Vol. 90, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 12, 1979 Pages 794-800

THE ISOLATION OF TWO DIPEPTIDE HYDROLASES FROM MOUSE BRAIN CYTOSOL

M.E.A. Reith and A. Neidle

Center for Neurochemistry, Rockland Research Institute, Ward's Island, N.Y. 10035

Received August 17,1979

SUMMARY: Two dipeptide cleaving enzymes from mouse brain cytosol were purified in parallel by ammonium sulfate precipitation followed by ultrafiltration and chromatography on diethylaminoethyl cellulose and hydroxylapatite. The enzymes appeared as non-overlapping peaks during the final step. The purification attained was 431-fold for enzyme I and 61-fold for enzyme II. The latter, from its specificity, pH optimum, and inhibition by SH is similar to glycylleucine dipeptidase (IUB 3.4.13.2) from other mammalian sources. Purification of enzyme I has not been reported previously. It is inhibited by EDTA and o-phenanthroline and stimulated by SH. Typical substrates are Ala-Ala, Lys-Ala, Trp-Gly and Pro-Ala. Some activity was found against peptides with 3 or more residues but none against peptides containing acidic or D-amino acids.

INTRODUCTION

Dipeptidases have been isolated from various mammalian sources including pig (1,2) and monkey (3) intestine, pig brain (4), and ascites tumor cells (5,6). These enzymes are similar to one another in substrate specificity, metal requirements, and response to inhibitors. In connection with a study of the degradation of ACTH fragments by mouse brain cytosol (7), we wanted to determine whether Ser-Met is cleaved by such an enzyme. As has been reported for several of these dipeptidases, the Ser-Met hydrolase proved to be unstable during purification. However, an increase in specific activity of 60-100 fold could be achieved by ammonium sulfate precipitation, ultrafiltration, and chromatography on DEAE and hydroxylapatite. When the latter column was monitored for activity using various peptides in addition to Ser-Met, it was found that a second dipeptidase

0006-291X/79/190794-07$01.00/0

had been copurified during the early steps of the isolation procedure. In the present communication, we compare the general characteristics and the substrate specificity of the two peptidases, which together constitute the major portion of the hydrolytic activity of mouse brain cytosol directed towards dipeptides with neutral or basic amino acid residues.

METHODS

Peptidase activity A fluorescence detector coupled to a batch sampling system was used for these determinations with o-phthalaldehyde in the presence of mercaptoethanol as the fluorogenic reagent (8). This method provides a sensitive assay for peptidase activity since the fluorescence yield of the released amino acids is greater than that of the initial peptide (often by a factor of 10 or more).

Incubation mixtures contained (except where noted) 1 mM peptide, 0.05 M phosphate buffer, and 5 µl of enzyme in a final volume of 0.15 ml. The reaction was stopped by the addition of 0.5 ml of 0.4 M borate buffer, pH 10, and further dilutions were made with the same buffer prior to fluorescence measurements. Incubations were carried out at 37°C. Appropriate 0 time blanks, control incubations, and amino acid and peptide standards were included in each experiment.

Enzyme purification All steps were carried out at 0-4°C in solutions containing 0.1 mM DTT. The brains from 5 mice (2.5 g) were homogenized in 12.5 ml of 1% NaCl and centrifuged at 35,000 g for 20 min. The supernatant solution was then fractionated with ammonium sulfate, and the material precipitating between 40 and 60% of saturation was dissolved in 10 ml of 0.02 M Tris buffer, pH 7 (Tris). The solution was concentrated to 0.5 ml at 60 lbs/in^2 using a PM-10 ultrafilter, (Amicon Corp.). 5 ml of Tris was then added and the ultrafiltration step repeated. The concentrated enzyme was diluted to 5.0 ml using the same buffer and applied to a column (5.0 x 1.2 cm) of DEAE. The column was eluted first with 9.0 ml of 0.05 M NaCl then with 20 1.5-ml portions of Tris containing a linear gradient of sodium chloride varying from 0.05 - 0.4 M. The 3 tubes containing the major portion of the recovered activity were combined and applied directly to a column of hydroxylapatite (3.0 x 1.2 cm). This column was eluted with 20 1-ml portions of buffer containing a linear gradient of phosphate ranging from 0 - 0.2 M at pH 7.0. Individual tubes containing appreciable peptidase activity were brought to 0.25 M sucrose and stored at -20°C. If the entire procedure could not be completed in one working day, the combined DEAE fraction was brought to 0.25 M sucrose and stored at -20°C overnight.

RESULTS AND DISCUSSION

The enzymatic formation of free amino acids from oligopeptides involves dipeptides as obligatory intermediates. In particular, we have been concerned with how the individual dipeptidases of mouse brain cytosol contribute to the

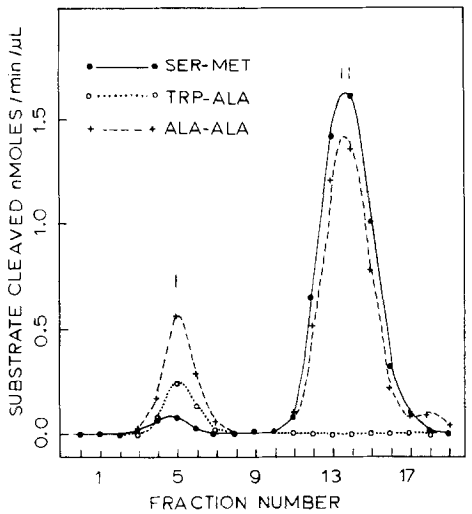

Fig. 1. Substrate specificity of cytosol dipeptide hydrolases separated on hydroxylapatite. Incubation mixtures contained: o——— o, 1 mM Ser-Met, 0.05 M borate buffer, pH = 8.3; +- - - -+, 1 mM Ala-Ala, 0.05 M phosphate buffer, pH = 8.0; o ·····o, 1 mM Trp-Ala, 0.05 M phosphate buffer, pH = 7.5 and 1 mM dithiothreitol.

metabolism of ACTH fragments by this preparation. When the degradation of ACTH-(1-4) was examined, ^{3}Ser-^{4}Met was found to be an intermediate, and isolation of the relevant dipeptidase was attempted. During the early stages of purification the activity appeared to be due to a single enzyme; however upon hydroxylapatite chromatography two peaks of activity were observed (Fig. 1).

A survey of the specificity of the enzymes of peaks I and II indicated that two enzymes with widely differing characteristics were present (Fig. 1). While Ser-Met was cleaved almost exclusively by enzyme II, Trp-Ala was split only by enzyme I. Ala-Ala was a good substrate for both. The enzyme purification procedure was then repeated,monitoring enzyme I activity with Trp-Ala and enzyme II with Ser-Met (Table 1). Until hydroxylapatite chromatography the two enzymes appeared to be purified in parallel, and even chromatography on DEAE did not suggest the presence of separate activities. The final step resulted in a

Table 1. Purification of dipeptide hydrolases from mouse brain

	n moles cleaved/min/mg protein	
Fraction	Trp-Ala	Ser-Met
35000g Supernatant	26.6	655
$(NH_4)_2SO_4$ 40-60%	82.1	1,121
DEAE	563	6,254
Hydroxylapatite fract. 5	11,660	4,178
Hydroxylapatite fract. 14	0	39,363

Incubation mixtures contained 1 mM Trp-Ala at pH 7.5 and 1 mM dithiothreitol, or 1 mM Ser-Met at pH 8.3. Fraction 5, the central portion of peak I had a specific activity 431 times that of the 35,000g supernatant. The total Trp-Ala hydrolase recovery in peak I was 33%. Fraction 14, the central portion of peak II, was 61-fold purified. Total recovery of Ser-Met hydrolase in the peak was 26%.

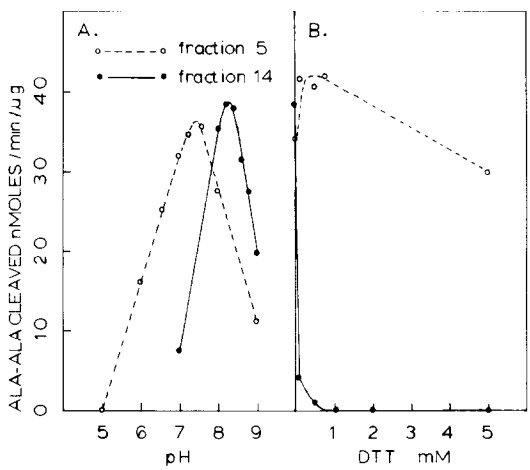

Fig. 2. Effect of pH and dithiothreitol on purified dipeptide hydrolases. Incubation mixtures contained: A, 1 mM Ala-Ala and 0.05 M phosphate buffer (pH 5-8) or 0.05 M borate buffer (pH 8-9); B, 1 mM Ala-Ala, 0.05 M phosphate buffer, pH = 7.5 and varying concentrations dithiothreitol.

purification of 431-fold for enzyme I and 61-fold for enzyme II. In other preparations purifications of up to 100-fold could be obtained for enzyme II. The yield of each was about 30%.

pH optima and the effects of inhibitors were compared using Ala-Ala as the substrate for both enzymes (Fig. 2, Table 2). Enzyme I was most active at pH 7.4; it was stimulated by low levels of DTT but somewhat inhibited by higher

Table 2. Effect of inhibitors on purified dipeptide hydrolases

	μmoles Ala-Ala cleaved/min/mg	
Inhibitor	Enzyme I	Enzyme II
-	27.6	34.5
Puromycin, 0.5mM	27.0	35.0
EDTA, 1mM	27.1	19.8
EDTA, 10mM	15.8	4.3
o-Phenanthroline, 0.5mM	2.6	5.6
Bestatin, 50μg/ml	0	0
Dithiothreitol, 1mM	32.8	2.1

Incubations were at pH 8 for 20 min (enzyme I) or 5 min (enzyme II).

concentrations (5mM) of the reagent. After storage at -20° C, increased activation by SH containing reagents was found. In contrast, enzyme II has optimal activity at a higher pH (8.3) and was strongly inhibited by DTT. Both were inhibited by bestatin, a microbial amino peptidase inhibitor (9), but not by puromycin. Both enzymes appear to be metal-requiring since o-phenanthroline and EDTA are inhibitory.

The striking effects of SH-containing compounds on some dipeptidases were reported previously; they were ascribed to SH groups acting as metal chelators (6). Among peptidases such inhibition,which is not often observed,may be useful for identifying sensitive enzymes in crude preparations. For example, we have found that the activity of enzyme II towards a given substrate can usually be predicted by the degree to which DTT inhibits its degradation by crude brain extracts. If this effect is due to an interaction between sulfhydryl groups and metal ions rather than to the reduction of a sensitive S-S bond at the active center, metal activation of a particular type (perhaps involving Zn) may be common to these enzymes.

The activity of the two enzymes towards a variety of peptides is summarized in Table 3. The specificity, SH inhibition, and other properties of enzyme II indicate it is related to a group of peptidases isolated from various mammalian sources (1-6).

Table 3. Substrate specificities of purified dipeptide hydrolases

Substrate	Source*	μmoles cleaved/min/mg			
		Enzyme I		Enzyme II	
		-DTT	+DTT	-DTT	+DTT
Ser-Met	1	3.5	4.0	45.7	0
Ala-Ala	2	27.6	31.4	34.5	0
Val-Val	2	0.4	0.4	39.2	0
Gly-Leu	3	3.5	4.4	19.3	0
Tyr-Tyr	2	3.5	3.5	1.7	0
Pro-Ala	4	23.9	25.2	2.4	0
Ala-Pro	4	16.8	5.9	0	0
Glu-Ala	1	0	0	0.3	0
Ala-Asp	1	1.5	1.5	0.3	0
Asp-Ala	1	0	0	0	0
Trp-Ala	5	9.2	9.9	0	0
Trp-Gly	6	4.2	7.0	0	0
Lys-Ala	1	15.3	19.1	0	0
His-Ala	1	5.7	6.0	0.2	0
Ala-His	1	10.3	10.4	1.6	0.1
Trialanine**	5	6.4	7.6	0	0
Pentaalanine	5	3.2	3.8	0	0
Leu-Gly-Gly	4	2.9	2.9	0	0
Gly-D-Leu	3	0	0	0.2	0.1
ACTH-(1-4)	7	2.4	3.6	0	0
ACTH-(4-10)	8	0.4	0.5	0	0
ACTH-(1-10)	9	0.6	0.7	0	0

Incubations were at pH 8 for 20 min (enzyme I) or 5 min (enzyme II), in the presence or absence of 1 mM DTT. *1, Vega; 2, Mann; 3, Cyclo; 4, Sigma; 5, Miles; 6, Chemalog; 7, Bachem; 8, Organon; 9, Peninsula. ** For this and other peptides with more than two residues, the figures represent μmoles of amino acid release/min/mg.

These enzymes are true dipeptidases with no action on peptides with 3 or more amino acid residues. They do not cleave acidic dipeptides, and they only rarely cleave those containing basic amino acids. The specificity of the mouse brain enzyme is consistent with this spectrum of activity. In the earliest description this enzyme was termed glycylleucine dipeptidase (1)(IUB 3.4.13.2).

The specificity of enzyme I is markedly different from that of enzyme II in respect to both substrate size and amino acid constituents. Thus, enzyme I hydrolyzes

dipeptides containing Trp or Pro, and also many peptides with basic amino acid residues that are not attacked by enzyme II. In addition, enzyme I displays activity against some peptides with from 3 to 10 amino acid residues (Table 3). Larger peptides (ACTH-(1-24), angiotensin II, and insulin A chain) were not degraded. The order of bond cleavage of the intermediate sized substrates has not been determined, except for Leu-Gly-Gly, where the enzyme acts as an aminopeptidase. Further purification of the preparation is now being carried out to determine whether all of their activities reside in a single enzyme. This question is of particular interest in regard to Ala-Pro hydrolysis since (i) this is the only enzyme I activity that is SH inhibited and (ii) in brain cytosol, its cleavage has been ascribed to the action of prolidase, an enzyme specific for substrates with an X-Pro sequence (10).

Peptidases with specificity similar to that of enzyme I have not been reported to be present in brain; however, a related enzyme may occur in rat intestinal mucosa (11) and the SH-dependent hydrolysis of typical enzyme I substrates has been reported in rat liver cytosol (12).

ACKNOWLEDGEMENT

The technical assistance of Mrs. L. Bazzuro is greatly appreciated.

REFERENCES

1. Smith, E.L. (1948) J. Biol. Chem. 176, 9-19.
2. Noren, O., Sjostrom, H., and Josefsson, L. (1973) Biochim. Biophys. Acta 327, 446-456.
3. Das, M., and Radhakrishnan, A.N. (1973) Biochem. J. 135, 609-615.
4. Neidle, A., and Chedekel, M. (1971) Trans. Am. Sci. Neurochem. 2, 98.
5. Hayman, S., and Patterson, E.K. (1971) J. Biol. Chem. 246, 660-669.
6. Patterson, E.K., Gatmaitan, J.S., and Hayman, S. (1973) Biochemistry 12, 3701-3709.
7. Reith, M.E.A., Neidle, A., and Lajtha, A. (1979) Arch. Biochem. Biophys. 195, 478-484.
8. Roth, M., and Hampai, A. (1973) J. Chromatogr. 83, 353-356.
9. Suda, H., Aoyagi, T., Takeuchi, T., and Umezawa, H. (1976) Arch. Biochem. Biophys. 177, 196-200.
10. Hui, K.-S. and Lajtha, A. (1978) Brain Res. 153, 79-85.
11. Schiller, C.M., Huang, T.I., and Heizer, W.D. (1977) Gastroenterology 72, 93-100.
12. Taylor, S.L., and Tappel, A.L. (1975) Can. J. Biochem. 53, 502-507.