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SEPARATION AND CHARACTERIZATION OF A DIPEPTIDYL AMINOPEPTIDASE THAT DEGRADES ENKEPHALINS FROM MONKEY BRAIN

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SUMMARY: A dipeptidyl aminopeptidase (a membrane-bound enzyme) which cleaved Met-enkephalin and released dipeptide (Tyr-Gly) was partially purified from monkey brain. A fraction containing both exoaminopeptidase and dipeptidyl aminopeptidase activity was obtained from DE-52 cellulose column chromatography. The dipeptidyl aminopeptidase activity in this fraction was not inhibited by addition of bestatin (300 μg/ml), while the exoaminopeptidase was strongly inhibited. Both enzymes were separated by AH-Sepharose 4B column chromatography. The molecular weight of the dipeptidyl aminopeptidase was calculated about 110,000. The enzyme activity was inhibited by addition of diisopropylfluorophosphate (DFP) or o-phenanthroline.

The opioid pentapeptides Met-enkephalin and Leu-enkephalin are rapidly hydrolyzed following their release and action, possibly by a specific enzymatic system (1,2). Recent attention has been focused on enzymes such as aminopeptidase, angiotensin converting enzyme and on enkephalinases which hydrolyze both pentapeptide and which have been solubilized from rat (3-9), bovine (10) and monkey brain (11). The properties of some aminopeptidases have been reported (7-11). Membrane-bound enzymes of rat brain have been reported (6) to show both exoaminopeptidase and dipeptidyl aminopeptidase activities. We have succeeded in separating of the exoaminopeptidase and dipeptidyl aminopeptidase from monkey brain extracts, which contain both enzyme activities and we describe here some properties of the dipeptidyl aminopeptidase.

MATERIALS AND METHODS

Chemicals. ([3,5-^{3}H]) Met-enkephalin (45 Ci/mmol) was purchased from New-England Nuclear. Met-enkephalin, puromycin and bestatin (13) from Sigma Chemical Co.: Porapak Q from Water Associates, Milford, Mass. were used for these experiments.

All other reagents were of the best commercial grade.

Tissue preparation. The brain was removed from a monkey (Macaca fasci-

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cularis) which was killed by exsanguination under ketamine hydrochloride anaesthesia. The preparation of enzymes was carried out by a modification of the procedure of Hersh (7). 50 g of the brain after removing cerebellum and lower brain stem were homogenized with 4 volumes of 25 mM Tris-HCl buffer pH 7.7, and centrifuged at 50,000 x g for 15 min. The pellet was washed again and suspended in 100 ml of 25 mM Tris-HCl buffer pH 7.7 containing 1% Triton X-100 and stirred at 37°C for 60 min. After centrifugation at 100,000 x g for 60 min, the supernatant was applied to a DE-52 cellulose column (3 x 5 cm) which was preequilibrated with the same buffer with Triton X-100. After washing with 500 ml of 25 mM Tris-HCl buffer pH 7.7 containing 100 mM NaCl, stepwise elution was carried out with 125, 150 and 200 mM NaCl contained in above buffer, respectively.

Enkephalinase activity. The enzyme activity hydrolyzing Met-enkephalin was assayed for activity with [^{3}H]Met-enkephalin by a procedure similar to that of Vogel and Altstein (12). An aliquot of test sample was placed in a solution containing 10^5 cpm of [^{3}H]Met-enkephalin, 10 μm unlabeled Met-enkephalin, and 25 mM Tris-HCl buffer pH 7.0, in a total volume of 100 μl. After incubation for 1 h at 37°C, 20 μl of 30% acetic acid was added and then 100 μl of reaction mixture was placed on a column containing 80 mg of Porapak Q. The [^{3}H]Tyr or [^{3}H]Tyr-Gly was eluted with 1 ml of water from Porapak Q column. After the addition of Aquasol-2 Scintilation fluid, the amount of radioactivity was determined. The reaction products after enzyme assays were also analyzed by thin layer chromatography (silica gel plate in ethylacetate-isopropanol-acetic acid-water (40:40:1:19) or chloroform-methanol-acetic acid-water (45:30:6:9) to determine amino acid components. Strips was then cut into 1/2 cm sections and radioactivity determined.

RESULTS

The Met-enkephalin degradation activity was assayed on each fraction obtained by DE-52 cellulose column. The enzyme activity was found only in an eluate with 25 mM Tris-HCl buffer containing 150 mM NaCl (Fig. 1). This fraction also showed exoaminopeptidase activity with Tyr- or Arg-β-naphthylamide as substrate.

By thin layer chromatography of the reaction products, it was shown that both Tyr and Tyr-Gly were released from Met-enkephalin. However, by addition of bestatin (300 μg/ml), the Met-enkephalin degradation activity was only partly inhibited (about 30-40% inhibition), while Tyr- or Arg-β-naphthylamide degradation was completely inhibited. Only Tyr-Gly was released from Met-enkephalin after treatment with this enzyme fraction in the presence of bestatin. From these results, it is suggested that the fraction obtained from DE-52 cellulose column contains two different enzymes with Met-enkephalin degradation activity, an exoaminopeptidase inhibited by bestatin and a dipeptidyl aminopeptidase. These were separated as described below.

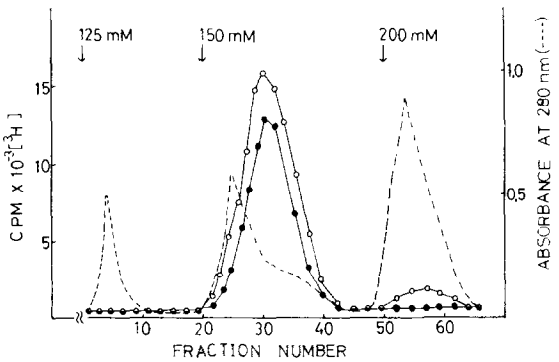

Fig. 1. Elution profile of membrane solubilized enzymes of monkey brain on DE-52 cellulose column chromatography. Fractions of 15 ml were collected and assayed for enzyme activity with Met-enkephalin as substrate in the presence (●) or absence (O) of 300 μg/ml of bestatin.

The fraction showing enzyme activity was applied to AH-Sepharose 4B (2 x 3.2 cm) equilibrated with 25 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl and gradient elution was performed with 150 to 400 mM of NaCl in Tris-HCl buffer. Two activities were separated (Fig. 2). The first peak showed dipeptidyl aminopeptidase activity and the second showed exoaminopeptidase activity.

For determination of the molecular weight of these enzymes, after the concentration by an Amicon ultrafiltration apparatus using a PM 10 membrane, the fractions showing enzyme activity were applied to Sephadex G-150 column

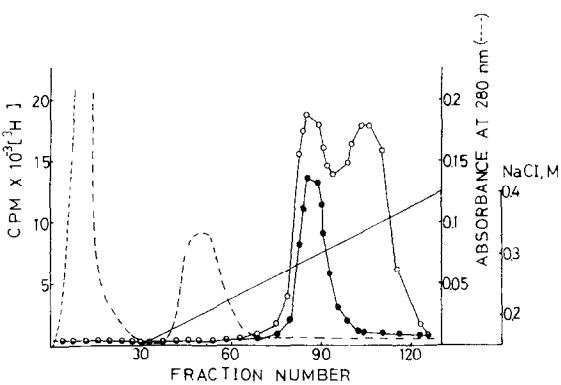

Fig. 2. Separation of dipeptidyl aminopeptidase and exoaminopeptidase by AH Sepharose 4B column chromatography. Fractions of 5 ml were collected and assayed for enzyme activity with Met-enkephalin as substrate in the presence (●) or absence (O) 300 μg/ml of bestatin.

(1.8 x 100 cm) equilibrated with 25 mM Tris-HCl buffer pH 7.0, and gel filtration was carried out with the same buffer. Two enzymes were eluted in the same fraction, and from this gel filtration pattern, the molecular weights were calculated nearly equal and about 110,000.

The detailed properties of the exoaminopeptidase are not reported here, but they appeared to be similar to those of the aminopeptidase which have reported previously (11). The activity of this enzyme was inhibited by bestatin (5 μg/ml) or by puromycin (100 μg/ml).

On the other hand, the dipeptidyl aminopeptidase was not inhibited by bestatin or puromycin (Table 1). Inhibitors of thiol, serine and carboxyl proteases, such as leupeptin, pepstatin, antipain and chymostatin did not inhibit the activity of the dipeptidyl aminopeptidase. It was inhibited by the addition of metal chelators such as EDTA and o-phenanthroline as reported for exoaminopeptidase (8-11). DFP was inhibitory at low concentrations (0.01 mM).

The dipeptidyl aminopeptidase activity was inactivated by heating to 100°C for 5 min, and was unstable in acid solution below pH 5.0 or in basic solution above pH 9.0; it was stable between pH 6.0 and pH 8.0.

DISCUSSION

Some enzymes that degraded enkephalins have been recognized in the soluble and membrane-bound fractions of rat, bovine and monkey brains (1-11). After the enzymes were solubilized by Triton X-100 from rat brain, activity was shown in a fraction from DE-52 cellulose chromatography (5-7). Some investigators (6) have reported that the enzyme in this fraction was a dipeptidyl aminopeptidase, releasing Tyr-Gly from enkephalins. It has been partially purified from rat brain and reported to be a dipeptidyl amino-peptidase which showed both exo- and dipeptidyl aminopeptidase activities.

We have attempted the separation of the enzyme by DE-52 cellulose column chromatography from an extract of monkey brain. This procedure yielded a single peak that released both Tyr and Tyr-Gly from Met-enkephalin. However, on addition of bestatin the enzyme activity was partly inhibited

Table 1

EFFECT OF INHIBITORS ON THE EXO- AND DIPEPTIDYL AMINOPEPTIDASE ACTIVITY

Inhibitor	Final Conc. (μg/ml)	Inhibition (%) Exoaminopeptidase	Dipeptidyl aminopeptidase
Leupeptin	10	0	0
Antipain	10	0	0
Pepstatin	10	0	5
Chymostatin	10	0	12
Bestatin	5	61	0
	100	100	0
Puromycin	100	78	0
DFP[1)]	0.01 mM	67.5	90
O-Phenanthroline	1 mM	97	92
EDTA	20 mM	72	58
Iodacetic acid	1 mM	0	0

1) Diisopropyl fluorophosphate

and the exoaminopeptidase activity (release of Tyr) became negligible. Bestatin is a powerful inhibitor for aminopeptidase (13). This suggested that the fraction contains two enzymes, one an exoaminopeptidase, and the other a dipeptidyl aminopeptidase. Gorenstein and Snyder (6) have suggested that two enzymes (aminopeptidase and enkephalinase B) were present in a fraction obtained by a similar method, but these enzymes were not characterized.

The two enzymes were separated each other by AH-Sepharose chromatography and identified as an exoaminopeptidase and a dipeptidyl aminopeptidase.

The exoaminopeptidase was inhibited by bestatin and puromycin. On the other hand, the dipeptidyl aminopeptidase was not inhibited by bestatin, puromycin and other inhibitors of microbial origin (14), but it was inhibited by metal chelators. This dipeptidyl aminopeptidase appears to be a metalloenzyme and not a member of the thiol, serine or carboxyl protease groups.

It may be concluded that there are at least two identified membrane-bound enzymes in monkey brain which are active to enkephalins, one an

exoaminopeptidase and the other a dipeptidyl aminopeptidase. Utilization of specific enzyme inhibitors is helpful in distinguishing between unidentified enzymes.

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