Biochimica et Biophysica Acta, 391 (1975) 388—395

BBA 67518

PURIFICATION AND CHARACTERIZATION OF AN AMINOACYL PROLINE HYDROLASE FROM GUINEA-PIG INTESTINAL MUCOSA

GERARD O'CUINN and P.F. FOTTRELL.

Department of Biochemistry, University College, Galway (Republic of Ireland)

(Received February 20th, 1975)

Summary

The purification of an aminoacylproline hydrolase from guinea-pig intestinal mucosa is described. The enzyme, which is an aminopeptidase has a molecular weight of 112 000 and is activated by manganese and inhibited by zinc.

Unlike other aminoacylproline hydrolases this enzyme displayed a broad substrate specificity. However, it was preferentially active against dipeptides containing proline in the C-terminal position.

Introduction

There has been increased interest in intestinal peptide hydrolases in recent years in view of their involvement in the final stages of mammalian protein digestion and their possible implications in protein malabsorption conditions. Previous studies from this laboratory showed that seven peptide hydrolases with broad substrate specificities are present in guinea-pig intestinal mucosa [1]. One of these enzymes, which was previously purified and characterised, hydrolysed relatively few peptides containing proline [2]. This report describes the purification and characterization from guinea-pig intestine of an aminoacylproline hydrolase previously [1] termed "γ" peptide hydrolase. The present enzyme is of particular interest because unlike other aminoacylproline hydrolases [3—5] it hydrolyses a variety of peptides which do not contain proline.

Materials and Methods

Peptide hydrolase assay

Aminoacylproline hydrolase activity (previously [1] termed γ peptide hydrolase) was measured using 5 mM L-Leu-L-Pro as substrate. Assays were conducted in 25 mM sodium tetraborate (pH 8.0) containing 0.5 mM $MnCl_2$. L-Leu-L-Leu hydrolase activity was measured as above except that 5 mM L-Leu-L-Leu was used as substrate and metal ions were omitted from the

assays. The latter substrate is readily hydrolysed by two of the other major peptide hydrolases in guinea-pig intestine. In each case the L-Leu released was measured using a coupled system containing L-amino acid oxidase, peroxidase and *O*-dianisidine as described previously [6]. Peptide hydrolase activity was expressed as nmol L-Leu released per min · ml^{-1} of enzyme solution.

Electrophoresis

The procedure for starch gel electrophoresis was the same as described previously [1]. L-Leu-L-Pro was used to detect the aminoacylproline hydrolase activity while L-Leu-L-Leu and L-Leu-L-Leu-L-Leu were used to detect other peptide hydrolases.

Purification procedure

All steps were conducted at 4° C. In a typical preparation 3 g of guinea-pig intestinal mucosa was homogenised in 30 ml of 0.3 M sucrose in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10 000 × *g* for 10 min and the supernatant recentrifuged at 34 000 × *g* for 20 min. The latter supernatant was adjusted to 30% saturation with respect to ammonium sulphate. Following centrifugation at 30 000 × *g* for 15 min the supernatant was brought to 50% saturation with ammonium sulphate. After centrifugation of this fraction at 30 000 × *g* for 15 min the resultant pellet was resuspended in 25 mM sodium tetraborate pH 8.0 and applied to a DEAE-cellulose column (DE-52) previously equilibrated with the same buffer. The aminoacylproline hydrolase activity was eluted from the column using a KCl gradient in a 25 mM sodium tetraborate buffer pH 8.0. Tubes containing aminoacylproline hydrolase activity were pooled and dialysed against 25 mM sodium tetraborate pH 8.0 with no salt present. The volume was reduced by vacuum dialysis and aliquots of the concentrated activity were added to a preparative polyacrylamide column (7%) (Shandon Scientific, London) and the component peptide hydrolases were separated by electrophoresis according to Davis [7]. Enzyme activities were eluted from the gel using a 25 mM sodium tetraborate buffer pH 8.0; flow rate 0.2 ml/min. Fractions containing aminoacylproline hydrolase activity were pooled and stored at −20° C. The enzyme was stable indefinitely at this temperature. Protein concentrations were determined using the Folin reagent [8]. Analytical polyacrylamide gel electrophoresis was employed according to the method of Davis [7]. Analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to Dunker and Ruechert [9]. Protein was located on these gels with Coomassie Blue [10].

Molecular weight determinations

The molecular weight of the aminoacylproline hydrolase was obtained by gel filtration on a Sephadex G-200 column (70 × 2 cm) calibrated with cytochrome *c*, bovine serum albumin, R-phycocyanin, R-phycoerythrin and small phycoerythrin [11]. The latter three biliproteins were gifts from Dr. P. O'Carra and Mr G. Downey of this laboratory.

Activation and inhibition studies

The various reagents were included in the assay buffer.

Substrate specificity

Peptides were purchased from Sigma Chemical Co. (London), Cyclo Chemical Co. (Los Angeles) and Bachem (Marina Del Rey, Calif.). Hexa-L-Leu was a gift from Dr T.J. Peters (Royal Postgraduate Medical School, London). Kinetic parameters were obtained from Michaelis-Menten plots. $MnCl_2$ was omitted from the assay when the latter studies were done. Further calculations on the kinetic data obtained were performed according to Fruton [12]. The activity of purified peptide hydrolase against Gly-L-Pro-L-Ala was determined with a Joel JLC-5AH amino acid analyser.

Results

Purification of aminoacyl proline hydrolase

The aminoacylproline hydrolase was separated from other intestinal peptide hydrolases such as "a" and "b" peptide hydrolases [1] and from most of the "α" peptide hydrolase [1,2] by ammonium sulphate fractionation. Although chromatography on DEAE-cellulose removed the remainder of the "α" peptide hydrolase the aminoacylproline hydrolase was significantly contaminated with other hydrolases, the "β" peptide hydrolases [1,13] (Fig. 1). Preparative polyacrylamide electrophoresis separated the "β" and aminoacyl proline hydrolases (Fig. 2). The final preparation of aminoacyl proline hydrolase was subjected to starch gel electrophoresis and a single band of peptide hydrolase activity was noted (Fig. 3). Likewise, when a concentrated sample of the same final enzyme preparation was examined by analytical polyacrylamide gel electrophoresis a single protein band was obtained. An antiserum to the purified enzyme preparation formed one precipitin band when allowed to diffuse on Ouchterlony plates against cytoplasm from guinea-pig intestinal mucosa.

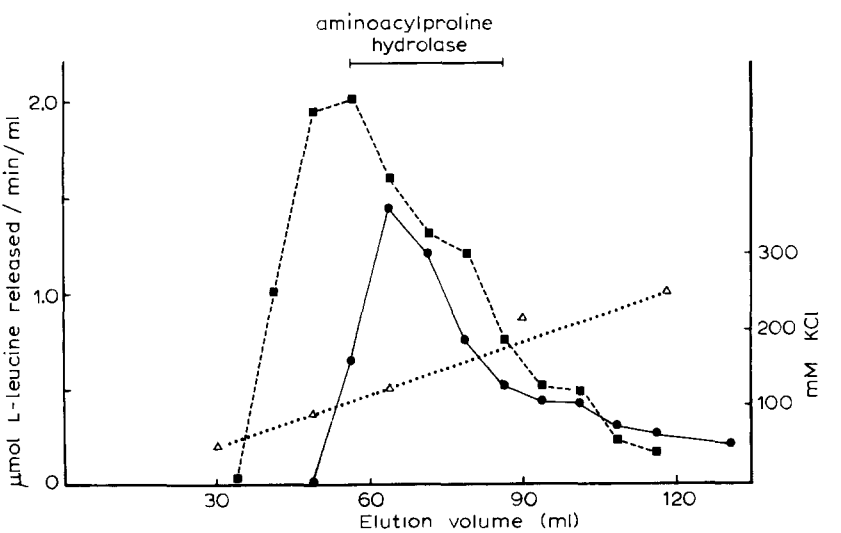

Fig. 1. Ion exchange chromatography (DEAE-Sephadex DE52) of the resuspended ammonium sulphate precipitate. Aminoacylproline hydrolase activity (●——●) was detected using L-Leu-L-Pro as substrate in the presence of 0.5 mM $MnCl_2$. L-Leu-L-Leu hydrolase activity (■- - - - -■) was assayed in the absence of metal. Peptide hydrolase activities were eluted from the column using a KCl gradient (△· · · · ·△).

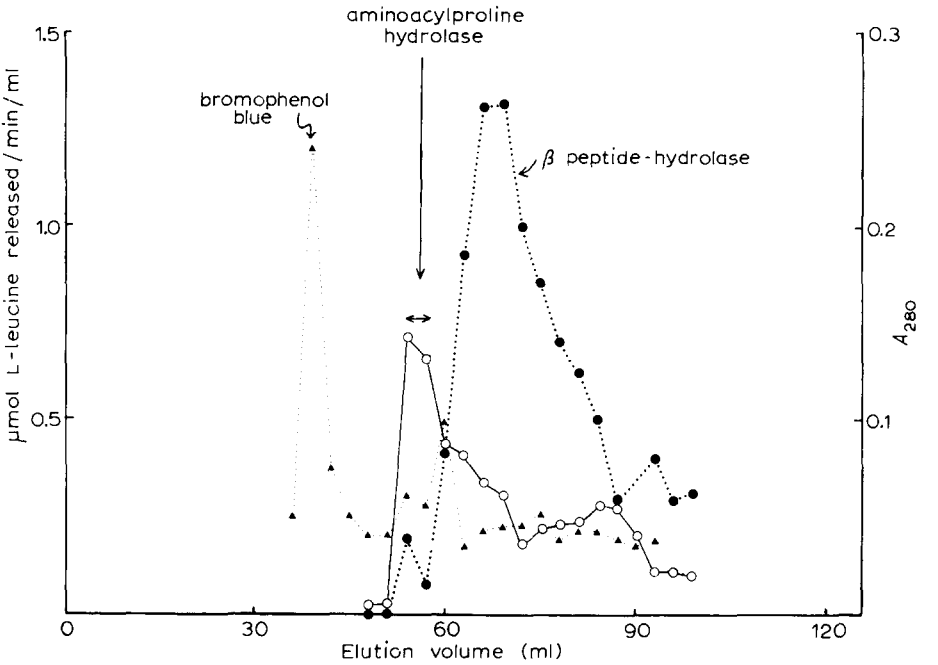

Fig. 2. Elution profile of DEAE fraction from a preparative polyacrylamide electrophoresis column. Aminoacylproline hydrolase activity (———), was determined using L-Leu-L-Pro as substrate in the presence of 0.5 mM $MnCl_2$. L-Leu-L-leu hydrolase activity (●· · · · · ●) was assayed in the absence of metal. Protein (▲. . . . . ▲) was determined using the Lowry et al., method [8].

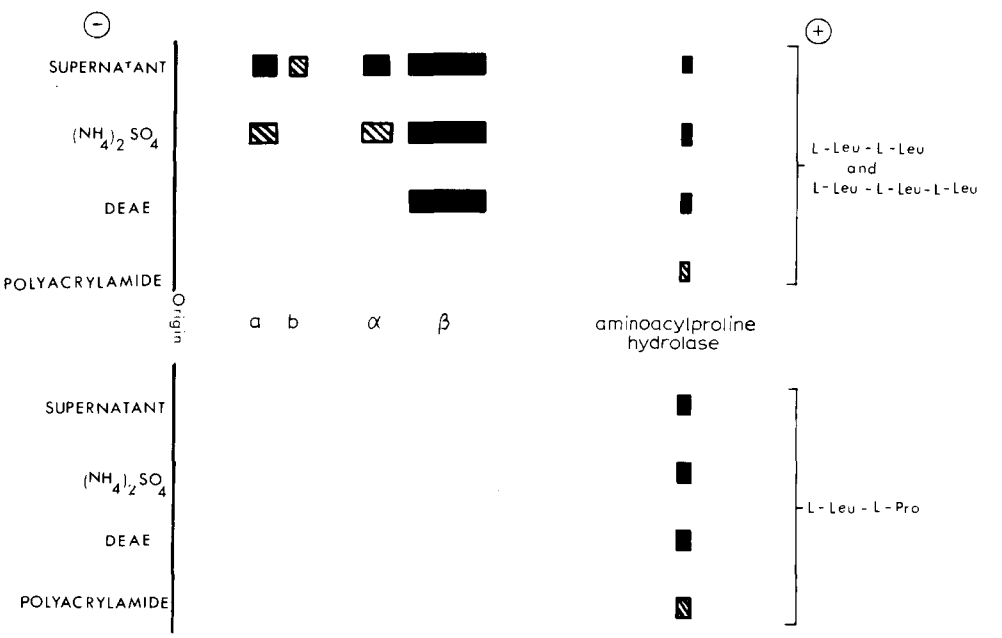

Fig. 3. Starch gel electrophoresis paterns of peptide hydrolases (using L-Leu-L-Leu and L-Leu-L-Leu-L-Leu as substrates) and aminoacylproline hydrolase (L-Leu-L-Pro) in the various fractions obtained during the purification procedure. The conditions for electrophoresis and detection of peptide hydrolases were described previously [1]. Relative intensities are indicated by shading.

TABLE I

PURIFICATION OF AMINOACYLPROLINE HYDROLASE FROM GUINEA PIG INTESTINAL MUCOSA

Step	Volume (ml)	Total protein (mg)	Total activity* (μmol/min)	Specific activity (μmol/min per mg protein)	Purification (fold)	Yield (%)
Crude extract	22.0	2057	572	0.28	1.00	100.1
Supernatant I	18.5	592	407	0.69	2.47	71.1
Supernatant II	17.0	476	391	0.82	2.90	68.3
Ammonium sulphate	12.0	276	246	0.89	3.20	43.0
DEAE-cellulose	33.0	12.5	90.7	7.21	25.90	15.8
Preparative polyacrylamide	5.6	1.6	12.6	7.5	26.90	2.2

* Enzyme activity was assayed with L-Leu-L-Pro as substrate (see text).

Confirmation that the precipitin line observed above was due to precipitation of aminoacylproline hydrolase with its respective antibody was provided by immunoelectrophoresis [14]. Table 1 shows that a 27-fold purification of the enzyme was achieved with a yield of 2%.

pH Optimum

The activity of aminoacylproline hydrolase with L-Leu-L-Pro as substrate was tested over a range of pH values in sodium tetraborate buffers of constant ionic strength and an optimum pH value of 8.0 was obtained. The influence of various buffers, at pH 8.0, on the activity of pure hydrolase was examined with L-Leu-L-Pro as substrate. Activity was higher in 25 mM tetraborate than in 25 mM Tris or 25 mM barbitone. The lowest activities were obtained with the latter buffer.

The effect of metals

A range of metals such as Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, Na^{+} and K^{+} were tested for their effect on the activity of peptide hydrolase with L-Leu-L-Pro as substrate. The activity of the enzyme was stimulated only by manganese whereas zinc was inhibitory. Maximum stimulation of hydrolase activity was obtained with 0.7 mM manganese.

Molecular weight

A molecular weight of 112 000 was calculated for the peptide hydrolase on the basis of its elution from a calibrated Sephadex G 200 column.

Electrophoresis of the purified enzyme on calibrated sodium dodecyl sulphate-polyacrylamide gels showed one band staining for protein which corresponded with a molecular weight of 57 000.

Inhibitors

The activity of the enzyme was not inhibited by *O*-phenanthroline (5 mM) but *p*-chloromercuribenzoate (0.1 mM) reduced its activity by 40%.

TABLE II

KINETIC DATA FOR THE HYDROLYSIS OF VARIOUS PEPTIDES BY AMINO ACYL PROLINE HYDROLASE

Substrate	K_m (mM)	V (nmol N-terminal residue released/ min/ml	k_{cat} (s^{-1})	$\frac{k_{cat}}{K_m}$ ($mM^{-1} \cdot s^{-1}$)	Relative reactivity
1 L-Met-L-Pro	0.70	263	5.260	17.51	470
2 L-Lys-L-Leu	0.35	37.4	0.748	2.13	136
3 L-Val-L-Pro*	1.22	102.9	2.058	1.68	107
4 L-Leu-L-Pro	2.30	181.0	3.620	1.57	100
5 L-Pro-L-Ile*	0.30	16.1	0.322	1.07	68
6 L-Tyr-Gly-Gly	0.80	31.2	0.624	0.78	49
7 L-Met-Gly-Gly	1.09	38.4	0.768	0.70	45
8 L-Phe-L-Pro	2.00	53.2	1.064	0.53	34
9 L-Lys-L-Phe	0.56	13.3	0.266	0.48	30
10 L-Pro-L-Tyr	1.11	23.7	0.474	0.43	27
11 L-Pro-L-Leu	1.25	20.0	0.400	0.32	20
12 L-Leu-L-Leu-L-Leu	1.66	22.2	0.444	0.27	17
13 L-Leu-L-Leu	5.00	55.7	1.114	0.22	14
14 Gly-L-Leu	1.50	11.5	0.230	0.15	10
15 Gly-L-Phe	1.42	10.0	0.200	0.14	8

* These substrates displayed inhibition at high substrate concentrations. V values for such substrates refer to velocities at optimum substrate concentration.

Substrate specificity

The hydrolysis of 14 peptides by the purified hydrolase was examined. K_m and V were derived from Michaelis-Menten plots; K_{cat} and K_{cat}/K_m (the specificity constant) were also calculated. A table of relative reactivities drawn up on the basis of the specificity constants with an arbitrary value of 100 being attributed to L-Leu-L-Pro is shown in Table II. The rate of hydrolysis of L-Pro-L-Met, L-Leu-L-Ala, L-Leu-β-naphthylamide and L-Leu-*p*-nitroanilide was too low to allow kinetic analysis, while another group of peptides (Table III) was not hydrolysed. The peptide hydrolase was free of endopeptidase activity on denatured cytochrome *c*. It was interesting to note that only the three substrates of lowest relative reactivity alone of those listed in Table II are hydrolysed to any appreciable degree by the β enzyme [13].

TABLE III

PEPTIDES NOT HYDROLYSED BY AMINOACYLPROLINE HYDROLASE

L-Leu-amide	α-L-Glu-L-Trp
L-Leu-L-Leu-amide	α-L-Glu-L-Tyr
L-Leu-D-Leu	L-Phe-L-Leu
D-Leu-L-Leu	L-Val-L-Phe
Hexaleucine	Z-Gly-L-Phe*
Gly-Gly-L-Leu	Z-Gly-L-Leu*
γ-L-Glu-L-Leu	

* Abbreviation: Z-, *N*-benzyloxycarbonyl.

Substrate competition studies

Studies were conducted on the ability of three substrates (L-Leu-L-Leu, L-Lys-L-Leu, and L-Pro-L-Tyr) from Table II to compete with L-Leu-L-Pro for the active site on the enzyme. In each case where two substrates were tested simultaneously with the enzyme the value recorded for the specific activity lay between the values obtained when each substrate was tested alone with the enzyme.

Aminopeptidase activity

When 5 mM Gly-L-Pro-L-Ala was incubated with the purified enzyme glycine alone was released as a free amino acid. This suggests that the aminoacylproline hydrolase acts as an amino peptidase on this substrate.

Discussion

The properties and substrate specificity of an aminoacylproline hydrolase active against a range of dipeptides and tripeptides are described in the present study. Although aminoacylprolines were good substrates with this peptide hydrolase, other dipeptides such as L-Lys-L-Leu and L-Pro-L-Ile were also readily hydrolysed. This enzyme was also capable of releasing the amino terminal amino acid from a tripeptide containing proline in the central position. Although L-Phe-L-Pro was not a good substrate for the present enzyme it is interesting that this was the only aminoacylproline hydrolysed by another guinea-pig peptide hydrolase termed "α" peptide hydrolase [2]. Likewise, peptides containing N-terminal glycine were very poor substrates for the present enzyme and for the "α" peptide hydrolase [2] but were excellent substrates for another peptide hydrolase, the "β" peptide hydrolase [13]. In common with the "α" peptide hydrolase the present enzyme hydrolysed L-Leu-*p*-nitroanilide and L-Leu-β-naphthylamide with difficulty, and it was inactive with substrates containing D-amino acids or blocked N-termini. The present enzyme was also inactive with substrates containing glutamic acid in the N-terminal position or amide derivatives of C-terminal groups.

In contrast to the "β" peptide hydrolase of guinea-pig intestinal mucosa the present aminoacylproline hydrolase was stimulated by Mn^{2+} ions which also activated the "α" peptide hydrolase from the same source. The present peptide hydrolase with a molecular weight of 112 000 is much smaller than other peptide hydrolases from guinea-pig intestine [2,13].

On the basis of its substrate specificity this present enzyme is different from the prolidase of Adams and Smith [3] and from other prolidases purified from pig intestinal mucosa [5,15] and swine kidney [4,16]. These latter enzymes displayed no significant activity against substrates other than aminoacylprolines. Hill and Schmidt [17], however, described a swine kidney prolidase capable of hydrolysing Gly-L-Pro-L-Leu.

The present enzyme is also unlike an enzyme from swine kidney microsomes termed *X*-prolylaminopeptidase [18]. This latter enzyme is active with peptides of different sizes and possesses an absolute requirement for a proline residue in the second position from the N-terminal end of the substrate. The *X*-prolylaminopeptidase is microsomal whereas the present enzyme is largely

cytoplasmic. Both *X*-prolylaminopeptidase and the present aminoacylproline hydrolase are capable of hydrolysing Gly-L-Pro-L-Ala but unlike the *X*-prolylaminopeptidase [19] the aminoacylproline hydrolase was not stimulated by bovine serum albumin in the presence of Mn^{2+} ions.

The present aminoacylproline hydrolase is therefore distinct from other related enzymes described in the literature. The enzyme is probably responsible for the hydrolysis of the majority of the aminoacylproline substrates presented to the guinea-pig intestinal mucosa and may also participate, together with other peptide hydrolases, in the hydrolysis of peptides which do not contain proline.

Acknowledgements.

We thank the Medical Research Council of Ireland and the Wellcome Trust for financial support.

References

1 Donlon, J. and Fottrell, P.F. (1972) Comp. Biochem. Physiol. 41B, 181–193
2 Donlon, J. and Fottrell, P.F. (1973). Biochim. Biophys. Acta 327, 425–436
3 Adams, E. and Smith, E.L. (1952) J. Biol. Chem. 198, 671–682
4 Davis, N.C. and Smith, E.L. (1957) J. Biol. Chem. 224, 261–275
5 Sjostrom, H., Noren, O. and Josefsson, L. (1973). Biochim. Biophys. Acta, 327, 457–470
6 Donlon, J. and Fottrell, P.F. (1971) Clin. Chim. Acta 33, 345–350
7 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
9 Dunker, A.K. and Ruechert, R.R. (1969) J. Biol. Chem. 244, 5074–5080
10 Diezel, W., Kopperschlager, G. and Hoffmann, E. (1972) Anal. Biochem. 48, 617–620
11 Nolan, D. and O'hEocha, C. (1967) Biochem. J. 103, 39–40 p
12 Fruton, J.S. (1970) in Advances in Enzymology (Nord, F.F., ed.), Vol. 33, 401–443, Interscience, New York
13 Piggott, C.O. and Fottrell, P.F. (1974) Biochem. Soc. Trans. 2, 1366–1368
14 O'Cuinn, G., Piggott, C.O. and Fottrell, P.F. (1975) FEBS Lett. 53, 164–168
15 Smith, E.L. and Bergman, M. (1944) J. Biol. Chem. 153, 627–651
16 Manao, G., Nassi, P., Cappugi, G., Camici, G. and Ramponi, G. (1972) Physiol. Chem. and Physics 4, 75–87
17 Hill, R.L. and Schmidt, W.R. (1962) J. Biol. Chem. 237, 389–396
18 Dehm, P. and Nordwig, A. (1970) Eur. J. Biochem. 17, 364–371
19 Dehm, P. and Nordwig, A. (1970) FEBS Lett. 9, 225–228