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PURIFICATION AND CHARACTERIZATION OF ONE OF THE FORMS OF PEPTIDE HYDROLASES FROM GUINEA-PIG SMALL INTESTINAL MUCOSA

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SUMMARY

One of the forms of peptide hydrolases occurring in guinea-pig small intestinal mucosa was purified and characterized. The enzyme, formerly termed "α" peptidase, is an aminopeptidase with an apparent molecular weight of 300 000 daltons. It is activated by Mn^{2+} and is not inhibited by *p*-chloromercuribenzoate or diisopropylfluorophosphate.

Kinetic parameters have been calculated to determine its substrate specificity. These results showed that this peptide hydrolase has a low order of specificity for dipeptides and tripeptides but preferentially hydrolysed peptides containing neutral or aromatic amino acid residues.

The kinetics of hydrolysis of some substrates indicated that there may be two active sites on the enzyme molecule; one of these sites being Mn^{2+}-dependent, the other Mn^{2+}-independent.

This aminopeptidase is compared to other well characterized aminopeptidases and its possible classification is also discussed.

INTRODUCTION

In a previous study[1] seven electrophoretically distinct peptide hydrolases (peptidases) were separated by starch-gel electrophoresis of extracts from guinea-pig small intestinal mucosa. Preliminary studies[1] on the substrate specificities and subcellular location of these forms suggested that they were capable of hydrolysing a broad range of dipeptides and tripeptides and that similar forms occurred throughout the intestinal epithelial cells with the greater proportion of the activities being located in the cytoplasm. Detailed information about the subcellular location and substrate specificities of these enzymes is essential for a thorough understanding of protein digestion and the aetiology of protein malabsorption conditions such as coeliac disease (gluten-induced enteropathy)[2].

Other workers have purified[3–6] or partially purified[7–12] intestinal peptide

Abbreviation: Cb_2-*N*-carboxybenzo.

hydrolases that are active against particular substrates. However, relatively little quantitative information is available on the substrate specificities of purified preparations of these enzymes. The purpose of this present investigation was to obtain definitive information concerning the substrate specificity and general properties of one of the forms of intestinal peptide hydrolases[1,13].

This report describes the purification and detailed characterization of a cytoplasmic peptide hydrolase from guinea-pig small intestinal mucosa, which has been termed "α" peptidase on the basis of its electrophoretic mobility on starch gels. A preliminary communication of part of these results has appeared[14].

MATERIALS AND METHODS

Peptide hydrolase assay

Enzyme activity was routinely assayed with 5 mM L-Leu–L-Leu in 25 mM sodium tetraborate buffer (pH 9.2), containing 0.5 mM $MnCl_2$ and the L-Leu released was measured with L-amino acid oxidase (EC 1.4.3.2) coupled with peroxidase (EC 1.11.1.7), as described previously[15]. Because some of the reagents tested in these studies interfered with the L-amino acid oxidase–peroxidase coupled assay system, peptide hydrolysis was also measured on a Jeol JLC-5AH amino acid analyzer, essentially according to Spackman *et al.*[16]. In these latter experiments peptide hydrolase activity was stopped by the addition of 1.53 ml of 0.01 M HCl prior to amino acid analysis. Peptide hydrolase activity was expressed as nmoles L-Leu released per min/ml of enzyme solution.

Purification procedure

In a typical preparation 1.5 g of guinea-pig intestinal mucosa was homogenized in 10 ml of cold isotonic saline by 6 up-and-down strokes in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10 000 $\times$ *g* for 10 min and the supernatant was recentrifuged at 34 000 $\times$ *g* for 20 min at 4 °C. A 3-ml sample of the supernatant was chromatographed on a Sephadex G-200 column (67 cm $\times$ 2.5 cm) at 4 °C. The flow rate of the eluant (25 mM Tris–HCl buffer (pH 7.6), containing 100 mM NaCl), was 15 ml/h. Fractions of 2.5 ml were collected and tested for protein (by absorbance at 280 nm) and for peptide hydrolase activity in the presence and absence of 0.5 mM $MnCl_2$. The location of the various multiple forms of peptide hydrolases in these fractions was determined by starch-gel electrophoresis with L-Leu–L-Leu and L-Leu–L-Leu–L-Leu as substrates[1].

Fractions containing α peptide hydrolase were pooled and brought to 40% (v/v) with acetone at -12 °C. The resultant precipitate was suspended in 50 mM veronal buffer (pH 8.6) and applied to a column (15 cm $\times$ 1.4 cm) of DEAE-Sephadex A-25, previously equilibrated with the latter buffer. Elution was performed using a NaCl gradient generated with a three-line peristaltic pump (Stalprodukter, Uppsala, Sweden); flow rate 12.5 ml/h. The active fractions (2.2 ml each) were pooled and stored at -20 °C.

Low protein concentrations were determined using the Folin reagent[17], whereas for higher concentrations a modified biuret method[18] was used. Polyacrylamide-gel electrophoresis was carried out by the method of Davis[19]. Protein was located in the polyacrylamide gels by staining with Coomassie Blue[20,21].

Molecular weight determinations

The molecular weight of the peptide hydrolase was estimated by gel filtration[22] on a Sephadex G-200 column (46 cm × 2 cm) calibrated with cytochrome *c*, egg albumin, bovine serum albumin (Serva, Heidelberg, Germany), R-phycocyanin and R-phycoerythrin; the latter two biliproteins were gifts from D. Nolan of this laboratory.

Activation and inhibition studies

The various reagents were included in the assay buffer. A range of spectrographically standardised metal salts were obtained from Johnson Matthey (London),

Substrate specificity and related studies

The analyzer method was also used to determine the exo-specificity of this peptide hydrolase and to examine and quantitate the hydrolysis products of substrates having amino acid residues which are not substrates for L-amino acid oxidase (*Crotalus adamanteus*)[23].

Peptides were purchased from Sigma Chemical Co. (London) and from Cyclo Chemical Co. (Los Angeles). Tetra-Gly, penta-Gly, hexa-Gly and hexa-L-Leu were gifts from Dr T. J. Peters (Royal Postgraduate Medical School, London). Tetra-L-Leu and L-Pro–L-Leu–L-Leu were synthesized by the solid phase technique[24]. The Thr (*tert*-butyl)-peptides together with *O-tert*-butyl-Thr were kindly donated by Dr Rolf Jost (Weizmann Institute of Science, Rehovot, Israel).

Kinetic parameters were determined from double reciprocal plots of velocities versus substrate concentrations[25]. All incubations were conducted in the presence of 0.5 mM $MnCl_2$. Further calculations based on these data were performed according to Fruton[26].

RESULTS

Purification and fundamental properties of α peptide hydrolase

The α peptide hydrolase was eluted from the Sephadex G-200 column between the main protein peak and an enzyme peak containing the other peptide hydrolases, as shown in Figs 1, 3a and 3b. Any contaminating peptide hydrolases were removed by the acetone treatment. The elution profile for the ion-exchange step is shown in Fig. 2. The final preparation of α peptide hydrolase was homogeneous by starch-gel electrophoresis (Figs 3a and 3b). Polyacrylamide-gel electrophoresis of a sample of purified enzyme concentrated 100-fold showed a single protein band.

The data in Table I indicate a 50-fold purification of the enzyme with a 12% yield. These latter calculations are based on an estimated 75% of the L-Leu–L-Leu–L-Leu hydrolase activity in the final supernatant being attributable to α peptide hydrolase (see Fig. 3b).

pH optimum

Preliminary data on the pH optimum of the purified hydrolase were obtained with a series of amine buffers having constant ionic strength[27]. A broad plateau over the range pH 8.9–9.7 was observed. The addition of 0.5 mM $MnCl_2$ did not alter this pattern. These results were confirmed using a range of sodium tetraborate buffers.

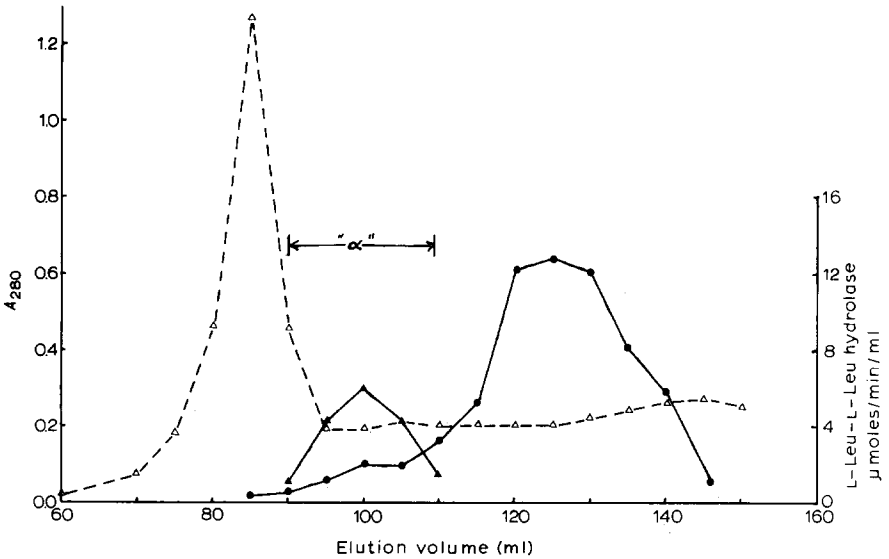

Fig. 1. Elution pattern of L-Leu-L-Leu hydrolase from Sephadex G-200 column as assayed in the absence (●—●) and presence (▲—▲) of 0.5 mM $MnCl_2$. The protein profile was monitored by measuring absorbance at 280 nm (△---△).

Molecular weight

The elution pattern of α peptide hydrolase from Sephadex G-200 was indicative of a relatively large molecule. A molecular weight of 300 000 daltons was determined from the calibrated Sephadex G-200 column.

Metal requirement

From a range of metal salts tested only divalent manganese showed any significant activation[14]. A 3-fold activation, compared with controls, was obtained

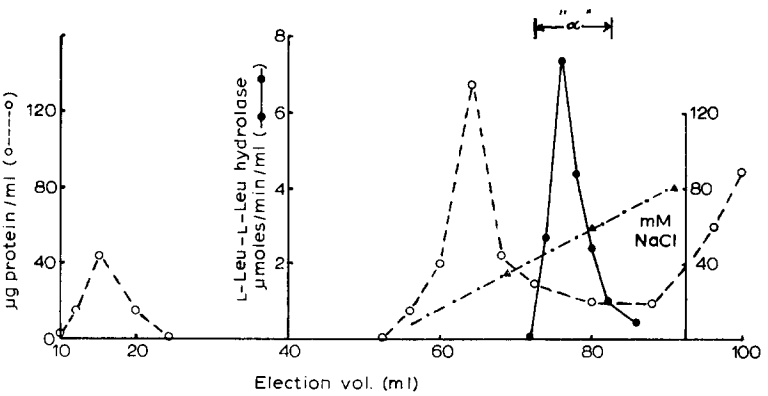

Fig. 2. Ion-exchange chromatography (DEAE-Sephadex A-25) of the resuspended cold acetone precipitate. Protein (○---○) was determined by a modified Folin-Lowry method[17]. L-Leu-L-Leu hydrolase activity (●—●) was assayed in the presence of 0.5 mM $MnCl_2$. The α peptide hydrolase was eluted with a NaCl gradient (▲—·▲).

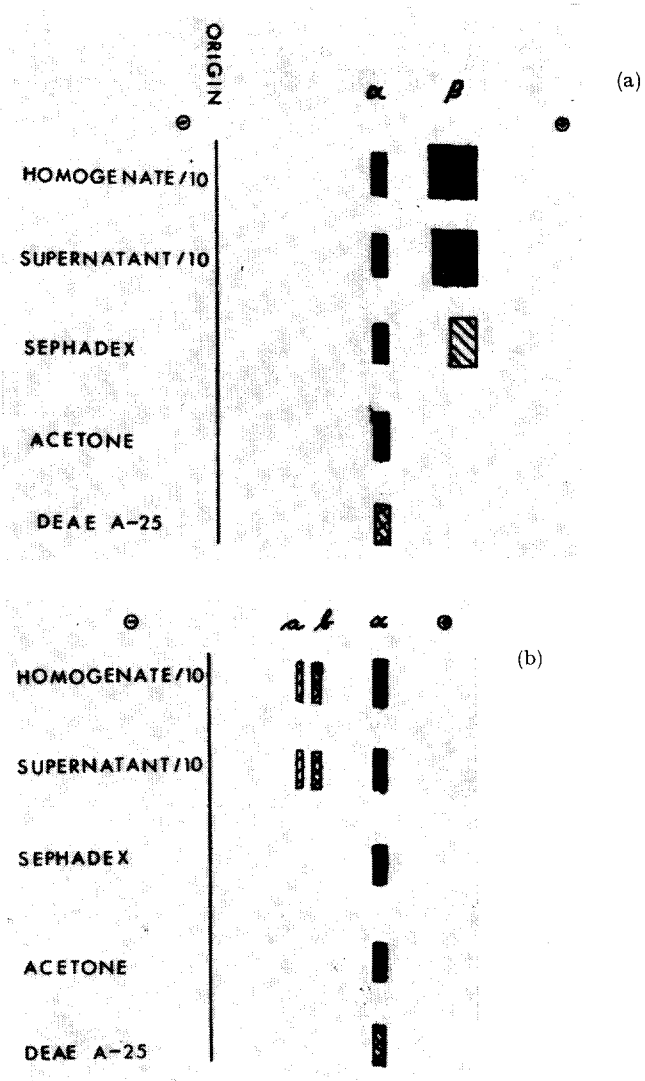

Fig. 3. a. Electrophoretic characterization of the L-Leu–L-Leu hydrolases present in the various fractions during the purification procedure. The electrophoretic and staining techniques have been described previously[1]. Relative intensties are indicated by shading. b. Electrophoretic characterization of the L-Leu–L-Leu–L-Leu hydrolases present in the various fractions during the purification procedure.

with either $MnSO_4$ or $MnCl_2$. The requirement for divalent manganese was also previously demonstrated in a series of experiments[14] with chelating agents, which complex transition elements with varying stability constants[28]. These chelating agents inhibited the unactivated enzyme to different degrees and also depressed the activation by 0.5 mM $MnCl_2$. The data in Figs 4 and 5 indicated that manganese interacted with the enzyme rather than the substrate. The dissociation constant for the metal was $3.3 \cdot 10^{-5}$ M (Fig. 5).

TABLE I

PURIFICATION OF α PEPTIDE HYDROLASE FROM GUINEA-PIG INTESTINAL MUCOSA

Enzyme activity was assayed, by the routine procedure, with L-Leu–L-Leu–L-Leu as substrate.

	Volume (ml)	*Total activity (μmoles/min)*	*Total protein (mg)*	*Spec. act. (μmoles/min per mg protein)*	*Purification (-fold)*	*Yield (%)*
Homogenate	9	344.25	186.75	1.84	1	100
Supernatant	7	249.9	71.75	3.48	1.9	72.6
Supernatant	3	80.33*	30.75	2.61*	1	100
Sephadex G-200	23	55.72	9.57	5.82	2.2	69.4
Acetone	21	32.13	4.56	7.05	2.7	40.00
DEAE-Sephadex A-25	13	12.71	0.195	65.17	25.0	15.82
					Overall 48-fold	11.5%

* Based on an estimated 75% of the L-Leu–L-Leu–L-Leu hydrolase being attributable to α peptide hydrolase.

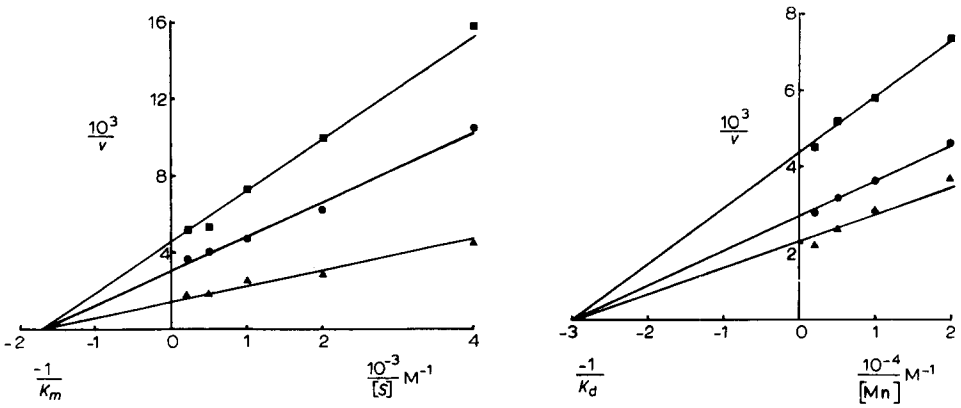

Fig. 4. Double reciprocal plots of velocities versus L-Leu–L-Leu concentrations in the absence of $MnCl_2$ (■—■) and in the presence of 0.05 mM (●—●) and 0.5 mM $MnCl_2$ (▲—▲). Peptide hydrolase activities are expressed as nmoles L-Leu released per min/ml.

Fig. 5. Double reciprocal plots of velocities *versus* $MnCl_2$ concentrations at 0.25 mM (■—■), 1 mM (●—●) and 5 mM L-Leu–L-Leu. Peptide hydrolase units as in Fig. 4.

Inhibitors

Purified α peptide hydrolase was not inhibited by the serine-protease inhibitor, diisopropylfluorophosphate (0.1 mM). Only 10% inhibition was observed with the unactivated enzyme in the presence of the sulphydryl-reagent, para-chloromercuribenzoate (0.1 mM). *p*-Chloromercuribenzoate inhibited the activated enzyme by 35%. Mercaptoethanol (5 mM) had no appreciable affect on the enzyme. Likewise, no inhibition occurred with iodoacetate (2 mM), 3-phenylpropionate (2 mM), tetranitromethane (50 μM), glycerol (1.37 M) or *n*-butanol (0.1 M).

Aminopeptidase activity

Preliminary evidence suggested that α peptide hydrolase might be an aminopeptidase. When 5 mM L-Met–L-Met–L-Ala was incubated with the purified enzyme L-Met was the first residue released. As hydrolysis proceeded, the concentration of

L-Met–L-Ala increased and this dipeptide then acted as a substrate, resulting in the release of L-Ala.

Substrate specificity

The kinetics of hydrolysis of 17 peptides together with L-Leu amide and L-Leu–β-naphthylamide by the purified α peptide hydrolase were examined. The following parameters were determined for the hydrolysis of each peptide: K_m, V, k_{cat} (V/E_0), k_{cat}/K_m (the specificity constant) and, finally, an index of the relative reactivity of each peptide was calculated based on the specificity constant, with an arbitrary value of 100 being assigned to L-Leu–L-Leu. (Table II). The rates of

TABLE II

KINETIC DATA FOR THE HYDROLYSIS OF VARIOUS PEPTIDES BY α PEPTIDE HYDROLASE

Substrate	K_m *(mM)*	V *(nmoles N-terminal residue released per min/ml)*	k_{cat} (s^{-1})	$\frac{k_{cat}}{K_m}$ $(mM^{-1} \cdot sec^{-1})$	*Relative reactivity*
L-Phe–L-Pro*	0.53	208.3	208.3	392.9	107.1
L-Leu–L-Leu	0.47	172.4	172.4	366.7	100.0
L-Lys–L-Phe**	(0.12)	(119.0)	(39.7)	(330.6)	(90.1)
L-Leu amide**	(0.09)	(78.4)	(26.1)	(290.3)	(79.2)
L-Leu–L-Tyr amide*	0.13	111.6	37.2	286.1	78.0
L-Val–L-Leu	0.25	157.3	52.4	209.7	57.2
L-Leu–L-Leu–L-Leu*	0.95	472.2	157.4	165.7	45.2
L-Leu–L-Tyr	0.52	233.8	77.9	149.8	40.9
L-Leu–L-Leu amide	1.0	441.1	147.0	147.0	40.1
L-Met–L-Met–L-Ala	0.5	204.0	68.0	136.0	37.1
L-Tyr–L-Tyr–L-Tyr*	0.15	59.5	19.8	132.2	36.0
L-Lys–L-Phe**	0.7	178.5	59.5	85.0	23.3
L-Leu–Gly–Gly	2.8	685.7	228.6	81.6	22.3
L-Phe–Gly**	(0.7)	(159.4)	(53.1)	(75.9)	(20.7)
L-Lys–L-Leu**	(0.7)	(141.7)	(47.2)	(67.5)	(18.4)
L-Leu–Gly	2.6	524.2	174.7	67.2	18.3
L-Tyr–Gly–Gly	0.15	29.8	9.9	66.1	18.0
L-Lys–L-Leu**	7	1180.5	393.5	56.2	15.3
L-Leu–L-Ala	7.1	1111.6	370.5	56.2	14.2
L-Leu amide	2.0	221.9	74.0	7.0	10.1
L-Phe–Gly**	2.7	263.5	87.8	32.5	8.9
L-Met–Gly–Gly	3.8	250.8	83.6	22.0	6.0
L-Leu–β-naphthylamide	2.5	122.8	40.9	16.4	4.5

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

** These substrates displayed two apparent K_m values (see Fig. 6). Bracketed values are based on extrapolations from double reciprocal plots.

hydrolysis of a further eight peptides and two amino esters were too low to permit kinetic analyses (Table III). A wide range of peptides and peptide derivatives not hydrolysed by the present enzyme are listed in Table IV. The α peptide hydrolase was free of endopeptidase activity on denatured cytochrome c (ref. 29). In the majority of cases peptide hydrolysis obeyed Michaelis–Menten kinetics. However, with four substrates, L-Lys–L-Leu, L-Lys–Phe, L-L-Phe–Gly and L-Leu amide, biphasic

TABLE III

PEPTIDES AND ESTERS WHICH ARE POOR SUBSTRATES FOR α PEPTIDE HYDROLASE

L-Phe–L-Leu
α-L-Glu–L-Leu
α-L-Glu–L-Tyr
α-L-Glu–L-Glu
α-L-Glu–L-Asp
α-L-Glu–L-Ala
Gly–Gly
L-Leu benzyl ester
L-Lys ethyl ester
L-Pro–L-Leu–L-Leu

plots were obtained (Fig. 6). Two apparent K_m values could be calculated from these plots for a substrate such as L-Lys–L-Leu (Fig. 6). Only the higher K_m value was observed when the latter substrate was hydrolysed in the absence of $MnCl_2$ (Fig. 6). The calculations in Table II for these substrates are based on the assumption that the observed K_m and V values relate to two independent sites on the enzyme molecule.

TABLE IV

PEPTIDES AND ESTERS NOT HYDROLYSED BY α PEPTIDE HYDROLASE

L-Leu–L-Pro amide	Gly benzyl ester
L-Leu–D-Leu	Gly *tert*-butyl ester
D-Leu–L-Leu	Gly methyl ester
L-Leu–L-Pro	Gly ethyl ester
D-Leu–Gly–Gly	Gly–Gly ethyl ester
D-Leu–D-Leu	Cb_z-Gly–L-Phe
L-Leu methyl ester	Cb_z-Gly–L-Leu
L-Leu ethyl ester	Cb_z-Gly–L-Trp
L-Leu *tert*-butyl ester	α-L-Glu–Trp
L-Leu–*p*-nitroanilide	α-L-Glu–β-naphthylamide
Gly–Gly–L-Leu	γ-L-Glu–β-naphthylamide
Gly–Gly–D-Leu	α-L-Glu–L-Pro
Gly–L-Leu	α-L-Glu–L-Glu
Gly–D-Leu	α-L-Glu–L-Val
Gly–D-Phe	L-Ala–L-Asp
Gly–Gly–Gly	Hippuryl–L-Phe
Tetraglycine	L-Pro–L-Leu
Pentaglycine	L-Pro–L-Ile
Hexaglycine	L-Pro–L-Tyr
Gly–L-Phe	L-Pro–L-Met
Gly–L-Tyr	L-Pro–L-Trp
Gly–L-Trp	L-Pro–L-Phe
Gly–L-Asp	L-Pro–Gly
Gly–L-Glu	L-Val–L-Phe
Gly–L-Val	L-Val *tert*-butyl ester
Gly–L-Pro	L-Tyr–L-Tyr–L-Tyr methyl ester
Gly–L-Lys	Tetra-L-Leu

α peptide hydrolase and leucine aminopeptidases

Jost *et al.*[30] have shown that threonine (*tert*-butyl)-peptides acted as strong competitive inhibitors of leucine aminopeptidases (EC 3.4.1.1) from swine kidney and bovine eye lens whereas aminopeptidase M (EC 3.4.1.2) from swine kidney microsomes was not inhibited[31]. Neither was the latter enzyme capable of degrading these peptides

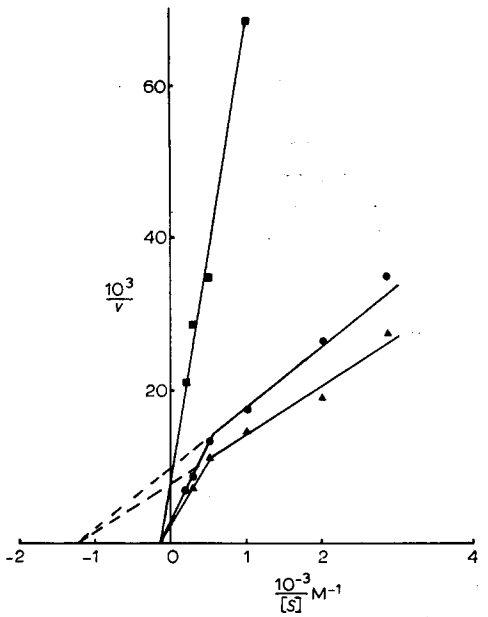

Fig. 6. Double reciprocal plots of velocities versus L-Lys–L-Leu concentrations in the absence (■—■) of $MnCl_2$ and in the presence of 0.25 mM (●—●) and 0.5 mM $MnCl_2$ (▲—▲). Peptide hydrolase units as in Fig. 4.

at a significant rate[31]. The present enzyme did not hydrolyse 5 mM H-(But)–Thr–Phe–OH or 5 mM H-(But)–Thr–Phe–Pro–OH. However, 1 mM H-(But)–Thr–Phe–OH and 0.5 mM H-(But)–Thr–Phe–Pro–OH inhibited the enzyme by 40–50%, but kinetic analysis showed that the inhibition was neither competitive, non-competitive or un-competitive (Fig. 7).

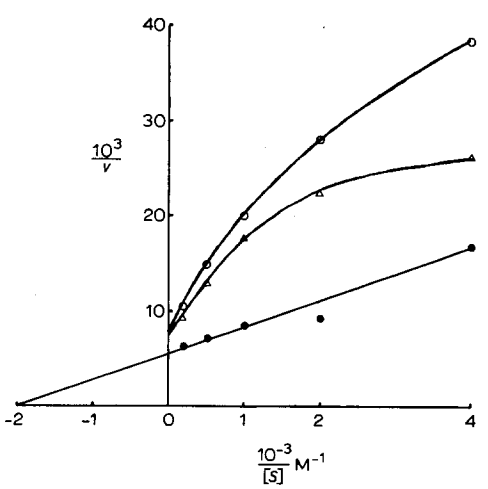

Fig. 7. Double reciprocal plots of velocities versus L-Leu–L-Leu concentrations in the presence of 1 mM H-(But)–Thr–Phe–OH (△—△) and 0.5 mM H-(But)–Thr–Phe–Pro–OH (○—○). Control activities are indicated by (●—●). Peptide hydrolase units as in Fig. 4.

DISCUSSION

This study has described a comprehensive investigation of the properties and specificity of one of the peptide hydrolases in guinea pig intestinal mucosa. Although intestinal peptidases have been purified[4–6] from species other than guinea pig, relatively little information was given about (1) homogeneity relative to other peptidases and (2) quantitative data on substrate specificity. The present enzyme had broad substrate specificity and hydrolysed a large number of dipeptides and tripeptides but not tetra- or hexa-L-Leu (Tables II and IV). The preferred substrates for the enzyme were dipeptides and tripeptides containing neutral, aliphatic or aromatic residues; one exception to this was L-Phe–L-Leu which was a poor substrate (Table III). Peptides containing prolyl residues, with the exception of L-Phe–L-Pro, were either poorly hydrolysed or not at all. Likewise, peptides containing D-amino acids, acidic amino acid residues or N-terminal glycine were not hydrolysed to any significant extent (Tables III and IV).

The majority of the peptides hydrolysed by α peptide hydrolase displayed simple Michaelis–Menten kinetics and were apparently hydrolysed at the same site on the enzyme molecule. For instance, when enzyme activity was measured simultaneously with two peptide substrates, the value obtained for the specific activity was intermediate between the original values obtained when the peptides were incubated alone with the enzyme.

A possible reason for the biphasic nature of double-reciprocal plots of velocities *versus* substrate concentrations for some substrates (Fig. 6) might be that two substrate-binding sites are present on the enzyme. One site has a low affinity for substrate and is not dependent on the presence of Mn^{2+} for catalytic activity. Whereas, a second site with relatively high affinity for substrate appears in the presence of Mn^{2+}.

Mn^{2+} was clearly the most effective metal activator of the enzyme[14]. The enzyme does not appear to be a true metalloenzyme[32] since in the absence of added manganese it was only inhibited 15% by 5 mM EDTA[14]. The inhibition of the unactivated enzyme by *o*-phenanthroline (5 mM), 8 hydroxyquinoline (2 mM) and diethyldithiocarbamate (2 mM) might be due to effects unrelated to the chelating properties of these compounds. The inhibition by *o*-phenanthroline, for instance, was non-competitive.

The present enzyme is not related to the pancreatic serine-proteases such as trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5) since it was not inhibited by excess diisopropylfluorophosphate[33]. Neither was the enzyme affected by 3-phenylpropionate or tetranitromethane; therefore, it is distinct from the carboxypeptidases[34–37] (EC 3.4.2.1 and EC 3.4.2.2).

The enzyme also had properties quite different from leucine aminopeptidase. For instance, L-leucinamide which is an excellent substrate for leucine aminopeptidase[38] was only 10% as reactive as L-Leu–L-Leu with the present enzyme (Table IV). Likewise, L-Leu–β-naphthylamide, the usual histochemical substrate for leucine aminopeptidase[39] was a very poor substrate for this enzyme; in contrast with leucine aminopeptidase the enzyme was not inhibited by glycerol or butanol[40]. Furthermore, this enzyme appears incapable of hydrolysing peptides with more than three amino acid residues[29].

Finally, this enzyme showed a striking difference compared with swine kidney

and bovine lens leucine aminopeptidase in its behavior towards Thr (*tert*-butyl)-peptides. These peptides markedly inhibited the enzyme but the kinetics of inhibition were unusual. The inhibition plots obtained suggested that the inhibitors reacted with a site or sites on the enzyme molecule other than the catalytic site (Fig. 7). For instance, when the data of Fig. 7 were replotted[41] a Hill coefficient of 1 was obtained for the H-(But)–Thr–Phe–OH inhibited curve at high substrate concentrations with a coefficient of 0.6 at low substrate concentrations. The transition in this plot occurred at approx. 50% satn ($S_{0.5}$)[41,42]. A Hill coefficient of 0.45 was obtained in the case of H-(But)–Thr–Phe–Pro–OH inhibition. These data suggest that these inhibitors induced a negative cooperativity effect[41].

This enzyme also differed from aminopeptidase M (EC 3.4.1.2)[43] in that a substrate for the latter enzyme, L-Leu–*p*-nitroanilide, was not hydrolysed and also in its behavior towards the Thr (*tert*-butyl)-peptides.

The α peptide hydrolase from guinea pig intestinal mucosa is therefore an aminopeptidase with properties distinct from leucine aminopeptidase (EC 3.4.1.1), aminopeptidase M (EC 3.4.1.2) or aminotripeptidases (EC 3.4.1.3)[44–46] and can be classified as another α-aminoacyl peptide hydrolase.

In conclusion these studies have shown that a peptide hydrolase capable of splitting tripeptides and dipeptides is present in the cytosol fraction of guinea pig intestinal mucosa and that the hydrolysis of di- and tripeptides produced during digestion could take place within the enterocytes of the small intestinal mucosa.

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