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PURIFICATION AND CHARACTERIZATION FROM GUINEA-PIG INTESTINAL MUCOSA OF TWO PEPTIDE HYDROLASES WHICH PREFERENTIALLY HYDROLYSE DIPEPTIDES

C.O. PIGGOTT and P.F. FOTTRELL

Department of Biochemistry, University College, Galway (Ireland)

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Summary

The purification and characterization of two peptide hydrolases from guinea pig intestinal mucosa is described. Both enzymes had very similar properties and preferentially hydrolysed dipeptides containing N-terminal glycine residues.

Introduction

Intestinal peptide hydrolases are responsible for the final stages of much of protein digestion in many omnivores and carnivores and may be implicated in protein malabsorption conditions [1,2]. In previous studies [3] from this laboratory seven electrophoretically distinct soluble peptide hydrolases were detected in guinea pig intestine and two of these enzymes were purified and characterized [4,5]. One hydrolysed a range of dipeptides and tripeptides but preferentially acted on peptides containing neutral or aliphatic amino acids [4]. The other enzyme preferentially hydrolysed peptides containing proline in the C-terminal position [5].

The present report describes the purification and characterization of two further soluble peptide hydrolases (previously [3] termed β_1 and β_2 peptide hydrolases) from quinea-pig intestinal mucosa. Unlike the aforementioned enzymes the present hydrolases preferentially split dipeptides, particularly, dipeptides containing N-terminal glycine.

Materials and methods

Enzyme assays. Enzyme activity was measured with 5 mM L-Leu-L-Leu in 0.1 M Tris · HCl buffer, pH 8.2 as previously described [6]. Hydrolysis of Gly-Gly was measured on a Joel JLC-5AH amino acid analyser.

Polyacrylamide gel electrophoresis. Preparative polyacrylamide gel electrophoresis was carried out using a commercial apparatus (Shandon Scientific Company, London). The gels were prepared according to a scaled-up modification of the analytical procedure of Davis [7]. Gel lengths of 6.0 cm and 9.5 cm were used for the separating and contact gels respectively. The separated proteins were eluted using 50 mM sodium tetraborate buffer, pH 8.4.

Analytical polyacrylamide gel electrophoresis was performed according to Davis [7] and proteins were stained using a modification of the amino acid oxidase - peroxidase zymogram stain previously described for starch-gel electrophoresis [3]. The modification involved incorporating 1% soluble starch in the overlay mixture.

Protein determination. The protein concentration of crude extracts was estimated by the Biuret method [8] using bovine serum albumen as standard. The elution of protein from chromatography and electrophoresis columns was monitored at 280 nm.

Purification procedure. All operations were carried out at 4°C.

Step 1. Fresh guinea-pig intestinal mucosa (14 g) was homogenized in a Waring blendor with 10 vol. of 50 mM sodium tetraborate buffer, pH 8.4. The homogenate was centrifuged at 30 000 × *g* for 30 min and supernatant was retained.

Step 2. Solid ammonium sulphate was added to 40% saturation to the buffered supernatant which was then allowed to stand for 30 min. The resulting precipitate was collected by centrifugation at 30 000 × *g* for 20 min and discarded. Solid ammonium sulphate was added to 65% saturation to the supernatant and after standing for 30 min the suspension was centrifuged at 30 000 × *g* for 30 min. The sediment was dissolved in 8 ml of 50 mM sodium tetraborate buffer, pH 8.4, and dialysed against two changes of the same buffer for 18 h.

Step 3. The dialysate was then passed through a column of DE-52 cellulose (1.5 cm × 25 cm) equilibrated with 50 mM sodium tetraborate buffer, pH 8.4. The column was washed for 19 h with 500 ml of equilibrating buffer and proteins were eluted with a linear gradient of NaCl (in 50 mM sodium tetraborate buffer, pH 8.4) from 0.00 to 0.35 M. Fractions rich in Leu-Leu hydrolase activity were pooled.

Step 4. Solid ammonium sulphate was added to 80% saturation to the pooled fractions rich in Leu-Leu hydrolase activity from Step 3. The precipitate, obtained by centrifugation at 30 000 × *g* for 20 min, was resuspended in 1.0 ml of 50 mM sodium tetraborate buffer, pH 8.4 and the solution was dialysed for 18 h against the same buffer.

Step 5. The dialysed material was then applied to a preparative polyacrylamide gel column and electrophoresis was allowed to proceed for 16 h at pH 8.1. The separated proteins were eluted using 50 mM sodium tetraborate buffer, pH 8.4.

Results

Purification of peptide hydrolases. Ammonium sulphate fractionation of the crude mucosal extract yielded a fraction which was essentially free from

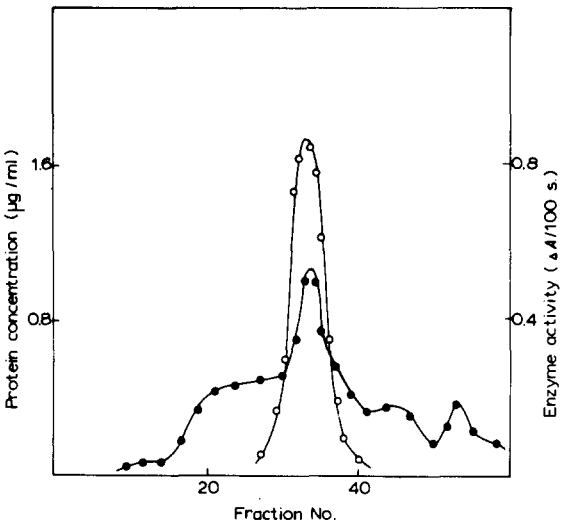

Fig. 1. Chromatography on DE-52 cellulose of the dialysed ammonium sulphate precipitate (from Step 2 in text). Protein (●———●) was measured by absorption at 280 nm, and enzyme activity (○———○) was estimated using L-Leu-L-Leu as substrate.

two other peptide hydrolases i.e. α and γ peptide hydrolases [3—5]. The ion-exchange step (Fig. 1) separated most of the remaining contaminating peptide hydrolase activities and the β_1 and β_2 peptide hydrolases eluted together as a single enzyme peak which indicated a close similarity between these two enzymes. Final separation of the enzymes was achieved by preparative polyacrylamide gel electrophoresis. The elution pattern, illustrated in Fig. 2, shows that the β_2 enzyme is the predominant species. Analytical polyacrylamide gel elec-

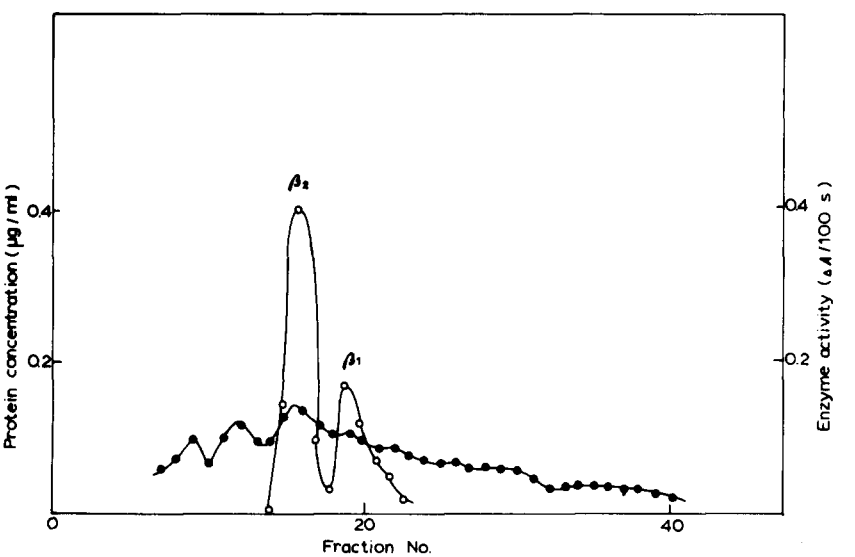

Fig. 2. Elution profile from polyacrylamide column of pooled DEAE fractions containing β_1 and β_2 peptide hydrolases. Protein (●———●) was monitored by recording the absorbance at 280 nm and peptide hydrolase activity (○———○) was measured using L-Leu-L-Leu as substrate.

TABLE I

PURIFICATION OF β_1 AND β_2 PEPTIDE HYDROLASES FROM GUINEA-PIG INTESTINAL MUCOSA

Enzyme activity was assayed as described in text with L-Leu-L-Leu as substrate.

	Volume (ml)	Total activity (μmol/min) $\times 10^{-4}$	Specific activity (μmol/min per protein) $\times 10^{-4}$	Total protein (mg)	Purification (fold)	Recovery (%)
Whole homogenate	78	7200	4	1764	1	100
Supernatant	60	4800*	8	600	1	100*
40–65% $(NH_4)_2SO_4$	8	4200	12.5	340	1.56	87
DEAE chromatography	37	1200	61	20	7.62	25
Preparative electrophoresis						
β_2 enzyme	9	50	46	1.2	5.75	1.0
β_1 enzyme	9	34	36	1.0	4.5	0.8

* Based on an estimated 70% of the L-Leu-L-Leu hydrolase activity in the supernatant being attributable to the β_1 and β_2 peptide hydrolases. The total L-Leu-L-Leu hydrolase activity of the supernatant is $6860 \cdot 10^4$ μmol/min.

trophoresis indicated that each of the purified peptide hydrolases consisted of a single protein band.

A summary of the purification procedure is given in Table I.

Stability. Solutions of the purified peptide hydrolases were repeatedly frozen and thawed without loss of activity. Frozen samples (– 20° C), retained enzyme activity for one year. However, after storage at 4° C only 10% of enzyme activity was retained after one week. Addition of glycerol, sucrose or ammonium sulphate failed to stabilize the enzymes at 4° C.

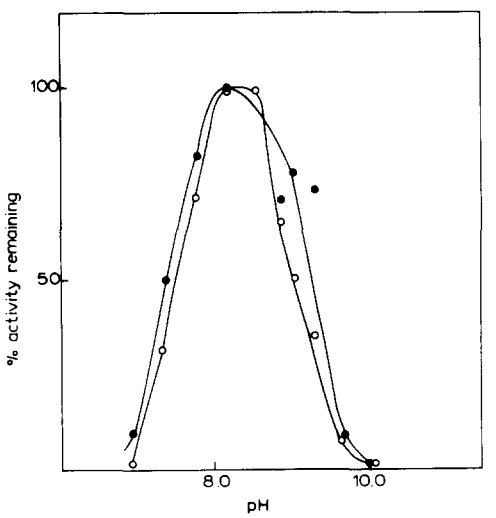

Fig. 3. pH optimum curves for the β_1 (○———○) and β_2 (●———●) peptide hydrolases. The buffer used was 0.1 M Tris · HCl and assays were performed with L-Leu-L-Leu at pH 8.4.

TABLE II

EFFECT OF METAL IONS ON THE β_1 AND β_2 PEPTIDE HYDROLASES FROM GUINEA-PIG INTESTINAL MICOSA

Activity was measured with L-Leu-L-Leu using 0.1 M Tris · HCl buffer, pH 8.2. Solutions of substrate and metal ions (0.5 mM) were prepared with this buffer. Enzyme ectivity in the presence of the various metals was expressed as a percentage of the value of the control tubes to which no metals were added.

Metal	% Activity	
	β_1 hydrolase	β_2 hydrolase
Control	100	100
$CoCl_2$	0	0
$ZnSO_4$	29	20
$MnSO_4$	29	20
$MgSO_4$	100	100
$CuSO_4$	0	0

pH optima and buffer effects. The effects of pH on the velocity of enzymic activity against L-Leu-L-Leu is shown in Fig. 3. The maximum velocity for both peptide hydrolases occurs at pH 8.2. The enzyme activity at the pH optimum was measured in three buffers (0.1 M), Tris · HCl, sodium barbitone and sodium tetraborate-boric acid. Maximum activity was observed with Tris buffer, while barbitone and borate buffers gave values representing 70% and 40% respectively of the activity in Tris buffer.

Inhibition by metals. Both peptide hydrolases were inhibited to the same degree by Co^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} (Table II). Mg^{2+} had no effect on enzyme activity.

Substrate specificities. Eleven dipeptides hydrolysed by purified β_1 and β_2 peptide hydrolases are shown in Table III. All of the peptides listed were hydrolysed by both enzymes with one exception, L-Phe-L-Ala, which was hydrolysed by β_2 but not by β_1. A wide range of peptides and peptide derivatives

TABLE III

SUBSTRATES HYDROLYSED BY β_1 AND β_2 PEPTIDE HYDROLASES FROM GUINEA-PIG INTESTINAL MUCOSA

Assays were performed in 0.1 M Tris · HCl buffer pH 8.2 using 5 mM substrate in all cases.

Substrates
Gly-L-Tyr
Gly-L-Phe
Gly-L-Trp
Gly-L-Leu
L-Leu-L-Leu
L-Leu-L-Ala
L-Leu-L-Tyr
L-Val-L-Leu
L-Phe-L-Leu
L-Val-L-Phe
L-Phe-L-Ala*

* Not hydrolysed by β_1 peptide hydrolase.

TABLE IV

PEPTIDES AND ESTERS WHICH ARE EITHER POOR SUBSTRATES OR ARE NOT HYDROLYSED BY THE β_1 AND β_2 PEPTIDE HYDROLASES.

Poor substrates	Substrates not hydrolysed
L-Leu-Gly	L-Leu-*p*-nitroanilide
L-Leu-L-Leu-L-Leu	L-Pro-L-Leu
L-Leu-Gly-Gly	γ-L-Glu-L-Leu
L-Pro-L-Met	L-Leu-L-Pro
L-Pro-L-Phe	L-Leu-D-Leu
L-Met-L-Met-L-Ala	D-Leu-L-Leu
L-Met-Gly-Gly	D-Leu-D-Leu
	Cbz-Gly-L-Leu
	Cbz-Gly-L-Phe
	Cbz-Gly-L-Trp
	L-Leu-L-Leu-amide
	Hippuryl-L-Arg
	Hippuryl-L-Phe
	L-Gly-D-Leu
	L-Phe-Gly

Abbreviation: Cbz, *N*-carboxybenzo.

were either poorly hydrolysed or not hydrolysed at all by β_1 and β_2 hydrolases (Table IV).

Both enzymes were free of endopeptidase activity on denatured cytochrome *c*.

Discussion

These studies show that β_1 and β_2 peptide hydrolases split a large number of dipeptides, several of which were also hydrolysed by other intestinal hydrolases such as the α and γ hydrolyses [4,5]. A notable feature of the β_1 and β_2 enzymes is their ability to hydrolyse peptides containing N-terminal glycine residues. Peptides such as the latter were not hydrolysed by the aforementioned α and γ [4,5]. Conversely, peptides containing proline were not hydrolysed by the present β_1 and β_2 enzymes whereas such substrates were preferentially hydrolysed by the γ enzyme which is an amino-acyl proline hydrolase [5]. The α peptide hydrolase has a broader substrate specificity than other hydrolases and was particularly efficient in hydrolysing tripeptides [4]. It seems therefore that although the four peptide hydrolases purified to date hydrolyse a wide variety of peptides, some degree of specificity exists with each enzyme and the enzymes appear to act in a complimentary fashion. The present hydrolases also showed some similarity to peptide hydrolases recently purified from monkey and pig intestinal mucosa [9,10]. For instance, these latter enzymes also hydrolysed dipeptides containing aromatic or neutral amino acids, including dipeptides containing glycine.

The present peptide hydrolases were unusual in so far as they were not activated by metal ions such as Mg^{2+}, Mn^{2+} or Co^{2+}. The latter metals in fact inhibited the hydrolases in contrast with the influence of these metals on other peptide hydrolases [4,5,9–12].

Finally, these studies strongly suggest that the two hydrolases in this report are very similar enzymes. For instance, both enzymes have similar specificities, stabilities, optimum pH values in different buffer systems and both behave in a similar fashion in the presence of various metal ions. Additional structural and immunochemical studies are in progress to further examine the extent of this similarity between the two hydrolases.

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