C was lower than the activities of F-A and B.

o-Phenanthroline, also known as an inhibitor of plasma kininases (11) and carboxypeptidase B (18) shows inhibitory effects on all fractions at a concentration of 0.5 mM. It was reported that the partially purified enzyme was inhibited about 50% by the compound, at 0.5 mM, in the bioassay system using rat uterus (16). Results obtained using guinea pig ileum also indicated that o-phenanthroline inhibited F-A, B and C by about 25, 40 and 25%, respectively, at a concentration of 0.1 mM (unpublished data). There were slight differences among the sensitivities of each fraction against the inhibitory effect of the compound in bioassay system. In relation to this, Kameyama et al. reported that peptides related to BK increased the contractile response by BK in smooth muscle (19). This observation explains the limitation of a bioassay system for the measurement of kinin degrading enzyme activity. Recently, we have designed a quantitative chemical assay system and attempted to elucidate the precise nature of F-A by comparing the results obtained with
both assay systems. The results will be published elsewhere.

In relation to these in vitro results, we observed that an intracerebral pretreatment with o-phenanthroline prolonged the effect of intracerebrally injected BK on rat electroencephalogram (16). These results suggest that the action of BK in the brain is limited by peptidases present in the central nervous system. The present results indicate that there are several heterogeneous BK degrading enzyme system in the rat brain. F-A which shows the highest specific activity and degrades the BK molecule at the Phe⁵-Ser⁶ peptide bond may be one of these enzymes. In considering the cleavage point of F-B and C, it is reasonable to postulate that Ser-Pro containing peptides are released in the rat brain and exert some pharmacological action on the central nervous system (16). Further, these data support the idea that Ser-Pro containing fragments prolonged pentobarbital induced sleeping time in mice, as shown in a previous report (10).

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