Biochemical Pharmacology, Vol. 35, No. 24, pp. 4499–4503, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

HYDROLYSIS OF NEO-KYOTORPHIN (Thr-Ser-Lys-Tyr-Arg) AND [Met]ENKEPHALIN-Arg^6-Phe^7 BY ANGIOTENSIN-CONVERTING ENZYME FROM MONKEY BRAIN

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(*Received* 22 *January* 1986; *accepted* 22 *May* 1986)

Abstract—Angiotensin-converting enzyme (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1) from monkey brain was partially purified 274-fold with 4.5% yield. The optimum pH of the enzyme was 8.2, and its K_m was 3.3 mM, with hippuryl-His-Leu as the substrate in 300 mM NaCl. Its molecular weight (M_r) was estimated to be approximately 260,000 by gel filtration on Sephadex G-200. On high-performance liquid chromatographic analysis, ACE hydrolyzed neo-kyotorphin (Thr-Ser-Lys-Tyr-Arg) with liberation of kyotorphin (Tyr-Arg), the [Met]enkephalin releasor. ACE also converted [Met]-enkephalin-Arg^6-Phe^7 to [Met]enkephalin; then the enzyme slowly hydrolyzed the resulting [Met]enkephalin. The K_m values of the enzyme for neo-kyotorphin and [Met]enkephalin-Arg^6-Phe^7 were 0.58 and 0.30 mM respectively. Thus, brain ACE may have a role in the formation of kyotorphin and [Met]enkephalin from their precursors but has little part in [Met]enkephalin degrading processes.

The analgesic dipeptide kyotorphin (Tyr-Arg) [1] is highly concentrated in the synaptosomal fraction of brain [2], and it releases [Met]enkephalin from brain and spinal cord by its selective depolarizing effect on enkephalinergic neurons [3]. The presence of "kyotorphin-containing neurons" suggests that kyotorphin may act as a neurotransmitter/neuromodulator. A novel analgesic pentapeptide, neo-kyotorphin (Thr-Ser-Lys-Tyr-Arg), has been isolated from bovine brain [4, 5]; it contains the kyotorphin sequence at its C-terminus. In a preliminary study, kyotorphin, Lys-Tyr-Arg, and Ser-Lys-Tyr-Arg showed no effect on monkey brain angiotensin-converting enzyme (ACE‡; dipeptidyl carboxypeptidase, EC 3.4.15.1), but neo-kyotorphin inhibited the ACE activity (IC_{50} 200 μM) [6]. The distinct ACE inhibition of neo-kyotorphin was suggested to correlate with its analgesia-inducing property.

[Met]enkephalin-Arg^6-Phe^7 contains the [Met]enkephalin sequence with the additional amino acid residues Arg-Phe [7, 8]. The heptapeptide is considered to be the precursor of [Met]enkephalin and/or has its own role as a neuropeptide. High amounts of [Met]enkephalin and [Met]enkephalin-Arg^6-Phe^7 are observed in the striatum [9] where there is high activity of ACE [10]. Since ACE hydrolyzes various peptides by releasing the C-terminal dipeptide, the enzyme was expected to have the ability to produce kyotorphin and [Met]enkephalin from their precursors.

In this report, we describe the purification of ACE from monkey brain and its catalytic properties with respect to neo-kyotorphin and [Met]enkephalin-Arg^6-Phe^7.

MATERIALS AND METHODS

Materials. [Met]- and [Leu]enkephalin, bradykinin potentiator B, and hippuryl-His-Leu were from the Protein Research Foundation (Osaka); [Met]enkephalin-Arg^6-Phe^7 was purchased from Bachem Feinchemikauden AG. Synthesized kyotorphin and neo-kyotorphin were supplied by Dr. K. Kitagawa, Tokushima University. Captopril was supplied by the Sankyo Co. (Tokyo). Phosphoramidon was supplied by Dr. H. Umezawa, Institute of Microbial Chemistry (Tokyo). All other reagents were of the highest quality available from commercial sources.

Enzyme assay. The ACE activity was assaying, measuring the amount of the liberated hippuric acid from hippuryl-His-Leu by the modified method of Hayakari *et al.* [11]. The reaction was initiated by addition of the enzyme and carried out at 37° for 30 min in 50 mM Tris–HCl buffer, pH 8.2, containing 300 mM NaCl and 5.6 mM hippuryl-His-Leu, in a total volume of 250 μl. The reaction was terminated by the addition of 15 μl of 1 N NaOH; after 30 min at room temperature, 1 ml each of 60 mM sodium phosphate buffer, pH 7.2, and 1% cyanuric chloride in ethylene glycol monomethyl ether (w/v) was added to the reaction mixture. After 15 min, the

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‡ Abbreviations: ACE, angiotensin-converting enzyme (EC 3.4.15.1); captopril, D-3-methylpropanoyl-L-proline; thiorphan, *N*-(DL-2-benzyl-3-mercaptopropionyl)glycine; and bradykinin potentiator B, Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro.

absorbance at 382 nm was determined. One milliunit (mU) of ACE activity was defined as the amount of enzyme that liberated 1 nmole of hippuric acid per min under the assay conditions.

Inhibitory effects of various compounds were studied using 0.85 mU of enzyme activity with the assay conditions described above.

HPLC analysis of reaction products. Incubation was carried out at 37° for specific periods in 50 mM Tris–HCl buffer, pH 8.2, containing 40 μg each of [Met]enkephalin (70 nmoles), [Met]enkephalin-Arg^6-Phe^7 (42 nmoles), or neo-kyotorphin (61 nmoles), with ACE preparation (0.85 mU), in a total volume of 100 μl. Each 20 μl of reaction mixture was added to 80 μl of column development solvent, as described below. Product analysis was performed by reverse-phase HPLC using a μBondapak C-18 column (0.39 × 30 cm). The column was equilibrated with acetonitrile–0.1% acetic acid containing 5 mM 1-pentanesulfonate 13:87 (v/v) for the product analysis from neo-kyotorphin, and 25:75 (v/v) for that from [Met]enkephalin or [Met]enkephalin-Arg^6-Phe^7 respectively. After application of the sample of hydrolyzed neo-kyotorphin (5 μl), [Met]enkephalin (10 μl), or [Met]enkephalin-Arg^6-Phe^7 (20 μl), the peptides were eluted isocratically at a flow rate of 1 ml/min at room temperature. The elution profiles from the product analysis of [Met]enkephalin-Arg^6-Phe^7 was reported previously [12]. The retention times of the peptides were neo-kyotorphin (11.0 min), kyotorphin (6.7 min), [Met]enkephalin-Arg^6-Phe^7 (18.1 min), [Met]enkephalin (7.2 min), and Tyr-Gly-Gly (3.5 min), determined with standard materials. The absorbance at 280 nm was monitored with a model 440 detector (Waters) at a setting of 0.005 full scale.

The K_m values of ACE for neo-kyotorphin and [Met]enkephalin-Arg^6-Phe^7 were determined by Lineweaver–Burk analysis, measuring the amount of produced kyotorphin or [Met]enkephalin. The reaction of neo-kyotorphin hydrolysis by ACE (0.85 mU) was carried out for 60 min, and [Met]enkephalin-Arg^6-Phe^7 hydrolysis by ACE (0.43 mU) was performed for 15 min.

Purification of ACE. All operations were performed at 4°. Monkey brain (*Macaca fascicularis*), without cerebellum, was stored at −80° until use. After thawing at 4°, a 50-g sample of monkey brain was homogenized with 5 vol. of 25 mM Tris–HCl buffer, pH 7.0 (buffer A), containing 0.25 M sucrose, followed by centrifugation at 1000 *g* for 10 min, and then the pellet was washed again. The combined supernatant fraction was centrifuged at 16,000 *g* for 30 min, and then the pellet was suspended in 100 ml of buffer A containing 1% Triton X-100 and stirred for 60 min. After centrifugation at 100,000 *g* for 60 min, the supernatant fraction was applied to a column (4.5 × 15.7 cm) of DE-52 cellulose (Whatman) that had been equilibrated with buffer A containing 0.1% Triton X-100. After washing the column with 550 ml of equilibration buffer, ACE was eluted with 100 mM NaCl in buffer A. The active fraction was dialyzed against 1 M ammonium sulfate in buffer A, and then applied to a column (3.3 × 12.5 cm) of phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals) equilibrated with the dialysis solution. The column was washed with 300 ml of the same buffer, and elution was performed at a flow rate of 100 ml/hr with 280 ml each of 0.5 M, then 0.25 M ammonium sulfate in buffer A. The ACE activity was found in the fraction of 0.25 M ammonium sulfate, and the active fraction was dialyzed against 1 mM potassium phosphate buffer, pH 7.0. The dialyzed solution was applied to a column (2.1 × 16 cm) of hydroxyapatite (Seikagaku Kogyo Co.) equilibrated with the dialysis buffer. After washing with 60 ml of the same buffer, the column was developed with a 600-ml linear gradient of 1–50 mM potassium phosphate buffer, pH 7.0, at a flow rate of 30 ml/hr. The active fraction was applied to a column (1.2 × 4.4 cm) of blue-Sepharose CL-6B (Pharmacia Fine Chemicals) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The ACE activity appeared in the breakthrough fraction, and then the active fraction was dialyzed against 1 mM potassium phosphate buffer, pH 7.0. The dialyzed solution was applied to a column (1.2 × 8.8 cm) of hydroxyapatite equilibrated with the dialysis buffer. After washing with 20 ml of the same buffer, the column was developed with a 100-ml linear gradient of 1–30 mM potassium phosphate buffer, pH 7.0, at a flow rate of 10 ml/min. The obtained ACE preparation did not possess enkephalin-degrading aminopeptidase or dipeptidyl aminopeptidase activities on the HPLC analysis with [Met]enkephalin as the substrate.

The protein concentration was determined by the method of Lowry *et al.* [13], with bovine serum albumin as the standard.

Determination of molecular weight. The molecular weight of the obtained ACE was estimated by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals) in 50 mM Tris–HCl buffer, pH 8.2. Ferritin (M_r 440,000), catalase (M_r 232,000), aldolase (M_r 158,000), and bovine serum albumin (M_r 67,000) were used as calibration standards.

RESULTS

By the procedures shown in Table 1, ACE was partially purified from monkey brain 274-fold with 4.5% yield. The molecular weight (M_r) of ACE was estimated to be approximately 260,000 by gel filtration on Sephadex G-200. The pH optimum of the enzyme for hippuryl-His-Leu was in the basic range (pH 8.2) and was determined in 50 mM Tris–HCl buffer containing 300 mM NaCl. The Michaelis constant (K_m) of the enzyme for hippuryl-His-Leu was 3.3 mM.

The effects of various compounds on the ACE activity are presented in Table 2. The enzyme activity was inhibited by the compounds that had been reported to be specific for ACE, i.e. captopril and bradykinin potentiator B. The enzyme was also inhibited by dithiothreitol and the metal chelators, EDTA or *o*-phenanthroline. But phosphoramidon and thiorphan, the specific inhibitors of "enkephalinase A" which hydrolyzes the Gly^3-Phe^4 bond of enkephalin, were not effective. Thus, the inhibitory effects of various compounds indicated that the enzyme obtained from monkey brain had the typical features of ACE.

Table 1. Purification of angiotensin-converting enzyme from monkey brain

Step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification rate (fold)
Triton X-100 extract	4298	5588	1.3	100	1.0
DEAE cellulose	642	2999	4.7	54	3.6
Phenyl-Sepharose	59	1590	27	28	21
Hydroxyapatite	6.3	788	125	14	96
Blue-Sepharose	2.7	588	218	11	168
Hydroxyapatite	0.7	249	356	4.5	274

Table 2. Inhibitory effects of various compounds on angiotensin-converting enzyme from monkey brain

Compound	Concentration (M)	Inhibition (%)
Captopril	1×10^{-7}	98
Bradykinin potentiator B	1×10^{-5}	93
Phosphoramidon	1×10^{-5}	31
Thiorphan	1×10^{-4}	23
EDTA	1×10^{-3}	98
o-Phenanthroline	1×10^{-3}	99
Dithiothreitol	1×10^{-3}	95

The effective ACE inhibition of neo-kyotorphin (IC_{50} 200 μM) suggested that the peptide may behave as a substrate for brain ACE. HPLC analysis directly indicated that neo-kyotorphin was hydrolyzed by ACE with the formation of free kyotorphin (Fig. 1). The K_m value of ACE for neo-kyotorphin was 0.58 mM.

Although [Met]- and [Leu]enkephalin (500 μM) were not effective inhibitors of ACE (5 and 18% inhibition, respectively), [Met]enkephalin-Arg^6-Phe^7 (IC_{50} 309 μM) inhibited brain ACE. To identify the products of ACE on [Met]enkephalin-Arg^6-

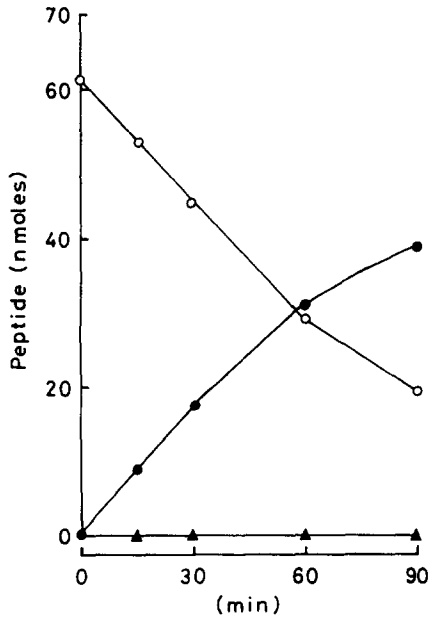

Fig. 1. Hydrolysis of neo-kyotorphin (61 nmoles) by angiotensin-converting enzyme. Key: Neo-kyotorphin (○), and kyotorphin in the presence (▲) and absence (●) of 1 μM captopril.

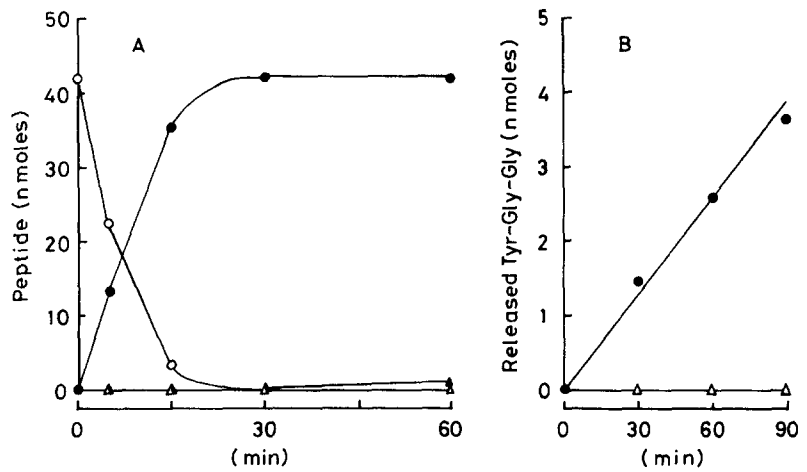

Fig. 2. (A) Hydrolysis of [Met]enkephalin-Arg^6-Phe^7 (42 nmoles) by angiotensin-converting enzyme. Key: (○) [Met]enkephalin-Arg^6-Phe^7; (●) [Met]enkephalin; (▲) Tyr-Gly-Gly; and (△) [Met]enkephalin or Tyr-Gly-Gly in 1 μM captopril. (B) Released Tyr-Gly-Gly from [Met]enkephalin (70 nmoles) with angiotensin-converting enzyme in the presence (△) and absence (●) of 1 μM captopril.

Phe^7, HPLC analysis was performed (Fig. 2A). [Met]enkephalin-Arg^6-Phe^7 was hydrolyzed to [Met]enkephalin by ACE within 30 min; then the further cleavage of [Met]enkephalin by ACE with release of Tyr-Gly-Gly progressed slowly. The same result was observed using [Met]enkephalin as the substrate (Fig. 2B). The apparent K_m value of ACE for [Met]enkephalin-Arg^6-Phe^7 was determined to be 0.30 mM by measuring the amount of produced [Met]enkephalin. In the enkephalin hydrolyzing analysis, neither tyrosine or Tyr-Gly, the products of enkephalin-degrading aminopeptidase [14] or dipeptidyl aminopeptidase [15], was observed. Captopril (1 μM) completely inhibited the hydrolysis of neo-kyotorphin, [Met]enkephalin, and [Met]enkephalin-Arg^6-Phe^7 by ACE.

DISCUSSION

In this study, ACE was partially purified 274-fold from monkey brain. The enzyme showed the typical features of ACE, since the enzyme was inhibited almost completely by 100 nM captopril and 10 μM bradykinin potentiator B, potent inhibitors of ACE. Weak inhibition was observed by 10 μM phosphoramidon and 100 μM thiorphan, specific inhibitors of "enkephalinase A", the presumed enkephalin-degrading dipeptidyl carboxypeptidase [16]. The reported IC_{50} values for phosphoramidon [17] and thiorphan [18] are 40 and 4 nM respectively. Recently, the resemblance of "enkephalinase A" to the neutral metalloendopeptidase has been reported [19]. The effective inhibition of captopril indicated that the enzyme obtained was also distinguished from brain cytosol peptidyl dipeptidase [20]. By HPLC analysis, the brain ACE preparation did not contain any enkephalin-degrading aminopeptidase or dipeptidyl aminopeptidase activities.

Brain ACE hydrolyzed neo-kyotorphin, producing free kyotorphin. It has been reported that neo-kyotorphin-induced analgesia is partially antagonized by naloxone pretreamtent (0.5 mg/kg mouse) [21]. The kyotorphin formation by ACE may have a part in the mechanism of neo-kyotorphin-induced analgesia. In contrast to the distribution of ACE activity, kyotorphin was observed in brain cortex at an 8-fold higher concentration than in the striatum [22]. Since brain aminopeptidase cleaves kyotorphin [23], and its highest activity exists in the striatum [16], the kyotorphin produced may be rapidly degraded by the dominant aminopeptidase activity, resulting in the relatively low concentration of kyotorphin in the striatum.

Without the influence of enkephalin-degrading enzymes, our results indicate that brain ACE converts [Met]enkephalin-Arg^6-Phe^7 to [Met]-enkephalin but has little part in the degradation processes of [Met]enkephalin. In contrast to the action of brain ACE, serum dipeptidyl carboxypeptidase shows rapid degradation of [Met]enkephalin [12]. At the present time, the cause of the different catalytic mode of those dipeptidyl carboxypeptidases is not clear. Previously, it was reported that the incubation of [Met]enkephalin-Arg^6-Phe^7 with striatal synaptic membranes produces [Met]enkephalin, and ACE was suggested as the first enzyme in the striatum that hydrolyzes the heptapeptide [24]. However, the co-existence of enkephalin-degrading aminopeptidase and "enkephalinase A" activities prevented the detailed analysis of the ACE action. Norman *et al.* [25] reported that the administration of a subanalgesic dose of an ACE inhibitor, either MK-422 or captopril, potentiates the analgesic response of a subanalgesic dose of [Met]enkephalin-Arg^6-Phe^7 *in vivo* [25]. Our results support their observations.

Brain ACE may prefer the C-terminal dipeptide portion of neo-kyotorphin and [Met]enkephalin-Arg^6-Phe^7 to those of other enkephalins. The K_m values of [Met]enkephalin-Arg^6-Phe^7 and neo-kyotorphin were 0.30 and 0.58 mM respectively. [Met]enkephalin-Arg^6-Phe^7 may possess more affinity for ACE than neo-kyotorphin. Since both [Met]enkephalin and its releaser kyotorphin are unlikely to be in one neuron, the physiological function of ACE will be decided by the demonstration of co-localization of these peptides and the enzyme in the same neurons. Thus, the enzyme has the potential to play a role in the enkephalinergic and kyotorphinergic systems.

Acknowledgements—We wish to thank Dr. T. Fujiwara, the National Institute of Health (Japan), for supplying monkey brain.

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