An Enkephalin-Degrading Aminopeptidase from Rat Brain Catalyzes the Hydrolysis of a Neuropeptide, Kyotorphin (L-Tyr-L-Arg)

Kenji AKASAKI* and Hiroshi TSUJI

Faculty of Pharmacy & Pharmaceutical Sciences, Fukuyama University, Higashimura-cho, Fukuyama, Hiroshima 729-02, Japan.

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We studied the hydrolysis of a neuropeptide kyotorphin (L-Tyr-L-Arg) by an enkephalin-degrading aminopeptidase purified from cytosol of rat brain *in vitro*. The purified enzyme was homogeneous as judged by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), gel filtration and isoelectric focusing. The aminopeptidase with an apparent molecular weight (M_r) = 98000 catalyzed the hydrolysis of Leu- and Met-enkephalins with K_m values of 125 and 142 μ M, respectively. The enzyme activity was inhibited by bestatin, amastatin and puromycin but not by pepstatin, leupeptin and phenylmethanesulfonyl fluoride (PMSF). Kyotorphin was degraded by the aminopeptidase at pH 7.0, and the V_{max} and K_m values were 9.2 μ mol/min/mg protein and 95 μ M, respectively. The K_m value for kyotorphin was compatible to those for Leu- and Met-enkephalins. Taken together, these results suggest a possible involvement of the enkephalin-degrading aminopeptidase in cytosolic degradation of kyotorphin in neuronal cells of rat brain.

Keywords aminopeptidase; kyotorphin (Tyr-Arg); enkephalin; bestatin; rat brain

Introduction

Kyotorphin is a neuropeptide isolated from bovine brain by Takagi *et al.*¹⁾ This dipeptide is considered to produce analgesia *via* a release of enkephalin.^{1,2)} In cytosol of synaptosomes (synaptosol), kyotorphin is formed from free amino acids and/or precursor proteins by specific enzymes.^{3,4)} This peptide is rapidly degraded in the homogenate and synaptosol of rat brain.^{3,5)} The degradation is inhibited by bestatin, a inhibitor for cytosolic aminopeptidases.⁶⁾ Little is known, however, about the kyotorphin-degrading enzyme(s) in rat brain.

A major aminopeptidase was purified from the soluble fraction of rat brain as an enkephalin-hydrolyzing enzyme.^{7,8)} The enzyme has M_r = about 100000, and exhibits a broad specificity for arylamidase substrates such as Arg- and Ala-naphthylamides. It has further been shown that the aminopeptidase activity is effectively inhibited by bestatin and analogues of bestatin.⁹⁾ Such an aminopeptidase reportedly exists in monkey, bovine and human brains.^{10–12)} Taken together, these results raised the possibility that the kyotorphin degradation in the synaptosol can be ascribed to the enkephalin-degrading aminopeptidase. However, whether or not this aminopeptidase actually catalyzes the hydrolysis of kyotorphin has not been determined as yet.

In the present study, we purified the enkephalin-degrading aminopeptidase from rat brain to an apparent homogeneity and investigated its action on kyotorphin.

Materials and Methods

Materials Kyotorphin, Tyr-4-methyl-7-coumaryl-7-amide (Tyr-MCA), Ala-MCA, and Phe-MCA were obtained from Sigma (St. Louis, MO). Arg-MCA, Leu-MCA, Bz-Arg-Phe-MCA, Gly-Pro-MCA, Met- and Leu-enkephalins, bradykinin, substance P, bestatin, amastatin, leupeptin, pepstatin, actinonin and alphamemine B were purchased from Peptide Institute, Inc. (Osaka, Japan). Diethylaminoethyl (DEAE)-Sephacel, Sephadex G-150 and ampholine (pH 3.5–9.0) were from Pharmacia & LKB (Uppsala, Sweden). TSK gel G3000 SW, TSK gel DEAE-5PW and TSK gel ODS 80 TM were obtained from Tosoh Co. (Tokyo, Japan). Other chemicals of reagent grade were from various commercial sources.

Enzyme Purification The enkephalin-degrading aminopeptidase was purified from crude extracts of rat brain by a modified method of Wagner *et al.*⁸⁾

Assay of Aminopeptidase Activity Enzyme (0.1 ml) was incubated for 10 min at 37°C in a medium (total volume of 1 ml) containing 50 mM

Tris-HCl buffer (pH 7.0), 2 mM 2-mercaptoethanol, and 0.1 mM Arg-MCA. Five mM 1,10-phenanthroline (2 ml) was added to terminate the reaction. The fluorescence of the liberated 7-amino-4-methylcoumarin was measured (λ_{ex} 380 nm; λ_{em} 450 nm) with reference to a fluorescence standard. The enzyme activity against other aminoacyl MCA derivatives was determined in the same way.

Analysis of Kyotorphin and Physiologically Active Peptides Degradation by Reversed-Phase High Performance Liquid Chromatography (HPLC) The purified enzyme was incubated with 100 μ M kyotorphin at 37°C for 10 min in a medium (total volume of 1.0 ml) containing 50 mM Tris-HCl (pH 7.0) and 2 mM 2-mercaptoethanol. Immediately after the reaction was terminated by cooling to 0°C, the mixture was applied to reversed-phase HPLC using a TSK gel ODS 80 TM column (7.5 $\times$ 0.8 cm). In determination of the kyotorphin degradation, the incubation mixture was subjected to the column equilibrated with 0.1 M KH_2PO_4 . The column was eluted with the equilibration buffer at a flow rate of 1.0 ml/min. Tyrosine and kyotorphin were monitored by an ultraviolet spectrophotometric detector (Tosoh model UV 8000) at 280 nm. For the other peptides, the column was equilibrated with 0.05% trifluoroacetic acid containing 20% acetonitrile. After application of the reaction mixture, elution was carried out with a linear gradient with acetonitrile concentrations from 20% to 80% at a flow rate of 1.0 ml/min.

Gel Electrophoresis Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 12% acrylamide gel according to the method of Laemmli.¹³⁾

Protein Determination Protein concentrations were determined by the method of Lowry *et al.*¹⁴⁾ using bovine serum albumin as a standard.

Results and Discussion

Enzyme Homogeneity The aminopeptidase was purified 820-fold from the soluble fraction of rat brain. On SDS–PAGE (Fig. 1) the purified enzyme preparation gave only a single protein band with an apparent molecular weight (M_r) of 98000. When the purified enzyme was applied to size exclusion HPLC on a column of TSK gel G 3000 SW, the enzyme protein was eluted in a position corresponding to M_r = 109000 (data not shown). In isoelectric focusing, the enzyme showed a single peak of the enzyme activity at pH 5.0 (data not shown). By these criteria, the aminopeptidase was judged to be homogeneous. The M_r values of our enzyme preparation by SDS–PAGE and gel filtration are very similar to those of the aminopeptidase reported by Wagner *et al.*⁸⁾

Effects of Inhibitors Effects of various protease inhibitors on the enzyme activity were tested, and the results obtained are summarized in Table I. Puromycin and

Fig. 1. SDS-PAGE of an Enkephalin-Degrading Aminopeptidase Purified from Rat Brain

Aminopeptidase purified from rat brain was analyzed by SDS-PAGE. Gel containing 2 μ g of the purified enzyme was obtained after Coomassie blue staining. M_r values of marker proteins are shown to the left of the gel: phosphorylase b (M_r =94000); bovine serum albumin (68000); ovalbumin (45000); and carbonic anhydrase (30000).

TABLE I. Effect of Protease Inhibitors on the Aminopeptidase

Inhibitor	Concentration (μ M)	Remaining activity (%)
No addition		100
Phenylmethanesulfonyl fluoride	1000	100
1,10-Phenanthroline	100	50
	500	0
Pepstatin	25	100
Leupeptin	15	100
Amastatin	0.48	50
Bestatin	0.78	50
Puromycin	2.0	50
Actinonin	0.12	50
Arphamenine B	6.9	50

1,10-phenanthroline inhibited the enzyme activity but inhibitors for serine, thiol, and aspartic proteases had no effect on the aminopeptidase activity, indicating that this enzyme falls into the category of metalloproteases. As expected, bestatin was a potential inhibitor for this enzyme; aside from this antibiotic, actinonin, amastatin and arphamenine B strongly inhibited the enzyme activity.

Hydrolysis of Kyotorphin by the Enkephalin-Degrading Aminopeptidase The purified enzyme exhibited the greatest hydrolyzing activity for Arg-MCA at pH 7.0 in the presence of 2-mercaptoethanol. Substrate specificity of the aminopeptidase against various aminoacyl MCA derivatives and biologically active peptides is cited in Tables II and III. The enzyme hydrolyzed Leu-MCA with the highest rate among the amino acid MCA derivatives tested, while it did not catalyze the hydrolysis of Gly-Pro-MCA and Bz-Arg-Phe-MCA at all. The aminopeptidase liberated the N-terminal Tyr from Met- and Leu-enkephalin with respective K_m values of 142 and 125 μ M (Table III); it also hydrolyzed delta sleep inducing peptide (DSIP). Substance P and bradykinin were resistant to hydrolysis by this ami-

TABLE II. Activity of the Aminopeptidase on MCA Derivatives

Substrate	K_m (μ M)	V_{max} (μ mol/min/mg protein)	V_{max}/K_m
Arg-MCA	6.4	8.6	1.35
Leu-MCA	7.1	40.1	5.16
Phe-MCA	25.6	17.0	0.66
Ala-MCA	15.4	4.3	0.28
Tyr-MCA	7.7	5.2	0.67
Bz-Arg-Phe-MCA		Not reactive	
Gly-Pro-MCA		Not reactive	

TABLE III. Activity of the Aminopeptidase on Biologically Active Peptides

Peptide	Amino terminus	K_m (μ M)	V_{max} (μ mol/min/mg protein)
Met-enkephalin	Tyr-Gly	142	26.8
Leu-enkephalin	Tyr-Gly	125	10.8
Kyotorphin	Tyr-L-Arg	95	9.2
Kyotorphin analogue	Tyr-D-Arg	Not reactive	
DSIP	Trp-Ala	10	2.5
Substance P	Arg-Pro	Not reactive	
Bradykinin	Arg-Pro	Not reactive

Fig. 2. Time Course of the Hydrolysis of Kyotorphin by the Enkephalin-Degrading Aminopeptidase

A reaction mixture (total volume of 1.0 ml) contained 50 mM Tris-HCl (pH 7.0), 2 mM 2-mercaptoethanol, 100 nmol kyotorphin and 500 ng of the purified enzyme. The mixture was incubated at 37°C for the time indicated. After the reaction was terminated by cooling to 0°C, amounts of tyrosine liberated were measured by reversed phase HPLC.

nopeptidase.

We next investigated whether or not the enkephalin aminopeptidase actually catalyzes degradation of kyotorphin *in vitro*. As shown in Fig. 2, kyotorphin was degraded by the purified enzyme in a linear reaction up to 15 min. At this time, about 85% of the initial substrates added were hydrolyzed by this enzyme. The degradation rate was dependent on the enzyme amounts and the concentration of kyotorphin. The apparent K_m and V_{max} values for kyotorphin were 95 μ M and 9.2 μ mol/min/mg protein (Table III). These values were compatible with those for Leu-enkephalin.

The enkephalin-degrading aminopeptidase showed a relatively broad specificity for arylamidase substrates. Arg-MCA is one of the favorable substrates for this enzyme; however, it does not exhibit the hydrolyzing activity for substance P and bradykinin in spite of the N-terminal Arg.

In addition, once D-Arg was substituted for L-Arg of kyotorphin (Table III), the enzyme did not demonstrate any hydrolyzing activity for the peptide. From these results, it is considered that the peptide conformation rather than the N-terminal amino acid residue is critical for the action of this enzyme. The enkephalin-degrading aminopeptidase has been shown to be widely distributed in rat tissues.¹⁵⁾ Such an enzyme has reportedly been purified from human skeletal muscles.^{16,17)} Together with the broad substrate specificity of this enzyme, these facts may reflect a central role of the aminopeptidase in degradation of di- or tripeptides presented at the final stage of general protein breakdown, as well as its possible function of controlling the kyotorphin level.

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