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ISOLATION AND CHARACTERIZATION OF AN ENKEPHALIN-DEGRADING AMINOPEPTIDASE FROM RAT BRAIN

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Summary

An enkephalin-degrading aminopeptidase from rat brain extracts has been purified to apparent homogeneity. This enzyme cleaves the N-terminal tyrosine from Leu-enkephalin and hydrolyzes some β-naphthylamides and *p*-nitroanilides of neutral, basic and aromatic, but not acidic, amino acids. The enzyme requires a free amino group on the substrate and has a neutral pH optimum. After dialysis against EDTA, the enzyme requires a divalent cation (Zn^{2+}, $Co^{2+} > Mn^{2+} > Mg^{2+}$) for activity. The enzyme is inhibited by puromycin, *o*-phenanthroline, *p*-chloromercuribenzoate and EDTA, but not by phenylmethylsulfonyl fluoride or a specific peptide inhibitor of leucine aminopeptidase. The aminopeptidase consists of two subunits and has a molecular weight of about 100 000.

Introduction

Enkephalins (Tyr-Gly-Gly-Phe-Met or -Leu) are of great interest because of their opiate-like properties [1] and their probable role as neurotransmitters or neuromodulators [2—4]. It is well known that their physiological activity is very transitory due to their rapid degradation by serum and brain enzymes [5,6]. The principal inactivating enzymes are believed to be aminopeptidases [5—8]. Inhibition of the enkephalin-degrading enzymes should prolong enkephalin activity and may be expected to produce neuropharmacological

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effects in vivo. In a continuing search for such inhibitors [9], we now report the purification to apparent homogeneity and some properties of a rat brain aminopeptidase that hydrolyzes Leu-enkephalin and several aminoacyl-β-naphthylamides. Although a number of rat brain peptidases have been reported [10—15], the enzyme described here has not been isolated and characterized previously.

Materials and Methods

Substrates. The β-naphthylamides of L-leucine, L-ariginine, L-aspartic acid, and L-tyrosine were purchased from ICN (Cleveland, OH). The L-leucine-*p*-nitroanilide and *N*-benzoyl-L-tyrosine-*p*-nitroanilide were obtained from Sigma (St. Louis, MO). Dr. B. Riniker (Ciba-Geigy AG, Basel) kindly supplied L-tyrosine-*p*-nitroanilide. Leu-enkephalin (enkephalin, 5-L-leucine) was purchased from Calbiochem (La Jolla, CA) and radioactive Leu-enkephalin ([*tyrosyl*-3,5-^{3}H]enkephalin (5-L-leucine)) was a product of Amersham (Arlington Heights, IL).

Inhibitors. Phenylmethylsulfonyl fluoride, *o*-phenanthroline, and *p*-chloromercuribenzoic acid were purchased from Sigma. Puromycin was obtained from Nutritional Biochemicals (Cleveland, OH). The leucine aminopeptidase inhibitor, H-(Bu^t)-Thr-Phe-Pro-OH [16], was synthesized at Ciba-Geigy AG (Basel) and obtained from Dr. B. Riniker.

Other chemicals. Fast Garnet GBC salt and DL-dithiothreitol were purchased from Sigma, and enzyme grade $(NH_4)_2SO_4$ was obtained from Schwarz-Mann (Orangeburg, NY). Porapak Q (polystyrene beads, 100—120 mesh) was purchased from Waters Associates (Milford, MA). DEAE-cellulose was Whatman grade DE-52.

Enzyme assays. The enzyme activity was routinely measured by either of two methods: (a) colorimetric determination of the hydrolysis of L-Tyr-β-naphthylamide after converting the liberated naphthylamine to a red dye, or (b) determination of the cleavage of the Tyr-Gly bond in Leu-enkephalin radiolabeled in the tyrosine residue. The [^{3}H]tyrosine released was separated from unreacted enkephalin on small polystyrene columns.

In routine assays, using Tyr-β-naphthylamide as substrate, a 10 mM stock solution of the substrate in dimethylacetamide was diluted 50-fold into 0.1 M Tris-HCl buffer (pH 7.4, final substrate concentration, 0.2 mM); to 1 ml diluted substrate, 5 or 10 μl enzyme was added to initiate the reaction; after 5—30 min, the reaction was stopped by adding 0.5 ml freshly prepared solution of Fast Garnet GBC salt (1 mg/ml in 1 M sodium acetate (pH 4.2)/10% Tween 20). After 15 min, the resulting color was measured at 530 nm, using β-naphthylamine as standard. The reaction was linear both with time (for more than 60 min) and enzyme concentration. Although the presence of up to 5% dimethylacetamide in the assay mixture did not affect enzyme activity, the highest concentration used was 2%. Assays with other β-naphthylamide substrates were performed in an analogous manner.

Aminoacyl-*p*-nitroanilide substrates were similarly dissolved in dimethylacetamide and diluted with 0.1 M Tris-HCl (pH 7.4). The reaction was initiated by adding the enzyme to 1 ml of this mixture and terminated with 0.1 ml

glacial acetic acid. The nitroaniline released was reacted to form a purple azo dye by adding 25 μl each of 1.8% $NaNO_2$, 9% ammonium sulfamate, and 1.8% *N*-1-naphthylethylenediamine dihydrochloride and allowing 10 min for color development. Absorbance was measured at 550 nm; *p*-nitroaniline was the standard.

For assays with enkephalin as substrate, radioactive Leu-enkephalin was added to appropriate concentrations of non-radioactive enkephalin in 10 mM Tris-HCl (pH 7.4), to give a substrate solution containing approx. 50 000 cpm in 50 μl. Incubations were carried out in Eppendorf microtest tubes (1.5 ml capacity) in a final volume of 100 μl. The reaction mixture consisted of 10 μl enzyme, 40 μl 10 mM Tris-HCl (pH 7.4), and 50 μl substrate solution (final concentration, 0.1 mM). After incubation for 10 min, reactions were stopped by adding 20 μl of 30% acetic acid, and 100 μl of the reaction mixture were then applied to washed Porapak Q columns (100 mg/column) [9]. Tyrosine was collected into scintillation vials by eluting the columns with two 1.0-ml portions of 10 mM Tris-HCl (pH 7.4); unreacted Leu-enkephalin was then eluted with two 1.0-ml portions of 50% ethanol. Scintillation fluid (15 ml Aquasol, New England Nuclear, Boston, MA) was added and the samples were counted in a Beckman liquid scintillation counter.

The assays with β-naphthylamides and nitroanilides were performed at 37° C; reactions using enkephalin as substrate were run at 30° C.

Protein concentration. The absorbance at 280 nm was used to measure protein concentration. A sample of the purified enzyme was dialyzed against distilled water, lyophilized and weighed; from this, the value $A^{1\%}_{280,1cm} = 8.3$ was calculated.

Polyacrylamide gel electrophoresis. A modification of the method of Laemmli [17] was used. The system consisted of 9% separating gel (pH 6.8) and pH 8.3 electrode buffer. All solutions contained 0.1% sodium dodecyl sulfate (SDS). Bovine serum albumin, ovalbumin, and the heavy and light chains of human γ-globulin served as molecular weight markers.

Ultracentrifugation. The enzyme solution from the second DE-52 column was concentrated and dialyzed against a solution containing 0.1 M NaCl, 0.01 M phosphate buffer (pH 7), 1 mM dithiothreitol, and 0.01 mM $ZnCl_2$. The sample was centrifuged in a Model E Spinco ultracentrifuge at 60 000 rev./min at ambient temperature (24° C) and sedimentation coefficients were determined using a Nikon microcomparator.

Preparation of brain extract. Frozen or fesh rat brains were homogenized briefly in a Waring Blendor with 10 vols. cooled, deaerated 0.01 M Tris-HCl (pH 7.4)/1 mM dithiothreitol and then homogenized by hand using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 27 500 × *g* for 15—20 min to give a postmitochondrial supernatant.

Results and Discussion

Purification of rat brain aminopeptidase

An enzyme which hydrolyzes both enkephalin and some aminoacyl-arylamides was purified as summarized in Table I. The procedure involved $(NH_4)_2SO_4$ fractionation, chromatography on DEAE-cellulose (Fig. 1), molec-

TABLE I

PURIFICATION OF RAT BRAIN AMINOPEPTIDASE

Starting material: 52 rat brains (93 g wet weight). Total protein was calculated from A_{280}, using the relationship $A^{1\%}_{280} = 8.3$ determined for purified enzyme. For specific activity Tyr-β-NA (L-Tyr-β-naphthylamide) was used as substrate.

Step	Total protein (mg)	Total activity (nmol/min)		Specific activity (nmol/min per mg)
		Leu-enkephalin	Tyr-β-NA	
Postmitochondrial supernatant	13 076	153 800	67 500	5.2
40—70% $(NH_4)_2SO_4$	1 236	121 000	53 600	48
First DEAE-cellulose	38	45 500	41 100	1 081
Sephadex G-100	5.2	25 400	26 000	5 000
Second DEAe-cellulose	2.1	14 200	13 100	6 240
Aged enzyme *	2.1	22 900	21 500	10 240

* Same material as 'second DEAE-cellulose' but assayed 3—30 days after purification.

ular sieving on Sephadex G-100 (Fig. 2), and rechromatography on DEAE-cellulose (Fig. 3). The purification procedure was simple and resulted in a 2000-fold enrichment of enzyme activity over the soluble brain extract used as the starting material.

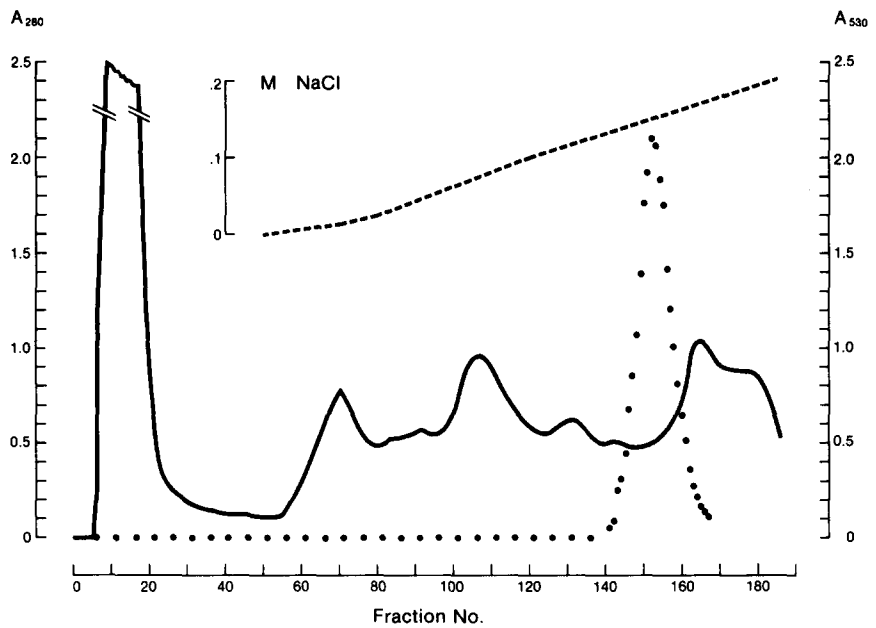

Fig. 1. Chromatography of brain aminopeptidase on DEAE-cellulose. The 40—70% $(NH_4)_2SO_4$ fraction of the postmitochondrial supernatant from 52 rat brains was dialyzed against 10 mM phosphate buffer (pH 7.0)/1 mM dithiothreitol, and 50 ml were applied to a Whatman DE-52 column (2.5 × 15 cm) equilibrated with the same buffer. After the column was washed, a salt gradient (0—0.25 M NaCl in buffer, 800 ml total) was instituted and the enzyme eluted in 6-ml fractions. The insert depicts the NaCl concentration calculated from conductivity measurements. The enzyme activity was measured with Tyr-β-naphthylamide as substrate and is expressed in arbitrary units (———, A_{280}; · · · · · ·, A_{530}).

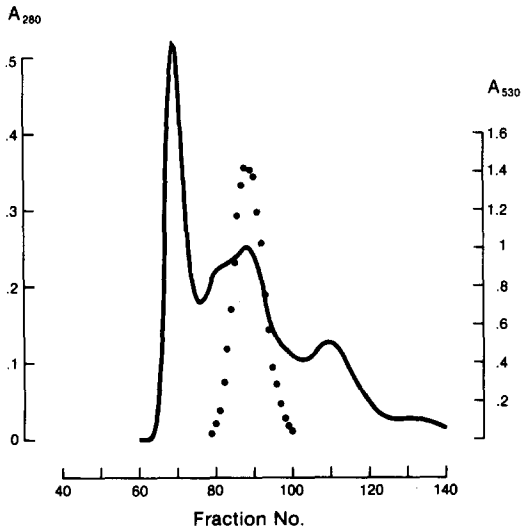

Fig. 2. Chromatography of brain aminopeptidase on Sephadex G-100. The active fractions from the first DEAE-cellulose column (Fig. 1) were pooled and concentrated to 3.6 ml with an Amicon pressure cell. This material was applied to a Sephadex G-100 column (2.5 X 98 cm) equilibrated with 10 mM phosphate buffer (pH 7.0)/1 mM dithiothreitol, and eluted with the same buffer. The enzyme activity was measured with Tyr-β-naphthylamide as substrate and is expressed in arbitrary units (———, A_{280}; · · · · ·, A_{530}).

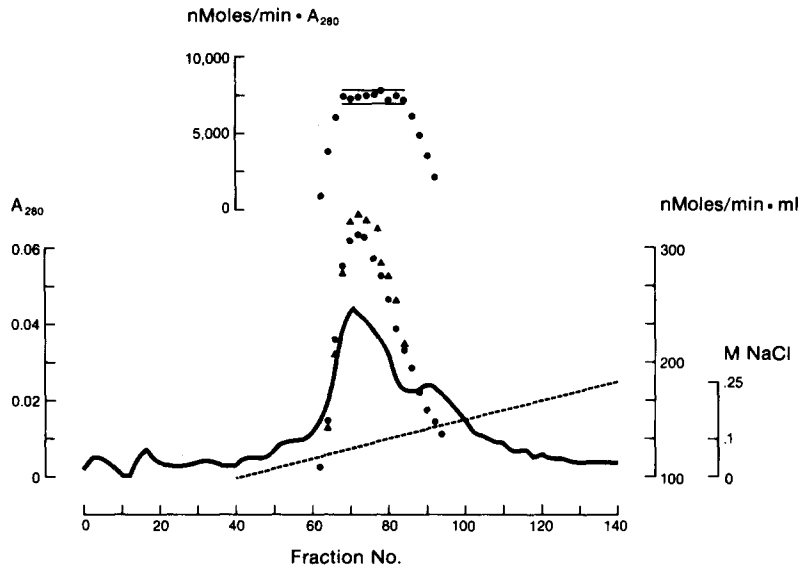

Fig. 3. Rechromatography of brain aminopeptidase on DEAE-cellulose. The active fractions from the Sephadex column (Fig. 2) were pooled and dialyzed against 50 mM phosphate buffer (pH 7.0)/1 mM dithiothreitol/0.01 mM $ZnCl_2$. The material was concentrated to approx. 5 ml and applied to a DE-52 column (1 X 5 cm) equilibrated with the same buffer. The enzyme was eluted in 3.5-ml fractions with a linear salt gradient (0—0.25 M NaCl in the same buffer, 400 ml total). The enzyme was assayed both with Tyr-β-naphthylamide (●) and with [^{3}H]Leu-enkaphalin (▲) as substrates. The insert depicts the specific activity calculated from the hydrolysis of Tyr-β-naphthylamide and the protein concentration (A_{280}) for each individual fraction. The fractions indicated between the horizontal bars in the insert were pooled and are referred to in the text as 'purified rat brain aminopeptidase'.

The activity against both Leu-enkephalin and L-Tyr-β-naphthylamide copurified throughout the procedure (Table I), except on the first DEAE-cellulose column. This may indicate that another enzyme (which hydrolyzes enkephalin, but not the naphthylamide) was removed in this step. In addition, the activities against the two substrates coincided exactly in the eluate from the last column (Fig. 3).

The final product had a constant specific activity across the peak eluted from the second DEAE-cellulose column (Fig. 3), and migrated as a single band when subjected to SDS gel electrophoresis. It was therefore judged to be homogeneous.

Stability

The enzyme rapidly lost activity in the absence of dithiothreitol. Purified enzyme, but not the original extract, lost activity upon freezing and thawing. In addition, the enzyme was irreversibly denatured below pH 5.5. However, it was completely stable for several weeks when stored refrigerated in a solution containing 50 mM phosphate buffer (pH 7.0), 1 mM dithiothreitol and 0.01 mM $ZnCl_2$. The activity actually increased upon 'aging' in this buffer (Table I): assayed immediately after elution from the second DEAE column, the enzyme had a specific activity of about 6200 nmol/min per mg against both Leu-enkephalin and L-Tyr-β-naphthylamide; 3 days and up to 30 days later, the specific activity was increased to about 10 000 nmol/min per mg (assayed with Leu-enkephalin or L-Tyr-β-naphthylamide under standard conditions).

Molecular properties

On Sephadex G-100 (Fig. 2) the enzyme behaved like a molecule with a molecular weight of about 100 000. Estimation of the subunit molecular weight using SDS gel electrophoresis, on two different batches of rat brain aminopeptidase, gave values of 48 000 and 50 000. In the unltracentrifuge, the purified enzyme sedimented with an uncorrected sedimentation coefficient of 6.6, compatible with a globular protein of 100 000 molecular weight: after about 28 min at 60 000 rev./min, a minor peak with a sedimentation coefficient of 4.2 was observed, perhaps indicating a partial dissociation into monomers at 24°C and the high pressure within the cell.

Enzymic properties

The purified rat brain aminopeptidase hydrolyzed Leu-enkephalin as well as naphthylamides of typical aliphatic (leucine), aromatic (tyrosine) and basic (arginine) but not acidic (aspartate) amino acids (Table II) it also hydrolyzed the *p*-nitroanilides of tyrosine and leucine. Leu-enkephalin and L-Tyr-β-naphthylamide were cleaved by the same activity of the enzyme, as indicated by the inhibition of the L-Tyr-β-naphthylamide hydrolysis by Leu-enkephalin. The K_i measured for Leu-enkephalin (0.07 mM) was close to the K_m (0.03 mM) measured directly (Table II).

The ratio (K_{cat}/K_m) is the second-order acylation rate constant [18] and is thus a useful parameter for the description of the efficiency of an enzyme-substrate system [19]. We have calculated (K_{cat}/K_m) for each of the substrates (Table II). Taking into account that studies with Leu-enkephalin were performed at 30°C (all other reactions were at 37°C), it is apparent that the

TABLE II

SUBSTRATE SPECIFICITY OF RAT BRAIN AMINOPEPTIDASE

	K_m (mM)	V per (nmol/min per mg)	(K_{cat}/Km) ($M^{-1} \cdot s^{-1} \times 10^5$)
L-Tyrosine-β-naphthylamide	0.08	16 100	3.4
L-Leucine-β-naphthylamide	0.03	15 000	8.3
L-Arginine-β-naphthylamide	0.5	52 000	1.7
L-Aspartyl-β-naphthylamide	—	0	0
L-Tyrosine-*p*-nitroanilide	0.2	1 800	0.15
N-Benzoyl-L-tyrosine-*p*-nitroanilide	—	0	0
L-Leucine-*p*-nitroanilide	0.2	16 300	1.3
Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) *	0.03	14 100	7.8

* Assayed at 30°C; all other assays were performed at 37°C.

enzyme prefers Leu-enkephalin to the other substrates tested (Table II). The best synthetic substrates were L-Leu-β-naphthylamide and L-Tyr-β-naphthylamide. Compared to the naphthylamides, the nitroanilides of tyrosine and leucine were six and 23 times less reactive, respectively (Table II).

While determining these kinetic parameters, it became apparent that all substrates were inhibitory at high concentrations. Thus, the 'maximal observable activity' (i.e. reaction rate at optimal substrate concentration) generally was about 2/3 of the V extrapolated from the double-reciprocal plots. Despite the fact that the extrapolated V for L-Arg-β-naphthylamide was higher than that determined for the other substrates, the 'maximal observable activity' was similar to that of the corresponding Leu and Tyr compounds; more important, the proteolytic coefficient (K_{cat}/K_m) ranked L-Arg-β-naphthylamide behind the other naphthylamides as a substrate for the rat brain aminopeptidase.

The enzyme required a free α-amino group on the N-terminal amino acid of the substrate, since it hydrolyzed L-Tyr-*p*-nitroanilide, but not the corresponding *N*-benzoyl derivative (Table II).

As shown in Fig. 4, the rat brain aminopeptidase was most active at neutral pH when assayed with L-Tyr-β-naphthylamide; a similar pH profile was obtained with a partially purified enzyme using Leu-enkephalin as substrate (not shown). The enzyme was slightly less active in phosphate than in Tris-HCl buffers.

A divalent cation was required for activity (Table III). Dialysis against EDTA completely inactivated the enzyme. Activity could be restored by the addition of various divalent cations; Co^{2+} and Zn^{2+} were the most effective. It must be noted, however, that all of the divalent cations tested inhibited the enzyme at 1 mM, whether or not it had previously been dialyzed against EDTA (Table III).

The rat brain aminopeptidase was inhibited by the metal chelators EDTA (Table III) and *o*-phenanthroline (Table IV). It was also sensitive to the sulfhydryl reagent *p*-chloromercuribenzoate (Table IV) and to divalent metal ions (Table III). The enzyme was not inhibited by phenylmethylsulfonyl fluoride, an inhibitor of serine proteases, and was only slightly affected by the specific leucine aminopeptidase inhibitor H-(Bu^t)Thr-Phe-Pro-OH [16] (Table

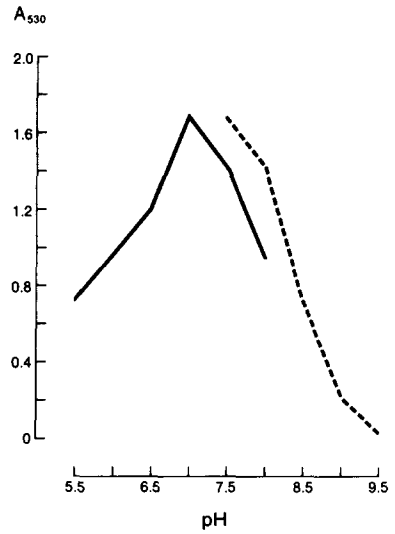

Fig. 4. pH dependence of rat brain aminopeptidase. Purified enzyme was assayed with 0.2 mM Tyr-β-naphthylamide as substrate in a series of 0.1 M phosphate buffers (———) and Tris-HCl buffers (- - - - -). Enzyme activities are expressed in arbitrary A_{530} units.

IV). Puromycin is the most potent inhibitor of rat brain aminopeptidase tested so far (Table IV) [9].

Comparison with other aminopeptidases

In many respects, the enzyme described here is similar to the kinin-converting aminopeptidase of human serum [20]. Both enzymes hydrolyze the naphthylamides of arginine and leucine but not of acidic amino acids. Both enzymes are sensitive to puromycin, *o*-phenanthroline and dialysis against EDTA; slight differences were observed in the restoration of activity with metal

TABLE III

EFFECTS OF METALS ON RAT BRAIN AMINOPEPTIDASE

The purified enzyme was dialyzed for 16 h against 0.1 M NaCl/0.05 M phosphate (pH 7.0) or against the same buffer containing 20 mM EDTA. The EDTA-treated enzyme was then dialyzed against the buffer alone to remove the chelating agent. Portions of the enzyme were then added to 0.1 M Tris-HCl (pH 7.4), and preincubated for 30 min with the salts indicated. Assays were performed with L-Tyr-β-naphthylamide as substrate. The figures shown under '0' salt concentrations and elsewhere in the table represent percent of activity versus undialyzed enzyme controls.

Salt added	Enzyme activity (% of control) at various concentrations of metal ions (mM)					
	Enzyme dialyzed against buffer only		Enzyme dialyzed against EDTA and buffer			
	0	1	0	0.01	0.1	1
$ZnCl_2$		<2		84	<2	<2
$CoCl_2$		5		93	43	5
$MgCl_2$	84	71	<2	6	18	31
$MnCl_2$		<2		36	47	4

TABLE IV

EFFECTS OF SOME INHIBITORS ON RAT BRAIN AMINOPEPTIDASE

Assays were carried out in 0.1 M Tris-HCl (pH 7.4), with 0.2 mM L-Tyr-β-naphthylamide as substrate.

Inhibitor	% inhibition	Remarks
o-Phenanthroline	50% at $1.3 \cdot 10^{-4}$ M	Preincubated 30 min
p-Chloromercuribenzoate	98% at 10^{-5} M	Dithiothreitol removed by dialysis of enzyme before testing
Phenylmethylsulfonyl fluoride	5% at 10^{-3} M	Preincubated 30 min
Puromycin	50% at $3.3 \cdot 10^{-6}$ M	With enkephalin as substrate, IC_{50} was $0.8 \cdot 10^{-6}$ M [9]
H-(Bu^t)Thr-Phe-Pro-OH	25% at 10^{-4} M	Highest concentration tested

ions. The molecular weight of the enzyme from serum (95 000) was also similar to that of the brain aminopeptidase.

On the other hand, our enzyme appears to differ from all of the brain aminopeptidases reported previously [10—15]. Kuhl and Taubert [14] and Fujimoto [15] did not attempt to purify their preparations, but reported properties widely different from those of our enzyme. Shikimi and Iwata [11] purified an enzyme by a procedure somewhat similar to ours; their enzyme was inhibited by sulfhydryl compounds and was activated by divalent metal ions and *p*-chloromercuribenzoate, thus differing markedly from our enzyme. Shikimi's enzyme would appear to be similar to the one described by Marks and Pirotta [12]. Marks et al. [10] reported on a series of rat brain 'arylamidases' and aminopeptidases, which they separated on DEAE-cellulose. These authors felt that their 'peak IV' contained more than one 'arylamidase' since it produced six bands upon gel electrophoresis. Their 'peak IV' preparation hydrolyzed both neutral and basic aminoacyl naphthylamides, with a pH optimum between 7 and 8, and was sensitive to *p*-chloromercuribenzoate, puromycin and EDTA; it thus probably contained the enzyme we describe here.

The rat brain aminopeptidase differs from the classical leucine aminopeptidase in its molecular weight (100 000 compared to 250—300 000), substrate specificity (leucine aminopeptidase does not hydrolyze basic terminal amino acids), and pH optimum (above 8 for leucine aminopeptidase) [21]; finally, the brain aminopeptidase described here is not sensitive to the leucine aminopeptidase inhibitor H-(Bu^t)Thr-Phe-Pro-OH [16].

The enzyme is also easily distinguished from aminopeptidases A, B and M. Aminopeptidase A is specific for acidic amino acid residues and aminopeptidase B is specific for basic amino acid residues and is sensitive to phenylmethylsulfonyl fluoride [22]; neither hydrolyzes L-Leu- or L-Tyr-β-naphthylamides. Aminopeptidase M has a higher molecular weight (280 000 with ten subunits) and is not affected by sulfhydryl reagents or divalent cations [23].

A recent report [24] described an enkephalin-degrading dipeptidyl peptidase in mouse brain. Although this enzyme has a reported K_m for enkephalins which is about 1000 times lower than the K_m of the aminopeptidase described here, it accounts for only about 20% of the enkephalin-degrading capacity of brain homogenates, determined at low enkephalin concentrations. The specific roles of these enzymes in the regulation of peptide activity in brain remains to be

established. Localization by immunofluorescence will be an important next step.

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