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PHARMACOLOGICAL SIGNIFICANCES OF PEPTIDASE AND PROTEINASE IN THE BRAIN—II

PURIFICATION AND PROPERTIES OF A BRADYKININ INACTIVATING ENZYME FROM RAT BRAIN

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Abstract—The enzyme inactivating bradykinin (kininase) in rat brain was partially purified by ammonium sulfate fractionation and chromatography on Sephadex G-100, DEAE-cellulose and CM-cellulose. The final preparation showed a single peak in the ultracentrifuge with a sedimentation constant of 4·55S.

This enzyme preparation inactivates bradykin by removing its ^{8}phenylalanine and C-terminal ^{9}arginine.

Enzyme activity was inhibited by chelating agents and SH-compounds and increased by SH reagents and Ca^{2+}. Ovomucoid trypsin inhibitor, tosylphenylalanine chloromethylketone (TPCK) and diisopropylfluorophosphate (DFP) had no effect. Among the analgesic antipyretic drugs tested, acetylsalicylic acid and aminopyrine activated the enzyme slightly.

PEPTIDASE and proteinase activities increase in many different pathological conditions.[1–3] In 1959, Chapman *et al.*[4] demonstrated that human cerebrospinal fluid from patients with disorders of the central nervous system, such as vascular headache of the migraine type or chronic schizophrenia, formed a vasodilatory polypeptide on incubation with plasma globulin. Moreover, bradykinin-like polypeptides have been found in the brain.[5] We reported the existence of an enzyme in rat brain which destroys bradykinin (kininase), and found that the effect of metal ions (zinc and cobalt ions) on it is different from their effect on the enzyme in plasma.[6]

This work was on the purification of the enzyme inactivating bradykinin and examination of the properties and specificity of the purified preparation.

MATERIALS AND METHODS

Biological assay

The methods of incubation and determination of kininase activity were as described previously.[6]

The specific activity was arbitrarily calculated from the time necessary to inactivate over 50 per cent of the bradykinin solution (2·5 μg/ml).[7] The linearity was observed between the arbitrarily defined specific activity and log dose of bradykinin from 0·5 to 5 μg/ml of bradykinin, 50 per cent inactivating time was inversely proportional to the concentration of enzyme preparation (0·04 mg protein/ml to 0·4 mg protein/ml). Synthetic bradykinin was obtained from the Institute for Protein Research, Osaka University.

Compounds and drugs

The following compounds were used: diisopropylfluorophosphate (DFP, Sigma Chemical Company, U.S.A.), *N*-tosylphenylalanine chloromethylketone (Seikagaku Kogyo Co. Ltd., Japan), ovomucoid trypsin inhibitor (Sigma Chemical Company, U.S.A.), and Trasylol® (a polypeptide extract of bovine origin, Bayer AG, Germany). All other chemicals were standard commercial preparations. Compounds used in experiments on kininase activity were of reagent grade and inorganic cations were used as their chlorides.

The following drugs were used: acetylsalicylic acid, aminopyrine, phenylbutazone (Fujisawa Pharmaceutical Co. Ltd., Osaka), acetophenetidin, antipyrine, quinine sulfate, caffeine, morphine hydrochloride, levallorphan tartrate (Takeda Chemical Industries Ltd., Osaka), diphenhydramine hydrochloride, methysergide (Sandoz Ltd., Basel).

Solutions of these compounds and drugs were adjusted to pH 7·6 with 40 mM Tris-HCl buffer, and were preincubated with the enzyme for 20 min, except that of DFP, which was incubated for 1 hr.

Protein determination

The chromatographic distribution of protein was determined from the absorption at 280 mμ, or by the method of Lowry *et al.*[8]

Chromatography

Diethylaminoethylcellulose (DEAE-cellulose) and carboxymethylcellulose (CM-cellulose) were obtained from Nakarai Chemicals, Ltd. (Kyoto). Sephadex G-100 (particle size 40–120 μ) was a commercial preparation from Pharmacia, Uppsala (Sweden), and was handled according to the manufacturers' directions. Chromatography was carried out at 0–4°.

Ultracentrifugation

Sedimentation analysis was performed with a Hitachi UCA-1A ultracentrifuge at 20°.

Paper chromatography and high voltage electrophoresis

Ascending chromatography was carried out using the upper phase of a mixture of butanol, acetic acid and water (4:1:5 v/v) with Toyo Filter Paper No. 51. After 5 hr, the paper was dried at 80° and then developed with 0·2% ninhydrin in ethanol at 80° for 10–15 min.

High voltage electrophoresis was carried out for 10 min at 4000 V on Toyo Filter Paper No. 51 (3 × 50 cm). The buffer used was pyridine–acetic acid (pH 6·5). The paper was dried at 80° and then developed with ethanolic ninhydrin solution as above.

RESULTS

Purification of the enzyme

The purification procedure is shown in Fig. 1 and Table 1.

Homogenate and 90,000 g *supernatant*

Adult male rats of the Wistar strain, weighing about 200 g, were used. After decapitation, brains were rapidly removed and as much blood as possible was carefully

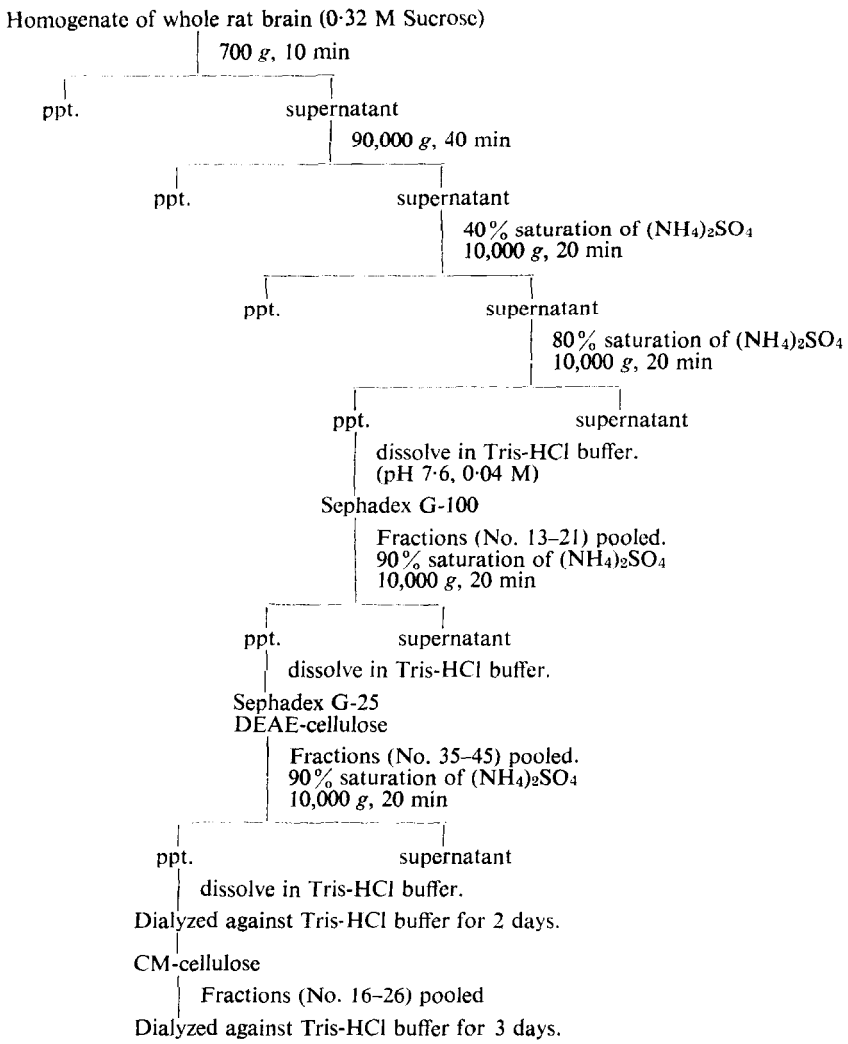

FIG. 1. Purification of rat brain kininase.

TABLE 1. SUMMARY OF ENZYME PURIFICATION

Purification stage	Volume (ml)	Amount of protein (mg)	Specific activity*	Activity†	Purification coefficient
Homogenate	160	1500	0·13	195	1·0
90,000 *g*, 40 min supernatant	150	310	0·44	137	3·4
$(NH_4)_2SO_4$ fractionation 40–80%	20	140	0·75	105	5·8
Sephadex G-100 pool	78	80	1·22	98	9·4
DEAE-cellulose pool	44	20	2·71	55	20·9
CM-cellulose pool	31	10	4·20	42	32·3

* Specific activity $= \dfrac{1}{t_{min} \times \text{enzyme concn in mg protein}}$.

t_{min}: time necessary to inactivate 50% of a bradykinin solution (2·5 μg/ml).

† Activity = mg protein × specific activity.

removed. The brains were homogenized in 9 vol. of 0·32 M sucrose solution at 0° in an all-glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 700 *g* for 10 min to remove the cell debris and any red blood cells present. The supernatant was recentrifuged at 90,000 *g* for 40 min.

Ammonium sulfate fractionation

Powdered ammonium sulfate was slowly added to this supernatant with stirring to give 40% saturation. The mixture was stood for 2 hr at 4°, and then centrifuged at 10,000 *g* for 20 min. Ammonium sulfate was added to the supernatant to 80% saturation, and the mixture was stood and centrifuged as before. The precipitate was dissolved in 40 mM Tris-HCl buffer, pH 7·6.

Sephadex G-100 chromatography

The solution was applied to a column (2·5 × 80 cm) of Sephadex G-100. Elution was carried out with 40 mM Tris-HCl buffer, pH 7·6. The flow rate was about 15 ml per hr and fractions of 9·7 ml were collected.

Figure 2 shows the distributions of protein and kininase activity. Fractions 13–21 with the highest activity toward bradykinin were combined and used in the following step.

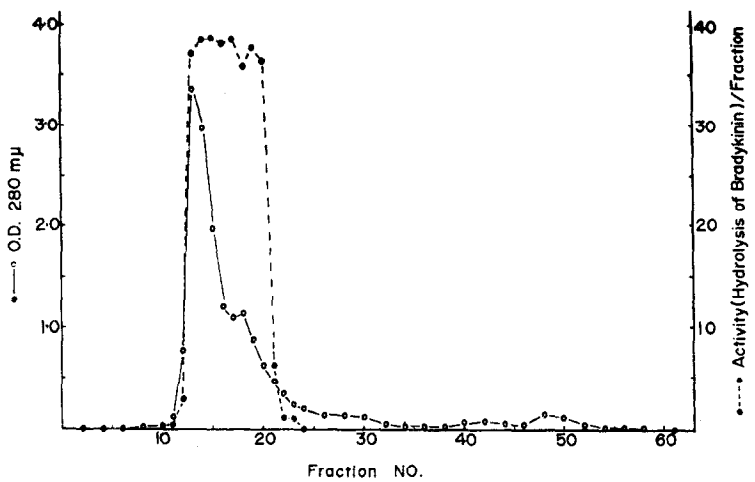

FIG. 2. Sephadex G-100 chromatography (2·5 cm × 80 cm). Sample, 20 ml; flow rate, 15 ml/hr; fraction volume, 9·7 ml.

DEAE-cellulose chromatography

Ammonium sulfate was added to the preparation to 90% saturation and the mixture was stood overnight and centrifuged at 10,000 *g* for 20 min. The precipitate was dissolved in 40 mM Tris-HCl buffer, pH 7·6, and the solution was deionized by passage through a Sephadex G-25 column (2·5 × 21 cm) eluting with 40 mM Tris-HCl buffer, pH 7·6. The fractions containing protein were pooled and applied to a DEAE-cellulose column (2·5 × 19·5 cm). The column was eluted with a linear gradient of zero to 0·5 M NaCl in the above-mentioned buffer. One hundred fractions of 4·6 ml were collected.

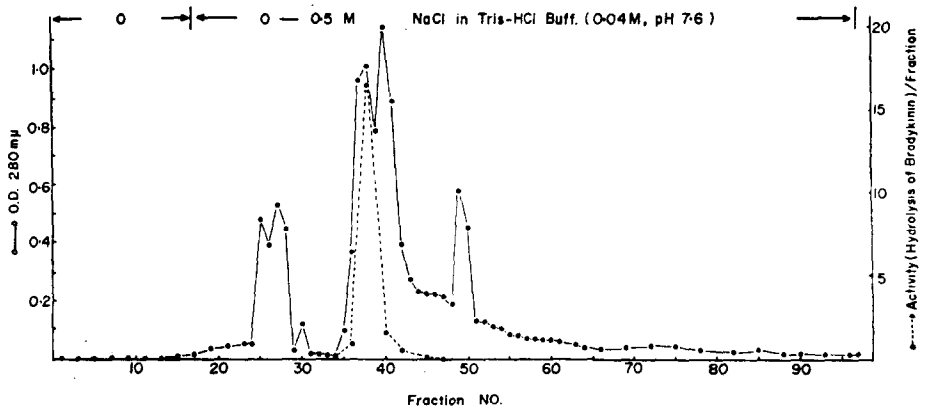

FIG. 3. DEAE-cellulose chromatography (2·5 cm × 19·5 cm). Sample, 50 ml; flow rate, 18 ml/hr; fraction volume, 4·6 ml.

The result of the chromatography is shown in Fig. 3. Fractions containing activity (Nos. 35–45) were pooled and purified further.

CM-cellulose chromatography

The pooled preparation was deionized by dialysis for two days against 40 mM Tris-HCl buffer (pH 7·6) at 4°. Then it was applied to a CM-cellulose column (2·5 × 22 cm) and eluted with a linear gradient of zero to 0·5 M NaCl in the dialysis buffer.

Figure 4 shows the distribution of protein and kininase activity. The fractions in tubes 16 to 26 in Fig. 4 were pooled and thoroughly dialyzed. This preparation was used in further characterization studies.

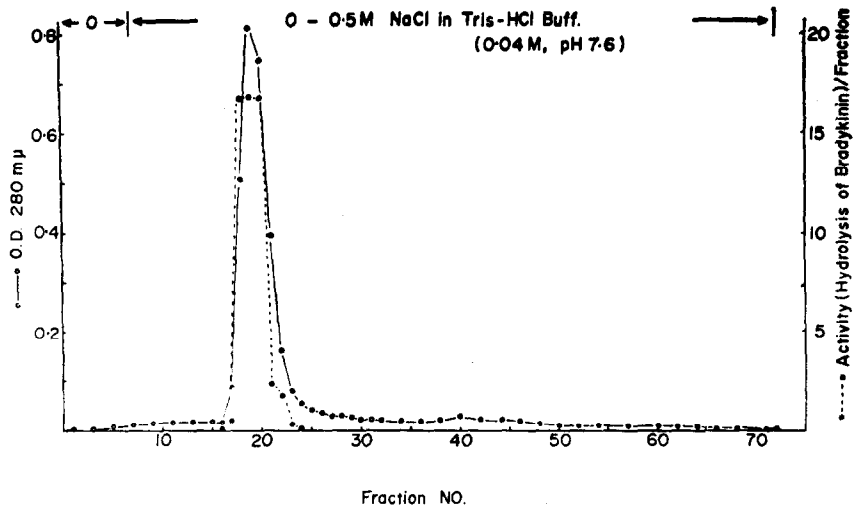

FIG. 4. CM-cellulose chromatography (2·5 cm × 22 cm). Sample, 11·2 ml; flow rate, 16 ml/hr; fraction volume, 4·6 ml.

Ultracentrifugation

The homogeneity of the purified enzyme preparation was tested by analytical ultracentrifugation. A solution of the enzyme preparation at a concentration of 0·42% in 40 mM Tris-HCl buffer (pH 7·6) was centrifuged at 55,430 r.p.m. at 20·0°.

The purified preparation showed a single peak in the ultracentrifuge with sedimentation constant of 4·55 S.

Enzymatic hydrolysis of bradykinin

One mg of bradykinin in 0·1 ml of water was incubated with 0·1 ml of the final enzyme preparation in 40 mM Tris-HCl buffer (pH 7·6) for 24 hr at room temperature. The solution of enzyme or bradykinin was left in the same condition as in the reaction mixture. The reaction products were subjected to paper chromatography and high voltage paper electrophoresis. No spontaneous reaction, e.g. might be induced by bacteria, was observed in this experimental condition.

Figures 5 and 6 show that bradykinin is hydrolyzed to release materials which migrate like arginine and phenylalanine. No single spot of heptapeptide, originating from bradykinin, was found in this experiment.

Effects of various compounds

The effects of various compounds on kininase activity are shown in Table 2. Strong inhibition is shown as *, and denotes a prolongation of the period required for breakdown of a certain amount of bradykinin from 13 min to 40 min or more. Less pronounced inhibition is shown as †, and no inhibition as ‡. Activation is indicated as §.

Glutathione and mercaptoethanolamine were inhibitory, whereas SH reagents, such as *p*-chloromercuribenzoate (PCMB), caused activation. Of the metal ions at the

TABLE 2. EFFECTS OF VARIOUS COMPOUNDS ON KININASE ACTIVITY OF RAT BRAIN

Compound	Concn (M)	Relative degree of inhibition
Cysteine	1×10^{-4}	†
Mercaptoethanolamine	1×10^{-4}	†
Glutathione	1×10^{-4}	†
NEM	1×10^{-4}	‡
PCMB	1×10^{-4}	‡, §
EDTA	1×10^{-4}	*
CDTA	1×10^{-4}	*
$CuCl_2$	1×10^{-4}	‡, †
$MgCl_2$	1×10^{-4}	‡, †
$CaCl_2$	1×10^{-4}	§, ‡
$CoCl_2$	1×10^{-4}	†
$ZnCl_2$	1×10^{-4}	†
$FeCl_3$	1×10^{-4}	*
Tosylphenylalanine chloromethylketone	1×10^{-4}	‡
DFP	5×10^{-6}	‡
Ovomucoid trypsin inhibitor	0·1 mg/ml	‡
Trasylol	180 KIE/ml	†

Symbols: * strong inhibition, † some inhibition, ‡ no inhibition, § activation.
Abbreviations used are: NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetic acid; CDTA, cyclohexanediaminetetraacetic acid; DFP, diisopropylfluorophosphate.
For details see text.

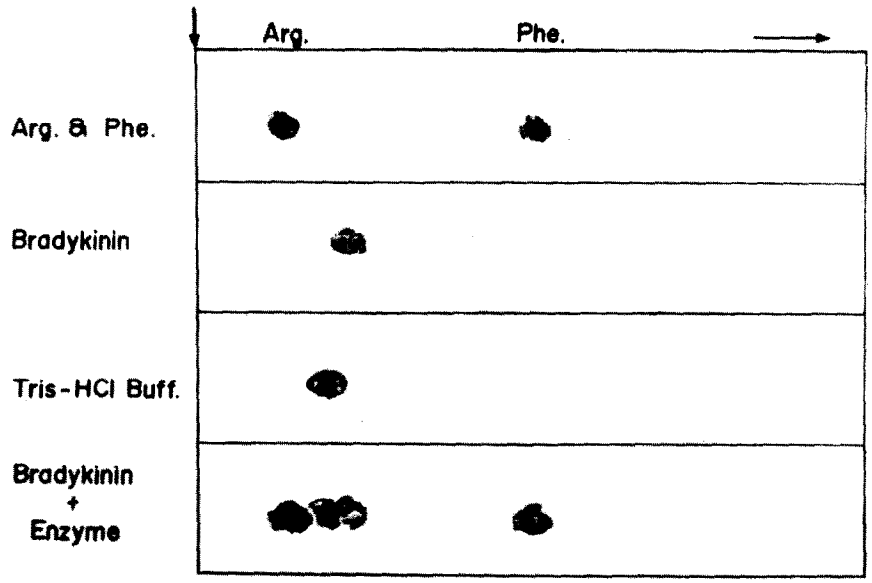

FIG. 5. Paper chromatography. Experimental conditions are given in Methods.

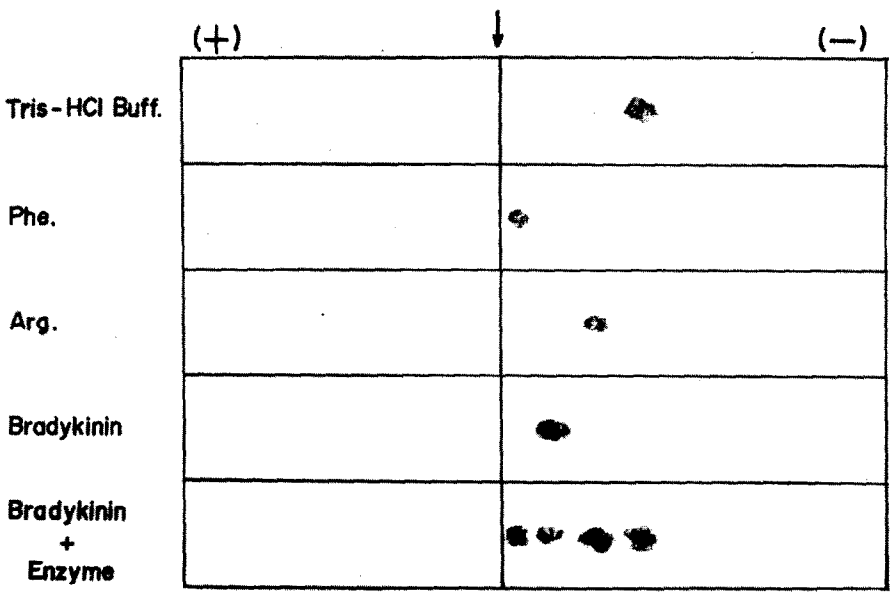

FIG. 6. High voltage paper electrophoresis. Experimental conditions are given in Methods.

concentrations tested, calcium ion caused activation of the kininase. Zinc and ferric ions and chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and cyclohexanediaminetetraacetic acid (CDTA) inhibited the enzyme activity. Ovomucoid trypsin inhibitor (0·1 mg/ml), DFP and specific chymotrypsin inhibitor, *N*-tosylphenylalanine chloromethylketone (TPCK) had no effect, whereas trasylol inhibited the enzyme at a concentration of 180 KIE/ml.

Effects of various drugs

The effects of following drugs on the kininase activity were tested at 1×10^{-5} M concentration, while morphine and levallorphan were at 1×10^{-6} M: acetylsalicylic acid, aminopyrine, acetophenetidin, antipyrine, phenylbutazone, quinine sulfate, caffeine, diphenhydramine, methysergide, morphine, levallorphan.

At the concentrations tested, neither enzyme, drugs or mixtures of enzyme and drugs, showed any effect on isolated guinea-pig ileum and the drugs had no detectable effect on the response to bradykinin activity. Among the analgesic antipyretic drugs tested, acetylsalicylic acid and aminopyrine increased bradykinin destruction slightly, whereas other drugs had no effect. The morphine antagonist, levallorphan inhibited kininase activity.

DISCUSSION

In 1962, Hooper[9] reported the enzymatic inactivation of some physiologically active polypeptides by a neural tissue extract, but these enzymes have mostly not been extensively purified or characterized. Recently, the isolation of the substance P-inactivating enzyme from bovine brain was reported.[10] However, no information was available on the isolation of a homogeneous enzyme which inactivates bradykinin in brain tissue.

We found the highest enzymatic inactivation of bradykinin in the supernatant on subcellular fractionation of rat brain.[6] The supernatant fraction of a preparation centrifuged at 90,000 *g* for 40 min was fractionated by salt fractionation and chromatographic procedures. This gave a preparation which behaved like a homogeneous protein on analytical ultracentrifugation with a sedimentation constant of 4·55 S. However, in starch-gel electrophoresis, it did not migrate as a single band. One main band having kininase activity and some inactive protein bands were also observed.[15] The relatively low degree of purification of final preparation could be one part due to the presence of these contaminated protein. To obtain an ultracentrifugally and electrophoretically homogeneous preparation, further purifications are now in progress.

Marks and Lajtha[11, 12] distinguished two different kinds of proteinase in rat brain, namely acid proteinase (pH optimum 3·4) and neutral proteinase (pH optimum 7·8) with molecular weights of approximately 60,000 and above 100,000, respectively. Riekkinen and Rinne[13] developed a method to distinguish the neutral (pH optimum 7·0) and alkaline (pH optimum 7·8) proteinases in the soluble fraction of rat brain, using Guroff's method[14] with some modifications. The molecular weights of the neutral and alkaline proteinase are 43,000 and 70,000, respectively. Our partially purified preparation had a pH optimum of 7·6. Alkaline proteinase is inhibited by calcium ion but not by EDTA.[13] The neutral proteinase purified from rat brain by Marks and Lajtha,[11, 12] exhibited a pH optimum at 7·6. This enzyme is activated by SH-compounds and inhibited by SH-reagents and the specific chymotrypsin inhibitor,

TPCK. Our enzyme preparation was not inhibited by calcium ion, SH-reagents or TPCK, but was inhibited by EDTA and SH-compounds. Moreover, casein and denatured bovine hemoglobin were not hydrolysed to any appreciable extent by our enzyme preparation.[15] This suggests that our enzyme preparation differs from the above-mentioned brain proteinase.

Very recently, Camargo and Graeff[16] reported on the properties of the enzyme inactivating bradykinin in rabbit brain homogenate. They found that 1 mM concentration of *p*-hydroxymercury benzoate completely inhibited the kininolytic activity and partial inhibition was observed in the calcium ion, whereas EDTA was ineffective. These different results might be due to the difference in the experimental animal used and degree of the purity in the enzyme preparation.

Zinc and cobalt ions activate the bradykinin destroying activity of human plasma fraction IV-1,[17] guinea-pig serum[18] and rat plasma,[6] whereas they inhibit the kininase from rat brain.

DFP and the specific chymotrypsin inhibitor, TPCK had no effect on kininase activity. This shows that histidine and the hydroxyl group of serine are probably not involved in the active center of this enzyme.[19-23] Ovomucoid trypsin inhibitor (0·1 mg/ml) which inhibits trypsin (0·09 mg/ml) completely,[15] had no effect on the enzyme, whereas trasylol with the same inhibitory activity[15] for trypsin as ovomucoid trypsin inhibitor (0·1 mg/ml), was inhibitory at a concentration of 180 KIE/ml. This implies that the present enzyme has a different proteolytic activity from trypsin.

In 1967, Yang and Erdös[24] reported two different kininases in human plasma. Kininase I cleaves hippuryl-L-lysine and inactivates bradykinin by breaking the carboxy terminal phe^8–arg^9 bond of the peptide, whereas kininase II does not hydrolyse hippuryl-L-lysine and cleaves the pro^7–phe^8 bond in bradykinin. In our work, free arginine and phenylalanine were released from bradykinin by the partially purified enzyme preparation, but no hydrolytic activity was observed with hippuryl-L-lysine.[15] Greenbaum and Yamafuji[25] reported that free arginine and phenylalanine are released from bradykinin by the catheptic preparation of beef spleen. However, their experimental system required cysteine and an acid pH value. These facts definitely differentiate their enzyme from ours.

The free arginine released from bradykinin might be the carboxy terminal arginine since the enzyme had no aminopeptidase activity,[15] and since phenylalanine was also released from bradykinin. In our experiment, no single spot of heptapeptide was found originating from bradykinin. However, it is likely that a heptapeptide would migrate with bradykinin in the present experimental conditions.

It has been reported that some drugs block the action of bradykinin *in vivo* and *in vitro*: Acetylsalicylic acid and other antipyretic drugs counteract the apnea or tachypnea[26, 27] and bronchoconstriction[28] caused by bradykinin. Visceral pain and formation of edema elicited by bradykinin, are also inhibited by narcotic analgesics[29] and some antiphlogistic agents,[30] respectively. The possibility that these drugs exert their effects by altering the rate of enzymatic destruction of bradykinin was investigated. In the present experiment, acetylsalicylic acid and aminopyrine showed the tendency to potentiate the enzymatic inactivation of bradykinin. Enzymatic activity was assayed on an isolated smooth muscle preparation, so it is unknown whether these drugs exert their effect directly on the enzyme. The present results are interesting in connection with the fact that intraventricular administration of synthetic bradykinin

causes extreme excitation followed by a period of depression and this excitation is specifically blocked by acetylsalicylic acid and aminopyrine.[31, 32] The mechanisms of activation and inhibition of the activity of this enzyme require further investigation.

The significance of kininase in the central nervous system should be better understood when the action of bradykinin on neural tissue and the effect of drugs on kininase are clarified.

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