Studies on Ox Brain Aminopeptidases

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1. Mn^{2+} -inhibited and Mn^{2+} -activated aminopeptidases have been observed in ox brain and separated from one another by DEAE-cellulose column chromatography. 2. The Mn^{2+} -inhibited enzyme has been purified 36-fold; it exhibits a specificity for tripeptide substrates, whereas the Mn^{2+} -activated aminopeptidase cleaves dipeptides as well as tripeptides. 3. Ammonium sulphate treatment generates a Mn^{2+} -stimulated aminopeptidase that is stable to dialysis against EDTA and water, in contrast with an endogenous Mn^{2+} -activated preparation that is irreversibly denatured by such dialysis against EDTA and water.

The role of peptidases in the total catabolism of protein is not yet fully understood. Peptidases of blood are known to act on the octapeptide pressor agent, angiotensin II, thereby decreasing and finally destroying its physiological activity (Hess, 1965; Nagatsu, Gillespie, Folk & Glenner, 1965). Some physical and chemical characteristics of the metal-ion-requiring leucine aminopeptidase derived from kidney cortex have been investigated (Spackman, Smith & Brown, 1955; Smith & Spackman, 1955). A peptidase not requiring metal ions in calf thymus has also been described (Fruton, Smith & Driscoll, 1948). Aminopeptidases and dipeptidases have been reported to be widely distributed in mammalian organs and body fluids, and have been found to vary greatly in substrate specificity and in metal ion requirements.

The presence of peptidase activity in brain has been known of for some time (Kies & Schwimmer, 1942). Pope & Anfinsen (1948) concluded that the L-alanylglycine-hydrolysing activity in rat and human brain was localized in the nerve-cell bodies (Pope, 1952, 1959). The effect of metal ions and EDTA on dipeptidase and tripeptidase activity of mouse brain extracts has been studied (Uzman, Rumley & van den Noort, 1960, 1961; van den Noort & Uzman, 1961; Uzman, van den Noort & Rumley, 1962), and the intracellular localization of peptidase activity in ox brain has also been reported (Brecher, 1963; A. S. Brecher & I. R. Koski, unpublished work). The amounts of peptidase activity in pathological tissue are known to differ from normal amounts of peptidase activity in human brain (Brecher, Oliphant & Sobel, 1966), human liver (Fleisher, Butt & Huizenga, 1958) and mouse ascitic fluid (Sylvén, Ottoson & Revesz, 1959). A characterization of the various amino-

peptidases in brain is desirable to evaluate ultimately changes in enzyme activities in pathological brain tissue and to interpret their significance.

The separation, partial purification and properties of Mn^{2+} -inhibited and Mn^{2+} -stimulated aminopeptidase activities in ox brain are reported in this paper. Preliminary reports have appeared elsewhere (Brecher, Sobel & Koski, 1966; Brecher & Sobel, 1966).

MATERIALS AND METHODS

Materials. Synthetic peptides for use as substrates (Mann assayed grade) were purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A., and were used without further purification. DEAE-cellulose (Cellex-D, 0.94 m-equiv./g.) was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and was treated as recommended by the manufacturer.

Enzyme preparation. All steps were carried out at 1–4°. Ox brains were obtained from a slaughterhouse and chilled within 15 min. of death. Within 2–4 hr. of death the brains were homogenized in a known volume of distilled water (usually 1:4, w/v) with a Teflon pestle and glass tube. Centrifugations were performed in a Spinco model L ultracentrifuge at 105 000g for 1 hr. to obtain supernatant fractions. EDTA at pH 8.5 was added to the supernatant fractions to a final concentration of 20 mM, and the fractions were dialysed against 20 mM-EDTA, pH 8.5, and then a minimum of three changes of water for a minimum total time of 24 hr. The dialysis residue was then subjected to $(NH_4)_2SO_4$ fractionation, and the precipitates obtained at 0–40%, 40–50%, 50–60%, 60–70% and 70–100% saturation were separated by centrifuging at 17 300g in a Servall centrifuge for 10–15 min., taken up in 20 mM-EDTA, pH 8.5, and dialysed against water.

Acetone-dried powders were prepared from minced brain or from water homogenates (1:1, w/v) by the method of Kies & Schwimmer (1942).

Column chromatography. DEAE-cellulose (15 g.) was
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washed three times with 500 ml. of the initial eluent (5 mm-tris-HCl prepared by diluting 1.0 M-tris-HCl adjusted to pH 8.0 with 1.0 N-HCl); the resin was allowed to settle between washings for 15 min. before the supernatant solution was poured off. The final slurry was poured into a chromatography column, 2.5 cm. internal diam., to a height of 17 cm.

A sample (5.5 ml.) of the 50–60% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction was placed on the column. Stepwise elution was carried out with 200 ml. of 5 mm-tris-HCl, pH 8.0, 310 ml. of 0.1 M-tris-HCl, pH 8.0, 250 ml. of 0.2 M-tris-HCl, pH 8.0, 260 ml. of 0.3 M-tris-HCl, pH 8.0, and 200 ml. of 0.4 M-tris-HCl, pH 8.0. Fractions (10 ml.) were collected with an automatic fraction collector at a flow rate of approx. 1 ml./min.

Determination of protein. Protein N was determined by the micro-Kjeldahl method where feasible. When limited amounts of protein were available, the method of Lowry, Rosebrough, Farr & Randall (1951) or E_{280} was used to estimate protein concentration. The two last-named methods were standardized by using the most closely related solution on which a micro-Kjeldahl determination had been performed.

Assay of aminopeptidase activity. Enzyme assays were conducted in a shaking water bath at 37°. Samples of 0.01 ml. of incubation mixture were removed at timed intervals and placed in 1 ml. of ninhydrin solution, according to the method of Robinson & Shaw (1960). The increase in α -amino N relative to time of incubation, resulting from the hydrolysis of a peptide bond (Moore & Stein, 1948), was used as a measure of the rate of reaction. Since equimolar concentrations of peptides and amino acids do not cause the same increase in colour with the ninhydrin reaction, standard curves were constructed relating the increase in colour yield to the appearance of specific amounts of free α -amino N, i.e. to the amount of hydrolysis of the peptide substrate.

The incubation mixture contained 0.20 ml. of 62.5 mM- or 31.3 mM-peptide in 50 mM-tris-HCl, pH 8.5, or 0.10 M-borate-NaOH, pH 8.5, and 0.05 ml. of enzyme preparation. The concentration of enzyme was adjusted so that the hydrolysis of substrate would proceed with zero-order kinetics. When the enzyme activity was assayed in the presence of a metal ion, the enzyme was preincubated with metal salt at a final concentration of 1 mM for 3 hr. before the addition of substrate. The concentration of metal salt was also adjusted to 1 mM in the final incubation mixture.

One unit of enzyme activity is defined as the amount of enzyme that hydrolyses 1 μ mole of peptide bonds/min. (or that amount of enzyme that causes an increase of 1 μ mole of free α -amino groups/min.). Specific activity is defined as the units of activity/mg. of protein. Initial velocity is given as units of enzyme activity in the assay medium.

RESULTS

Enzyme assay. The hydrolysis of L-leucylglycylglycine is directly proportional to the enzyme concentration. The specific activity of the enzyme preparation remains constant with various concentrations of enzyme (Table 1).

Identification of the hydrolysis products of the tripeptides produced by soluble supernatant fractions with paper chromatography with butan-2-ol-formic acid-water (15:3:2, by vol.) indicated that the peptides were cleaved at the N-terminal end of the molecule. Leucine and glycylglycine were observed upon hydrolysis of L-leucylglycylglycine; glycine and L-leucylglycine were identified as the split products of glycyl-L-leucylglycine. No C-terminal cleavage of the tripeptides was observed by paper chromatography.

Methods of aminopeptidase preparation. During the determination of the most effective means of extracting aminopeptidase activity from brain, both homogenates and acetone-dried powders were prepared in or extracted with water, salts and buffers. Acetone-dried powders contained only 40% of the L-leucylglycylglycine-cleaving activity of homogenates. Water and 50 mM-tris-hydrochloric acid, pH 8.5, extracted more enzyme activity from acetone-dried powders than did 2% (w/v) sodium chloride. Water, 0.32 M-sucrose and 0.2 M-borate-sodium hydroxide, pH 8.0, extracted more activity when used as homogenizing media than did 0.2 M-tris-hydrochloric acid, pH 8.0, and 2% (w/v) sodium chloride. Sodium chloride was the poorest extracting medium for use with an acetone-dried powder or homogenate.

Table 1. *Effect of enzyme concentration on the hydrolysis of L-leucylglycylglycine*

The incubation mixture contained 0.20 ml. of 62.5 mM-L-leucylglycylglycine in 50 mM-tris-HCl, pH 8.5, and 0.05 ml. of suitably diluted enzyme. Incubations were carried out at 37° and samples were taken at timed intervals for measurement of increase in α -amino N.

Preparation	Protein (mg./assay)	Amino groups (μ moles released/min./ mg. of protein)
Freeze-dried 105 000g supernatant fraction	0.071	0.86
	0.035	0.89
	0.312	1.71
50–60% Satd. $(\text{NH}_4)_2\text{SO}_4$ fraction	0.069	1.73
	0.035	1.73
	0.017	1.78

Table 2. *Effect of storage on the stability of the L-leucylglycylglycine-splitting enzyme system*

The incubation mixture contained 0.20 ml. of 62.5 mM-L-leucylglycylglycine in 50 mM tris-HCl, pH 8.5, and 0.05 ml. of suitably diluted enzyme. Incubations were carried out at 37° and samples were taken at timed intervals for measurement of increase in α -amino N.

Preparation	Storage (days)	Amino groups (μ mole released/min./mg. of protein)
Freeze-dried 105 000g supernatant fraction (resuspended and stored frozen)	18	0.89
	39	0.80
	53	0.87
	99	0.86
	218	0.82
105 000g Supernatant fraction (refrigerated under toluene)	1	0.64
	60	0.66

Table 3. *Effects of dialysis, Mn^{2+} and ammonium sulphate on the hydrolysis of L-leucylglycylglycine*

m-EDTA, pH 8.5, was added to each enzyme preparation to give a final concentration of 20 mM. The preparations were dialysed against 20 mM-EDTA, pH 8.5, for 6 hr. followed by a minimum of three changes of redistilled water for 24 hr. Portions of enzymes were then preincubated at 0–4° in a final concentration of 1 mM- $MnCl_2$ for 3 hr., after which the enzymes were assayed for peptidase activity.

Preparation	Dialysis	α -Amino groups liberated (μ moles/min./mg. of protein)	
		Without Mn^{2+}	With Mn^{2+}
105 000g Supernatant fraction from ox brain (1)	—	0.92	0.92
	(1) EDTA + water	0.39	0
	(2) —	1.10	1.60
	(2) EDTA + water	0.89	0
	(3) —	0.60	0.96
	(3) EDTA + water	0.30	0
50–60% Satd. $(NH_4)_2SO_4$ fraction from ox brain (3)*	EDTA + water	1.62	3.48

* The 50–60% saturated $(NH_4)_2SO_4$ fraction was taken up in 20 mM-EDTA and dialysed against water.

Stability of aminopeptidase activity to storage. The L-leucylglycylglycine-hydrolysing activity of the 105 000g supernatant fraction of a water homogenate is stable to storage frozen or in the refrigerator under toluene (Table 2). However, no dipeptidase activity towards glycyl-L-leucine, L-alanylglycine or diglycine was observed in a 105 000g supernatant fraction stored frozen for 42 days.

Dialysis and salt effects on metal ion sensitivity of peptidase activity. The aminopeptidase activity assayed with L-leucylglycylglycine is sensitive to metal ions and dialysis (Table 3). The 105 000g supernatant fraction contains peptidase activity that may be variously stimulated from 0 to 60% by 1 mM- Mn^{2+} . Dialysis of such enzyme preparations against EDTA followed by water effects a loss of 40–60% of the tripeptidase activity. The resultant peptidase activity is inhibited by Mn^{2+} . Whereas dialysis of the 105 000g supernatant fraction against water alone has sometimes caused a loss of Mn^{2+} stimulation, it has never produced a preparation markedly inhibited by Mn^{2+} . Fractionation of the EDTA- and water-dialysed preparations with ammonium sulphate gives a purification of non- Mn^{2+} -stimulated peptidase as well as a

generation of Mn^{2+} -activated peptidase activity. The influence of buffers and salts on such an enzyme preparation is given in Table 4. Tris-hydrochloric acid and sodium chloride-sodium hydroxide solutions at pH 8.5 and at low molar concentrations (50 mM) increase the aminopeptidase activity not requiring metal ions as well as Mn^{2+} -stimulated peptidase activity. Tris-hydrochloric acid at a higher concentration (0.2 M) enhances the aminopeptidase activity not requiring metal ions and has no further effect on the Mn^{2+} -stimulated activity. However, 0.2 M-sodium chloride adjusted to pH 8.5 with 5 mM-sodium hydroxide enhances aminopeptidase activity not requiring metal ions and exerts an inhibition on aminopeptidase activity in the presence of Mn^{2+} .

Purification and characterization of two aminopeptidases

The separation of Mn^{2+} -stimulated and Mn^{2+} -inhibited aminopeptidase activities in the 50–60% saturated ammonium sulphate fraction was accomplished by DEAE-cellulose column chromatography (Fig. 1). Approx. 75% of the non- Mn^{2+} -stimulated

aminopeptidase activity applied to the column is isolated in fractions 57–61 of the 0.2M-tris-hydrochloric acid eluate. The hydrolysis of L-leucylglycylglycine by these fractions is inhibited by Mn^{2+} . The most active Mn^{2+} -inhibited amino-peptidase has a specific activity 36 times that of similar activity in the EDTA- and water-dialysed

105 000g supernatant fraction. Another amino-peptidase, which is Mn^{2+} -stimulated, is recovered in two tubes of the 0.3M-tris-hydrochloric acid eluate. The stepwise purification is given in Table 5.

Characteristics of the isolated amino-peptidases. The peptidases isolated by DEAE-cellulose chromatography have been characterized on the basis of their reactivity with peptide substrates in the presence and absence of metal ions. The activities are compared with those enzyme preparations obtained in each step of the purification process (Table 6). These data indicate that: (1) leucine amino-peptidase-type activity is lost on dialysis; (2) a tripeptidase activity that is inhibited by Mn^{2+} is observed in the dialysis residue; (3) ammonium sulphate treatment generates a metal-ion-requiring amino-peptidase; (4) Mn^{2+} -stimulated and Mn^{2+} -inhibited amino-peptidases are separable from one another by DEAE-cellulose chromatography.

The 0.2M-tris-hydrochloric acid eluate contains no leucine amino-peptidase-type activity as measured by the hydrolysis of L-leucylglycine. It does hydrolyse the three tripeptides tested. The hydrolysis of triglycine and L-leucylglycylglycine is partially inhibited by Mn^{2+} . The 0.3M-tris-hydrochloric acid eluate hydrolyses L-leucylglycine and glycyl-L-leucylglycine in the presence of Mn^{2+} , increases the rate of hydrolysis of L-leucylglycylglycine in the presence of Mn^{2+} , and is unreactive to triglycine. The 0.2M-tris-hydrochloric acid

Table 4. *Effect of concentration of sodium chloride and tris on L-leucylglycylglycine-splitting activity*

The 50–60%-saturated $(NH_4)_2SO_4$ fraction of an EDTA- and water-dialysed 105 000g supernatant fraction was diluted 20-fold in the salts indicated below in the presence and absence of 1 mM- $MnCl_2$. The enzyme preparations were preincubated at 0–4° for 3 hr. before assay at 37°.

Treatment	Mn^{2+}	Amino groups liberated (μ moles/min./ mg. of protein)
Water	—	1.6
Water	+	3.5
0.05M-Tris, pH 8.5	—	2.0
0.05M-Tris, pH 8.5	+	4.3
0.05M-NaCl, pH 8.5	—	2.0
0.05M-NaCl, pH 8.5	+	5.1
0.2M-Tris, pH 8.5	—	3.8
0.2M-Tris, pH 8.5	+	4.3
0.2M-NaCl, pH 8.5	—	2.9
0.2M-NaCl, pH 8.5	+	1.6

Fig. 1. Separation of amino-peptidases by DEAE-cellulose column chromatography. —, Protein; ----, amino-peptidase activity in the absence of Mn^{2+} ; -.-, amino-peptidase activity in the presence of Mn^{2+} . The enzyme was eluted stepwise with increasing concentrations of tris-HCl, pH 8.0, as described in the text. The activity unit is defined as μ moles of amino groups liberated/min. during the hydrolysis of 50 mM-L-leucylglycylglycine.

Table 5. *Purification of the L-leucylglycylglycine-splitting enzymes*

The enzyme preparations obtained during purification were preincubated at 0–4° for 3 hr. in the presence and absence of 1 mM-MnCl₂. The incubation mixture contained 0.20 ml. of 62.5 mM-peptide in 50 mM-tris-HCl, pH 8.5, with and without 1 mM-MnCl₂, and 0.05 ml. of enzyme. Incubations were carried out at 37° and samples were taken at timed intervals for measurement of increase in α -amino N.

Preparation	Mn ²⁺	Amino groups liberated (μ moles/min./mg. of protein)	Purification	Units	Recovery (%)
Homogenate in water (1:4)	—	0.13	—	—	—
	+	0.20	—	—	—
105 000g Supernatant fraction	—	0.82	—	—	—
	+	0.88	—	—	—
EDTA- and water-dialysed 105 000g supernatant fraction	—	0.36	1	2990	100
	+	0	—	—	—
50–60%-Satd. (NH ₄) ₂ SO ₄ fraction	—	1.41	4	1300	44
	+	2.76	—	2540	—
Fraction 57 (0.2 M-DEAE eluate)	—	13.15	36	975	33
	+	8.34	—	617	—
Fraction 82 (0.3 M-DEAE eluate)	—	1.48	—	95	3
	+	3.25	—	209	—

Table 6. *Specificity of L-leucylglycylglycine-splitting enzymes towards di- and tri-peptides*

The enzyme preparations were preincubated at 0–4° for 3 hr. in the presence and absence of 1 mM-MnCl₂. The incubation mixture contained 0.20 ml. of 62.5 mM-peptide in 50 mM-tris-HCl, pH 8.5, with and without 1 mM-MnCl₂, and 0.05 ml. of enzyme. Incubations were carried out at 37° and samples were taken at timed intervals for measurement of increase in α -amino N.

		Peptide hydrolysed (μ moles/min./mg. of protein)									
Fraction.....		105 000g supernatant fraction		EDTA- and water-dialysed 105 000g supernatant fraction		50-60%-Satd. (NH ₄) ₂ SO ₄ fraction		Fraction 57 (0.2M-DEAE eluate)		Fraction 82 (0.3M-DEAE eluate)	
Mn ²⁺		-	+	-	+	-	+	-	+	-	+
L-Leucylglycine		0.18	0.43	0	0	0.43	1.90	0	0	0	3.54
Glycyl-L-leucylglycine		0.31	0.85	0.19	0.10	0.98	2.94	5.13	5.45	0	2.36
Glycylglycylglycine		0.20	0.15	0.16	0.03	0.67	0.61	4.81	3.53	0	0
L-Leucylglycylglycine		0.82	0.88	0.36	0	1.41	2.76	13.15	8.34	1.48	3.25

eluate requires a tripeptide chain length and exhibits a preference for leucine over glycine residues as the *N*-terminal amino acid residue. The 0.3 M-tris-hydrochloric acid eluate, which is Mn²⁺-stimulated, also acts preferentially on *N*-terminal leucine residues.

DISCUSSION

The recovery of almost all of the aminopeptidase activity in the supernatant fraction of brain homogenates is consistent with the findings of Pope & Anfinsen (1948), Brecher (1963) and Uzman *et al.* (1962), who found soluble peptidase activity in mammalian brain. The aminopeptidase activity is stable for months when stored frozen or refrigerated under toluene. However, dipeptidase activity is

lost on storage for long periods and may be more susceptible to inactivation by aging (Robinson & Shaw, 1960; Smith, 1948) and by some purification procedures (Bergmann & Smith, 1941). Tetra-glycine is not cleaved by the enzyme preparations. Since only very slight hydrolysis of tetrapeptides consisting of at least three glycine residues has been reported by Fruton *et al.* (1948) in studies on an aminotripeptidase, and by Smith & Spackman (1955) with leucine aminopeptidase, it appears that such tetrapeptides are less susceptible than di- and tri-peptides to hydrolysis by aminopeptidases.

Mn²⁺-inhibited and Mn²⁺-stimulated aminopeptidases have been separated from one another by DEAE-cellulose chromatography. The Mn²⁺-inhibited enzyme, which behaves similarly to a hog intestinal mucosal preparation (Smith &

Bergmann, 1944), acts on tripeptides but does not hydrolyse dipeptides that are classically cleaved by leucine aminopeptidase (Smith & Spackman, 1955). The Mn^{2+} -stimulated aminopeptidase cleaves both dipeptides and tripeptides. Both peptidases exhibit a preference for *N*-leucyl peptides.

In addition to the presence of the Mn^{2+} -inhibited tripeptidase, there are indications for the presence of two Mn^{2+} -stimulated aminopeptidases. The Mn^{2+} -stimulated peptidase activity observed in 105 000g-supernatant solutions is irreversibly inactivated on dialysis against EDTA followed by water. The second enzyme, obtained during purification by salting out with ammonium sulphate, taking up in EDTA and dialysing against water, is not inactivated by such treatment. These results suggest the generation or activation of a new enzyme activity by treatment with ammonium sulphate, and may explain the recovery of more than 100% of starting activity in the ammonium sulphate fractionation step of leucine aminopeptidase purification by Spackman *et al.* (1955). Patterson, Hsiao & Keppel (1963) have also recovered more peptidase activity than originally observed by an ammonium sulphate fractionation during the purification of peptidase activity from ascites-tumour cells.

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