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HYDROLASES IN THE MUCOSA OF RAT SMALL INTESTINE FOR PHENYLALANINE-CONTAINING DIPEPTIDES

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SUMMARY

A qualitative survey, made with a high-voltage paper electrophoresis screening technique, showed that mucosa of rat small intestine catalyzes the hydrolysis of 15 dipeptides containing 7 different amino acids A recently described assay method was used for the quantitative determinations of hydrolase activities for Gly–L-Phe, Gly–L-Try, L-Met–L-Phe, L-Ala–L-Phe, L-Leu–L-Phe, Gly–L-Tyr, L-Phe–L-Met, L-Phe–L-Phe, L-Phe–Gly and L-phenylalanine amide in rat intestinal mucosa The L-Ala–L-Phe hydrolase activity was studied extensively It is rapidly inactivated above 45°, is unable to catalyze the hydrolysis of L-Ala–L-Phe diketopiperazine and D-Ala–D-Phe, is only slightly active with D-Ala–L-Phe or L-Ala–D-Phe and is inhibited by *p*-chloromercuribenzoate but not by DFP or *β*-phenylpropionic acid. The soluble L-Ala–L-Phe hydrolase activity of intestinal mucosa requires a divalent cation, probably Zn^{2+}, for full activity and is distinct from the mucosal L-leucyl-*β*-naphthylamidase, Gly–L-Ala–L-Phe aminopeptidase, and L-Phe–Gly hydrolase activities In mucosal homogenates approx 90% of the activity of the L-Ala–L-Phe hydrolase, and of 6 of the other dipeptide hydrolases studied, is in the soluble fraction The specific activity of soluble L-Ala–L-Phe hydrolase in intestinal mucosa is 30-fold higher than gastric, 4-fold greater than colonic and slightly less than renal specific activity Specific activity in intestinal mucosa is 4-fold greater than in the remaining layers of the intestinal wall Specific activities of hydrolases for L-Ala–L-Phe or L-Phe–Gly in mucosa from upper, middle and lower small intestine show no significant regional differences L-Ala–L-Phe hydrolase activity of rat intestinal mucosa increases almost 4-fold between approximately the 7th and 25th days of life Fasting a rat for 48 h does not significantly change the specific activity of L-Ala–L-Phe or L-Phe–Gly hydrolase of intestinal mucosa or kidney

* The term "peptide hydrolase activity" has been used in this manuscript instead of dipeptidase because peptide hydrolase is the term approved by the Enzyme Commission[1] and because none of the enzymatic activities to be discussed has been proven strictly to be a dipeptidase

INTRODUCTION

Although the mucosa of mammalian small intestine is rich in peptide hydrolase* activities, the function of peptide hydrolysis in the intestinal mucosa is not unequivocally established It seems likely, however, that peptide hydrolases play an essential role in the final phase of protein digestion, a function analogous to the role of disaccharidases in carbohydrate digestion Several intestinal dipeptide hydrolase activities have been distinguished from one another, and a few have been partially purified and studied[2] We have investigated the L-Ala–L-Phe hydrolase activity of rat intestinal mucosa and have compared it to other intestinal hydrolase activities for compounds containing aromatic amino acids This work was facilitated by the development of a method[3] for quantitative measurement of the hydrolysis of dipeptides containing L-phenylalanine, L-tyrosine or L-tryptophan

EXPERIMENTAL PROCEDURE

Sprague–Dawley rats (230–300 g males) were fasted overnight and were decapitated At 4° the small intestine was flushed with 100 ml of cold 0 25 M sucrose, was opened lengthwise, was rinsed with sucrose solution and was blotted Mucosa, scraped off with a spatula, was homogenized in 0 25 M sucrose (5–10 ml per g wet weight) in a Potter–Elvehjem homogenizer fitted with a teflon pestle Other tissues were homogenized in a similar manner High-speed supernatant solutions were prepared by centrifugation for 1 h at 105 000 × *g* in a Model L Spinco ultracentrifuge at 0° In the remaining manuscript "mucosal high-speed supernatant" is used to indicate a solution prepared in this manner from an homogenate of intestinal mucosa in 0 25 M sucrose

Carboxypeptidase A (twice crystallized) was obtained from the Worthington Biochemical Corp and lyophilized *Crotalus adamanteus* snake venom from Ross Allen Reptile Institute (Silver Springs, Fla). Chemicals were reagent grade, and water was twice distilled from glass vessels

For gel filtration chromatography a column (2 5 cm × 82 8 cm) of Sephadex G-200 (Pharmacia Fine Chemicals) was equilibrated and eluted at 4° with 0 01 M sodium phosphate buffer (pH 7 0) at a flow rate of 15 ml/h A desalting column (0 9 cm × 14 cm) was prepared with Sephadex G-25 (fine) and was equilibrated and eluted at 4° with 0 005 M Tris–HCl buffer (pH 7 0) at a flow rate of about 0 3 ml/min Anion-exchange chromatography was performed with a column (1 0 cm × 35 cm) of DEAE-cellulose (Selectacel, Type 70, 0 87 mequiv/g, from Schleicher and Schuell Co , Keene, N H) equilibrated at 4° with 0 005 M Tris–HCl buffer (pH 7 0)

Enzyme substrates and amino acid standards

Except for compounds noted below, all the amino acids, amides, dipeptides and L-leucyl-β-naphthylamide–HCl were obtained from Mann Research Laboratories Fox Chemical Co (Los Angeles, Calif) supplied Gly–L-Ala–L-Phe, D-Ala–L-Phe, L-Ala–D-Phe and D-Ala–D-Phe Cyclo Chemical Corp (Los Angeles, Calif) supplied L-Ala–L-Phe diketopiperazine The compounds were of the highest purity offered Each one was analyzed by subjecting 0 1 μmole to high-voltage paper electrophoresis at 56 V/cm for 45 min in 6 8% formic acid (pH 1 9) at 25–29° (High-voltage electro-

phoresis apparatus Model DW supplied by Gilson Medical Electronics, Middleton, Wisc and equipped with a special cooling system) Contamination by a ninhydrin-reactive compound of approx 0 1% would have been detected. Impurities were seen only with Gly–L-Phe and L-Met–Gly. Each preparation produced faint spots corresponding to its components Contamination was no greater than 0.3% Substrates were prepared for quantitative enzyme assays as 0 02 M aqueous solutions (L-Ala–L-Phe was 0 05 M and, because of limited solubility, L-Phe–L-Phe was 1 7 mM)

For routine assays solutions of L-Ala–L-Phe were used within 3 days of preparation, others were used on the day of preparation However, aqueous solutions of L-Ala–L-Phe were stable for at least 19 weeks at 4° Extract of rat intestinal mucosa catalyzed hydrolysis of 99 2% of the substrate in a solution stored this long This showed that during storage the dipeptide had not cyclized significantly, since rat intestinal mucosa is unable to hydrolyze the cyclic compound L-Ala–L-Phe diketopiperazine (see under RESULTS)

Qualitative screening test for peptide hydrolase activities

To screen a solution for many peptide hydrolase activities, we used high-voltage paper electrophoresis adapted to permit analysis of up to 85 samples on a single paper strip[3] A buffered substrate solution (0 02 M substrate in 0 2 M Tris–maleate buffer, pH 7 5) was mixed with an equal quantity of solution to be assayed, the mixture and appropriate controls were incubated for 15 to 30 min at 37°, and the reaction was stopped by placing the tubes in boiling water for 3 min The mixtures were cooled, and 0 01-ml samples were applied to paper for electrophoresis The dried strip was stained with ninhydrin[4], and the degree of hydrolysis was estimated to the nearest 20%

Quantitative assay for hydrolases of substrates containing L-phenylalanine, L-tryptophan or L-tyrosine

Quantitative measurements were made of hydrolase activities for Gly-L-Phe, Gly–L-Try, L-Met–L-Phe, L-Ala–L-Phe, L-Leu–L-Phe, Gly–L-Tyr, L-Phe–L-Met, L-Phe–L-Phe, L-Phe–Gly and L-phenylalanine amide In the assay[3] the amount of aromatic amino acid liberated was determined using an enzymatic spectrophotometric method[5] The standard incubation mixture contained 10 μmoles of substrate (0 835 μmole in the case of L-Phe–L-Phe), 100 μmoles of Tris–maleate buffer (pH varied with substrate, as indicated below), enzyme, and other additions when indicated in a total volume of 1 0 ml For mucosal high-speed supernatants, the approximate quantity of protein used was 4 μg for Gly–L-Phe hydrolase, 20 μg for L-Phe–L-Met, L-Phe–L-Phe and Gly–L-Tyr hydrolases, 70 μg for L-Phe–Gly hydrolase and L-phenylalanine amidase and 7 μg for the other hydrolases The reaction was carried out at 37° and was stopped after 15 min by addition of 0 1 ml of 60-62% $HClO_4$ In 2–5 min, 0 1 ml of 9 M KOH solution was added After centrifugation at 1600 $\times$ g for 10 min, aliquots of each supernatant were transferred to two silica cuvettes (1 0-cm light path) with a 1 2-ml capacity and were assayed for liberated L-phenylalanine, L-tyrosine or L tryptophan according to the protocol on page 412 (additions listed in ml)

The contents of each cuvette were mixed, and the absorbance of the experimental solution was read against the control solution at 308 nm (330 nm for tryptophan) Then 0.1 ml of snake venom solution (10 mg/ml) was added to each cuvette,

	Control	*Experimental*
0 2 M phosphate buffer (pH 6 5)	0 3	0 3
2 0 M sodium arsenate (pH 6 5)	0 5	
1 0 M sodium borate in 2 0 M sodium arsenate (pH 6 5)		0 5
Supernatant solution	0 2	0 2

the solutions were mixed and absorbance readings were made at 5-min intervals, beginning 10–20 min after addition of venom, until there was no further significant increase in absorbance This ordinarily occurred between 15 and 30 min The value for absorbance usually rose rapidly during the first 10 min and then continued to increase very slowly for a prolonged period of time The point at which the rate of increase in absorbance declined to its lowest value was selected for calculation of amino acid concentration On occasion, when values for absorbance rose to a maximum and then fell, the peak value was selected for the calculation A linear relation was observed between quantity of phenylalanine added to the cuvette and absorbance, over a range between 0 and 0 14 μmole of phenylalanine The absorbance was 0 8 when 0 14 μmole of phenylalanine was present

A unit of hydrolase activity was defined as that amount of enzyme required to catalyze the release of 1 μmole of phenylalanine, tyrosine or tryptophan under standard conditions Specific activity was defined as enzyme units per mg of protein

The same procedure was used to assay for carboxypeptidase A activity with *N*-Z–Gly–L-Phe as substrate The standard incubation mixture contained 10 μmoles of substrate, 150 μmoles of Tris–maleate buffer (pH 7 5), enzyme and other additions as indicated in a total volume of 1 0 ml

Nonenzymatic hydrolysis of L-phenylalanine amide during incubation and after addition of $HClO_4$ was reduced to below 0.6% by rapid neutralization of the $HClO_4$

In some experiments with phenylalanine-containing substrates, we added enzyme inhibitors, metal ions or other compounds to the hydrolase incubation mixture Except for 1 mM $CuCl_2$ or $MnCl_2$, none of these additions interfered with the enzymatic spectrophotometric assay for liberated L-phenylalanine under conditions of these experiments Addition of EDTA to the aliquot of hydrolase incubation mixture removed for the phenylalanine assay overcame the difficulty caused by $CuCl_2$ and $MnCl_2$ High-speed supernatants of rat intestinal mucosa and rat kidney did not release L-phenylalanine from the tissue proteins in the incubation mixture or metabolize L-phenylalanine added to the mixture

Leucyl-β-naphthylamidase assay

Hydrolysis of L-leucyl-β-naphthylamide was assayed according to the method of GOLDBARG *et al* [6] by incubation for 2 h at 37° and pH 7.0 A unit of activity was defined as the amount of enzyme required to catalyze the release of 1 nmole of β-naphthylamine under standard conditions

Other procedures

Protein was determined by the method of LOWRY *et al* [7] Pooled frozen human serum served as a standard The use of crystalline bovine albumin as a standard

would have resulted in values for protein concentration 1.15-fold greater than those we obtained. Nitrogen determinations (used for only one experiment) were made with a micro-Kjeldahl method

RESULTS

Survey of peptide hydrolase activities of rat intestinal mucosa

Mucosal high-speed supernatant was incubated with each of the substrates listed in Table I With 4 compounds, no hydrolysis was detected Cleavage of the remaining 19 varied between 100% and nearly 0%.

TABLE I

QUALITATIVE SCREENING TEST FOR PEPTIDE HYDROLASE ACTIVITY IN RAT INTESTINAL MUCOSA

High-speed supernatant of mucosa from the entire small intestine was incubated for 30 min at 37° with buffered (pH 7 5) substrate solution The reaction was stopped by heating the incubation tube in boiling water, and the extent of hydrolysis was estimated by use of the high-voltage paper electrophoresis technique

*Hydrolyzed**	*No hydrolysis detected*
Gly–L-Phe	*N*-Z–L-Ala–L-Phe
Gly–L-Leu	*N*-Z–Gly–L-Phe
Gly–L-Ala	β-Ala–L-His
L-Ala–L-Phe	L-Ala–L-Phe diketopiperazine
L-Leu–L-Phe	
Gly–L-Tyr	
L-Leu–L-Ala	
L-Leu–Gly	
L-Leu–L-Tyr	
L-Met–Gly	
Gly–L-Ala–L-Phe**	
L-Ala–Gly	
Gly–Gly	
Gly–L-Pro	
L-Phe–Gly	
L-Leucine amide	
L-Leucyl-β-naphthylamide	
L-Phenylalanine amide	
L-Pro–Gly	

* Listed approximately in decreasing order of degree of hydrolysis
** Refers to hydrolysis of the amino-terminal peptide bond

Quantitative studies of intestinal L-Ala–L-Phe hydrolase activity

Enzyme assay Rate of liberation of phenylalanine from L-Ala–L-Phe was linear with respect to time for at least 30 min (Fig 1A) Rate of release of free aromatic amino acid from 8 other dipeptides which were used for quantitative assays and from L-phenylalanine amide was linear with respect to time for at least 15 min. For each substrate, the reaction rate varied as a linear function of amount of mucosal protein over the ranges used in these experiments Results for L-Ala–L-Phe are shown in Fig 1B

pH optima The pH values for optimal hydrolysis of each substrate used for the quantitative assays were L-Ala–L-Phe, 7 5, Gly–L-Phe, 8 25, L-Leu–L-Phe, 8 5,

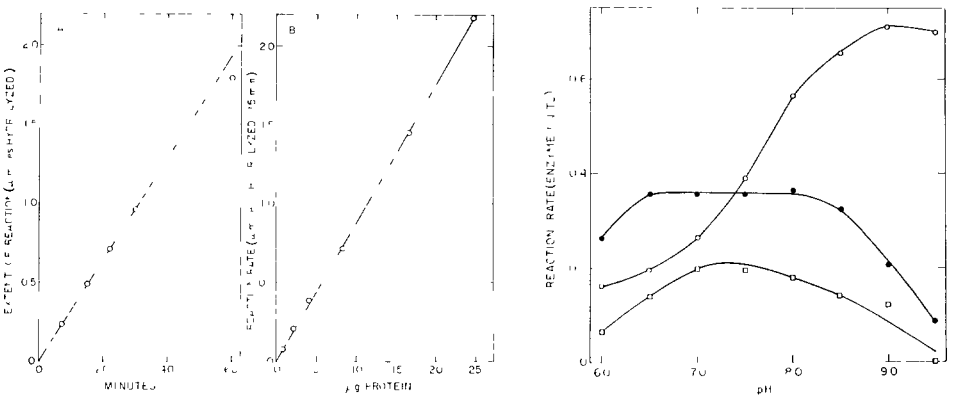

Fig 1 Hydrolysis of L-Ala–L-Phe plotted as a function of (A) incubation time and (B) amount of tissue protein in incubation mixture The preparation was high-speed supernatant of mucosa from rat small intestine, the standard assay mixture contained 10 μmoles of substrate

Fig 2 Reaction rate plotted as a function of pH for representative peptide hydrolase activities in mucosal high-speed supernatant from rat small intestine The curve for L-Ala–L-Phe (●—●) is similar to that determined for L-Phe–Gly The curve for L-Met–L-Phe (○—○) is similar to those obtained for L-Leu–L-Phe, L-Phe–L-Met, and L-phenylalanine amide The curve for Gly–L-Tyr (□—□) is similar to those obtained for Gly–L-Phe, L-Phe–L-Phe, and Gly–L-Try The buffer used was 0 1 M Tris–maleate except for incubations at pH 9 5, and occasionally pH 9 0, when 0 1 M glycine–NaOH buffer was used instead Values for activity determined with the latter buffer were corrected in relation to those determined with Tris–maleate buffer, on the basis of a comparison of activities made with each buffer at pH 8 5

L-Met–L-Phe, 9 0, L-Phe–L-Phe, 8 25, L-Phe–Gly, 7 5, L-Phe–L-Met, 9 0, Gly–L-Tyr, 7 25, Gly–L-Try, 7 5, and L-phenylalanine amide, 8 5 (Fig 2) The standard assay was performed at the pH optimum in each case

Stability The L-Ala–L-Phe hydrolase activity of frozen (−18°) mucosal high-speed supernatant was 100% of the original activity at 14 days, 80% at 30 days, 50% at 100 days and 30% at 270 days When maintained at 4° the enzyme activity was more stable at pH 7 than at pH 6 or 8 At pH 7 (1 0 mM Tris–HCl buffer) the activity was less stable in the presence of 0 05 mM $MnCl_2$, $BaCl_2$ and $ZnCl_2$ than it was in 0 05 mM $MgCl_2$ or buffer alone At pH 7 L-Ala–L-Phe hydrolase activity was very unstable when exposed to temperatures of 26° or greater (Fig 3)

Metal requirement L-Ala–L-Phe hydrolase activity of mucosal high-speed supernatant was not affected by the presence of Zn^{2+} (5–10 000 nM) or 5 μM Mg^{2+}, Mn^{2+} or Co^{2+} in the assay incubation medium It was inhibited 84% by 0 3 mM Zn^{2+} Dialysis of the supernatant for 16 h at 4° against 0 25 M buffered sucrose did not reduce the hydrolase activity below that of an undialyzed control However, it was inhibited by chelating agents as follows EDTA (5–10 000 μM), approx 45%, 1 mM 1,10-phenanthroline, 98%, and 1 mM α,α′-dipyridyl, 26% (Table II)

The inhibition by 10 μM EDTA was almost completely overcome by an optimal (18 μM) concentration of $ZnCl_2$ (Fig 4) The same concentration of $FeCl_2$ and $CoCl_2$ restored activity to 92% and 76%, respectively, of the initial value, whereas the chloride salts of Cd^{2+}, Sn^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Cu^{2+} and Mn^{2+} did not reverse the inhibition by EDTA

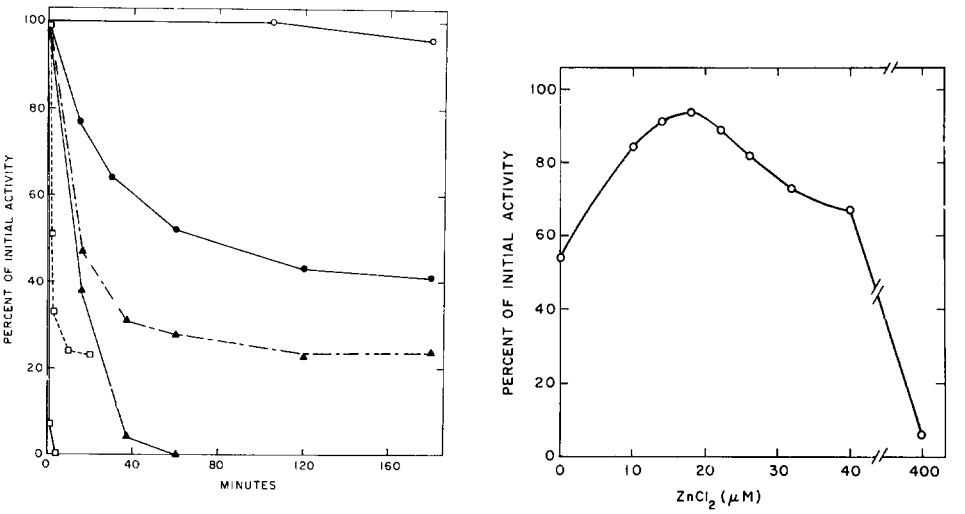

Fig 3 Stability of hydrolase activities for L-Ala–L-Phe (———), L-Phe–Gly (— — —), and L-leucylnaphthylamide (- - - -) at 26° (○), 40° (●), 45° (▲), and 60° (□) Mucosal high-speed supernatant from rat small intestine was incubated at the indicated temperatures in 0 005 M Tris–HCl buffer (pH 7 0) Remaining hydrolase activity was determined at the times shown and expressed as a percentage of the initial activity

Fig 4 Effect of Zn^{2+} on L-Ala–L-Phe hydrolase activity in the presence of EDTA Mucosal high-speed supernatant was assayed in 10 μM EDTA *plus* the various concentrations of $ZnCl_2$ indicated The hydrolase activity is expressed as per cent of that obtained when neither EDTA nor $ZnCl_2$ was present

TABLE II

EFFECTS OF CHELATING AGENTS ON PEPTIDE HYDROLASE ACTIVITIES OF RAT INTESTINAL MUCOSA

Aliquots of mucosal high-speed supernatant were assayed in the absence and presence of chelating agents Results are expressed as per cent of the activities obtained when no chelator was added to the standard assay

Substrate	*Additions to assay mixture*				
	α,α'-Dipyridyl (1 mM)	*1,10-Phenanthroline (1 mM)*	*EDTA (10 mM)*	*EDTA (10 μM)*	*EDTA (10 μM) + $ZnCl_2$ (18 μM)*
	% of initial activity				
L-Ala–L-Phe	74	2	54	54	95
Gly–L-Phe	67	4	23	22	101
L-Phe—L-Phe	64	12	79	79	99
L-Phe–Gly	71	18	77	66	111
L-Leu–L-Phe	91	44	88	79	90
L-Phe–L-Met	94	44	94	92	99
L-Phe amide	90	78	67	70	116

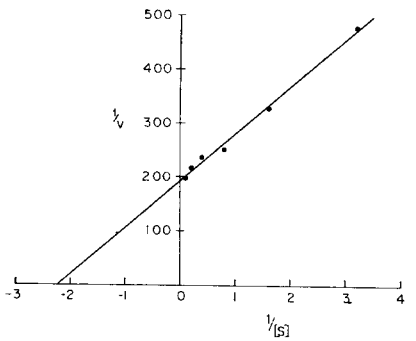

Fig 5 L-Ala–L-Phe hydrolase activity expressed by a reciprocal plot as a function of substrate concentration Enzyme source, rat mucosal high-speed supernatant v, reaction velocity as μmoles/min, [S], substrate concentration as mM The value for K_m derived from these data is 0 45 mM

These results suggest that there is a metal requirement for intestinal L-Ala–L-Phe hydrolase activity and that the cofactor is either Zn^{2+}, Fe^{2+} or Co^{2+} The marked inhibition by 1,10-phenanthroline, a strong Zn^{2+} chelator[8], supports the possibility that Zn^{2+} is the cofactor

The effects of chelating agents on intestinal hydrolase activities for 6 other substrates (Table II) were similar to those observed for L-Ala–L-Phe hydrolase activity in that 1,10-phenanthroline was more inhibitory than either EDTA or α,α'-dipyridyl, and EDTA was as inhibitory at 10 μM as it was at 10 mM The less marked inhibition of L-Leu–L-Phe and L-Phe–L-Met hydrolase and L-phenylalanine amidase activities by the 3 metal chelators could be the result of lower affinity (see ref 8) of the chelating agents for metal ions at the more alkaline pH (8 5–9 0) at which these assays were carried out

Kinetics The value for K_m for the hydrolysis of L-Ala–L-Phe was determined twice and found to be 0 45 (Fig 5) and 0 3 mM

Effect of enzyme inhibitors Neither 1 mM DFP, an esterase inhibitor, nor 1 mM β-phenylpropionic acid, a carboxypeptidase inhibitor, had any effect on the hydrolase activities for L-Ala–L-Phe, Gly–L-Phe, L-Leu–L-Phe, L-Phe–L-Phe, L-Phe–Gly, L-Phe–L-Met or L-phenylalanine amide However, 1 mM p-chloromercuribenzoate reduced the activity of these hydrolases by 83–99%, suggesting that one or more reduced

TABLE III

PARTIAL PURIFICATION OF THE L-Ala–L-Phe HYDROLASE ACTIVITY OF RAT INTESTINAL MUCOSA

Enzyme preparation	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*
I Whole homogenate	4940	165	30
II High-speed supernatant	4600	71 9	64
III 50–70% satd $(NH_4)_2SO_4$ fraction	1980	18 7	106
IV Gel filtrate	1960	16 0	122
V DEAE-cellulose eluted with 0 1 M NaCl in buffer	870	4 1	212

sulfhydryl groups may be necessary for the hydrolase activities In the absence of *p*-chloromercuribenzoate, 1 mM cysteine did not change the activity of L-Ala–L-Phe hydrolase

Enzyme purification Only a 7-fold purification of L-Ala–L-Phe hydrolase activity was achieved (Table III) Among the factors hindering purification was enzyme instability Mucosal homogenate (I) (0 25 M sucrose) was centrifuged at 105 000 × *g* for 1 h and the supernatant solution (II) was fractionated by addition of a cold neutralized $(NH_4)_2SO_4$ solution The fraction precipitating between 50 and 70% saturation with $(NH_4)_2SO_4$ (III), dissolved in 0 005 M Tris–HCl buffer (pH 7 0), contained approx 90% of the recovered activity This fraction was desalted by gel filtration (IV) and was applied to DEAE-cellulose (by use of a batch technique) which was eluted with equilibrating buffer and then with 0 1 M NaCl in that buffer (V) Recovery of activity was approx 18% Since fraction V was inhibited 40% by 5 mM EDTA, the low recovery was not due to the loss of metal cofactor

Structural requirements There was no detectable phenylalanine release from *N*-Z-L-Ala–L-Phe by either the mucosal homogenate or high-speed supernatant Specific activity of more than 0.4 unit/mg protein would have been detected Incubation of L-Ala–L-Phe, L-Ala–D-Phe, D-Ala–L-Phe and D-Ala–D-Phe with the mucosal high-speed supernatant under standard conditions gave specific activities of 104, 6, 1 and 0 (less than 0 1) units/mg of protein, respectively The importance of the L-configuration of both amino acids is apparent

Distinction from other enzyme activities A mucosal high-speed supernatant containing 82 5 units/mg protein of L-Ala–L-Phe hydrolase activity had 0 5 unit/mg protein of carboxypeptidase A activity, whereas pig pancreas carboxypeptidase A containing 1640 units/mg protein of carboxypeptidase activity had no detectable L-Ala–L-Phe hydrolase activity In addition, 7 mM *β*-phenylpropionic acid inhibited pig carboxypeptidase A activity 98% but did not inhibit rat intestinal L-Ala–L-Phe hydrolase activity Thus L-Ala–L-Phe hydrolase activity of rat intestinal mucosa appears distinct from carboxypeptidase A activity.

The intestinal L-Ala–L-Phe hydrolase activity is not attributable to leucylnaphthylamidase High-speed supernatants contained 90% of the homogenate L-Ala–L-Phe hydrolase activity but only 15% of the leucylnaphthylamidase activity. During incubation of a high-speed supernatant at 60° for 2 min, L-Ala–L-Phe hydrolase activity decreased 99% whereas leucylnaphthylamidase decreased only 49% (Fig 3)

L-Ala–L-Phe hydrolase activity was distinguished from hydrolases for three other phenylalanine-containing compounds When the mucosal high-speed supernatant was incubated at 45°, the hydrolase activities for Gly–L-Phe, L-Leu–L-Phe, L-Met–L-Phe, L-Phe–L-Phe and L-Phe–L-Met decreased at the same rate as L-Ala–L-Phe hydrolase activity (latter shown in Fig 3), but L-Phe–Gly hydrolase activity was more stable (Fig 3) After high-speed centrifugation, the supernatant retained 90% of the L-Ala–L-Phe hydrolase activity but less than 50% of the L-Phe–Gly hydrolase and L-phenylalanine amidase activities Partial separation of peptide hydrolase activities was achieved by using column chromatography on DEAE-cellulose (Fig 6) 2-ml fractions were collected and fractions 10, 20, 30, 40 and even-numbered fractions from 42 to 146 were assayed for hydrolase activity by the high-voltage paper electrophoresis technique The elution patterns for L-Phe–Gly hydrolase and aminopeptidase activity (amino terminus of Gly–L-Ala–L-Phe) were similar

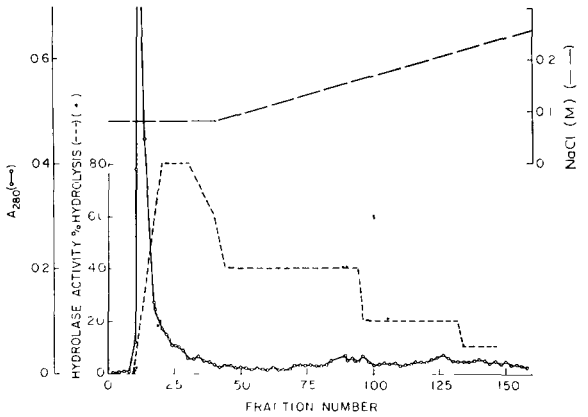

Fig 6 Anion-exchange chromatography of two peptide hydrolase activities in rat intestinal mucosa A fraction precipitating from high-speed supernatant at 50–70% saturation with $(NH_4)_2SO_4$ was desalted using gel filtration and applied to a column of DEAE-cellulose The column was eluted at 4°, first with 80 ml of 0 08 M NaCl in 0 005 M Tris–HCl buffer (pH 7 0) and then with 300 ml of the same buffer containing increasing amounts of NaCl (elution rate, 60 ml/h) The linear NaCl concentration gradient (— —) was produced according to the method of PARR[9] The appearance of protein in the column effluent (○—○) was monitored by determination of $A_{280\ nm}$ Hydrolase activity was estimated to the nearest 20% of total substrate cleaved by use of the high-voltage paper electrophoresis technique Values given as 10% indicate estimates clearly below 20% but greater than zero The pattern of elution for L-Ala–L-Phe hydrolase activity ( ) was similar to that for Gly–L-Phe hydrolase activity (not shown) and distinct from that of aminopeptidase activity for Gly–L-Ala–L-Phe (- - - -) and that of L-Phe–Gly hydrolase activity (not shown)

but distinctly different from those for L-Ala–L-Phe and Gly–L-Phe hydrolase activities (Fig 6) These results indicate that L-Ala–L-Phe hydrolase activity is distinct from the enzyme(s) which catalyzes cleavage of L-phenylalanine amide, L-Phe–Gly and the amino-terminal peptide bond of Gly–L-Ala–L-Phe The presence of a second smaller peak of L-Ala–L-Phe hydrolase activity in fractions 20 and 30 from the DEAE column (Fig 6) suggests that more than one enzyme may be capable of hydrolyzing L-Ala–L-Phe

Chromatography on Sephadex G-200 failed to separate L-Ala–L-Phe hydrolase activity from Gly–L-Leu, L-Leu–Gly, Gly–L-Pro or Gly–L-Tyr hydrolase activities, suggesting that if more than one enzyme is involved the proteins do not differ greatly in size

Localization of intestinal peptide hydrolase activity

Subcellular Mucosal high-speed supernatants (0 25 M sucrose) contained 70–100% (usually slightly over 90%) of the L-Ala–L-Phe, Gly–L-Phe, L-Leu–L-Phe, L-Met–L-Phe, L-Phe–L-Phe and L-Phe–L-Met hydrolase activities in the whole homogenate Thus at least 90% of the activity of each of these mucosal hydrolases is present either in soluble form or bound loosely to a cell organelle This agrees with results reported for other intestinal peptide hydrolases[10–12] In contrast, the mucosal high-speed supernatant contained only 43% of the total L-Phe–Gly hydrolase and 35% of the total L-phenylalanine amidase activities

Intramural In the small intestine the concentration of soluble L-Ala–L-Phe

TABLE IV

L-Ala–L-Phe HYDROLASE ACTIVITY OF VARIOUS RAT TISSUES

Tissue high-speed supernatant	*L-Ala–L-Phe hydrolase activity (units/mg protein)*		
	Rat 1	*Rat 2*	*Rat 3*
Stomach, mucosa	1 7		
Small intestine*, mucosa	60		56
Proximal small intestine		41	
Mid small intestine		63	
Distal small intestine		49	
Small intestine, wall remaining after removal of mucosa			15
Large intestine, mucosa	14		
Kidney			60
Spleen			18
Liver			14
Heart			5

* Pylorus to ileocecal valve

hydrolase activity is approx 3-fold greater in mucosa than in remaining wall (Table IV, rat No 3) In the contents of small intestine L-Ala–L-Phe hydrolase activity (units per g of material homogenized) was less than 10% of mucosal activity

Regional In mucosa of stomach and large intestine L-Ala–L-Phe hydrolase activities were 3 and 20%, respectively, of that in small intestinal mucosa (Table IV, rat No 1) In 4 rats studied (representative experiment, Table IV, rat No 2), activities in the proximal and distal thirds of the small intestine were 75 ± 17% (mean ± S D) and 88 ± 16%, respectively, of that in the middle segment Similar results were obtained for L-Phe–Gly hydrolase activity None of the differences was statistically significant ($P > 0$ 05), but the suggestion of slightly greater activity in the middle and distal segments is consistent with results reported for other peptide hydrolase activities[13–15].

Peptide hydrolase activity in other rat tissues

The L-Ala–L-Phe hydrolase specific activities in high-speed supernatants from heart, liver, spleen and kidney were 9, 25, 33 and 108%, respectively, of the specific activity of intestinal supernatant (Table IV, rat No 3) In 7 rats, mean activities for high-speed supernatants of kidney and intestinal mucosa were 78 5 ± 16 1 (S D) and 58 3 ± 8 4 units/mg protein, respectively ($P < 0$ 02) For L-Phe–Gly hydrolase, specific activities were 8 5 ± 0 7 for kidney and 4 9 ± 0 8 units/mg protein for intestinal mucosa ($P < 0$ 01) High-speed supernatant of kidney contained 100% of the L-Ala–L-Phe hydrolase and 44% of the L-Phe–Gly hydrolase activity present in the whole homogenate, results which are essentially identical to those obtained for intestinal mucosa Kidney homogenate had higher L-phenylalanine amidase specific activity than homogenate of small intestinal mucosa (1 5 compared to 1 1 units/mg protein) However, much less of the kidney total activity remained in the supernatant after high-speed centrifugation (7 *vs* 35%)

TABLE V

PEPTIDE HYDROLASE ACTIVITIES IN INTESTINAL MUCOSA OF ADULT RATS

Mucosa from the entire small intestine of adult rats was homogenized in 0 25 M sucrose solution and an aliquot was centrifuged at 105 000 × *g* for 1 h Quantitative determinations of hydrolase activities for the indicated substrates were performed with both whole homogenates and high-speed supernatants

Substrate	*Peptide hydrolase activity (units/mg protein)*					
	Whole homogenate			*High-speed supernatant solution*		
	Mean ± S D	*Range*	*Number of animals*	*Mean ± S D*	*Range*	*Number of animals*
Gly–L-Phe	67		1	162 ± 82	82–249	4
Gly–L-Try				138	125–151	2
L-Met–L-Phe	30		1	70 ± 30	43–106	4
L-Ala–L-Phe	31 ± 5	25–41	8	70 ± 18	43–113	27
L-Leu–L-Phe	31		1	70 ± 29	33–102	4
Gly–L-Tyr				47 ± 20	25–65	3
L-Phe–L-Met	14		1	35 ± 8	27–42	3
L-Phe–L-Phe	11		1	28 ± 11	17–39	3
L-Phe–Gly	3 8		1	4 5 ± 0 8	2 9–5 7	8
L-Phe amide	1 2	1 1, 1 2	2	0 57 ± 0 28	0 30–0 85	3

Effects of fasting and age on peptide hydrolase activity

Fasting. Values for hydrolase activities in mucosal high-speed supernatants from normal adult rats are presented in Table V The effect of a 48-h fast was determined for L-Ala–L-Phe and L-Phe–Gly hydrolase activities in intestine and kidney

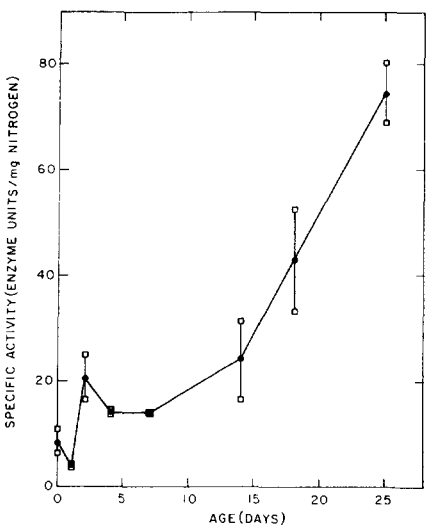

Fig 7 Neonatal development of rat intestinal L-Ala–L-Phe hydrolase activity 2 animals from a litter of 16 were sacrificed at the indicated times, the entire small intestine of each was homogenized in 0 03 M potassium phosphate buffer (pH 6 9) and centrifuged at 5000 × *g* for 15 min at 4° The supernatants were frozen and assayed simultaneously 7 days after the last pair of rats was killed Individual (□) and mean (●) values for specific activity are shown

Although each activity decreased slightly in each organ, the differences were not statistically significant

Intestinal L-Ala–L-Phe hydrolase activity during postnatal development Between the 7th and 25th days after birth, intestinal L-Ala–L-Phe hydrolase activity in the rat increases more than 4-fold (Fig 7) This change is similar to those reported for amino tripeptidase (Gly–Gly–Gly) and leucyl-β-naphthylamidase in rat small intestine[16]

DISCUSSION

Our screening studies revealed that rat intestinal mucosa can catalyze hydrolysis of 15 dipeptides in a group chosen randomly To our knowledge, 10 of these were not tested previously[10,14,16–21] for hydrolysis by rat intestinal mucosa. Our quantitative studies were restricted to substrates containing aromatic amino acids, particularly phenylalanine, because little is known about their hydrolases and because we hoped to determine whether dipeptides containing an aromatic amino acid are hydrolyzed by a single enzyme To our knowledge, neither the 9 dipeptide hydrolase activities we studied nor the phenylalanine amidase activity were previously examined quantitatively in preparations of rat intestinal mucosa Consequently, our results cannot be evaluated in relation to published data, but comparison with results obtained for other dipeptide hydrolase activities of rat intestinal mucosa[10,14,16,19–21] reveals a similar range of values for optimum pH (pH 7–9) and the same order of magnitude of specific activities, approx 10–100 μmoles hydrolyzed per 15 min per mg protein

Despite the difficulties we encountered with enzyme purification, we showed that L-Ala–L-Phe hydrolase of rat intestinal mucosa is distinct from the mucosal L-leucyl-β-naphthylamidase, Gly–L-Ala–L-Phe aminopeptidase and L-Phe–Gly hydrolase, and also from pig pancreas carboxypeptidase A Thus, more than one intestinal enzyme catalyzes the hydrolysis of phenylalanine-containing dipeptides We also obtained information about the substrate requirements of L-Ala–L-Phe hydrolase activity Crude enzyme did not hydrolyze the cyclic dipeptide, L-Ala–L-Phe diketopiperazine or D-Ala–D-Phe, and had minimal activity against L-Ala–D-Phe and D-Ala–L-Phe

Most dipeptide hydrolase activities studied have had a divalent metal ion requirement The metal producing greatest activation varies, depending on the source of the enzyme[2], the buffer used in the assay system[2] and, possibly, the substrate used[22] The cations most commonly required are Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+}(ref 22) At least one dipeptide hydrolase, from pig kidney, is a zinc metalloprotein[23]; Gly–L-Leu hydrolase from human uterus and rat muscle is strongly activated by Zn^{2+} (ref 2), and leucylnaphthylamidase and L-Leu–Gly hydrolase activities from intestinal mucosa may be Zn^{2+}-dependent[11] Our observations suggest that Zn^{2+} is a cofactor for L-Ala–L-Phe hydrolase activity of rat intestinal mucosa The failure of dialysis to decrease the enzyme activity and the inability of divalent cations to increase the activity in the absence of EDTA, indicate that the metal cofactor may be tightly bound to the enzyme protein[22] Inhibition by *p*-chloromercuribenzoate suggests that L-Ala–L-Phe hydrolase may be similar to zinc-containing enzymes in which the metal ion is covalently bound to mercaptide groups[24] High concentrations of Zn^{2+} markedly

inhibit the activity, an effect which has been demonstrated for several metal-enzyme systems[22,25] and which is apparently not widely appreciated, though emphasized before[22]

In most cases studied[10,11], 80–95% of the activity of peptide hydrolases in intestinal mucosal homogenates was in the soluble fraction The remaining activity was thought to be attached to the microvillus membrane[11] It is important to confirm this for other peptide hydrolases and to make additional efforts to prove that the association of these enzymes with microvillus membrane is not an artifact of membrane isolation We found that approx 90% of homogenate activity was in the soluble fraction for 6 of the 7 peptide hydrolase activities studied In contrast, less than 50% of the L-Phe–Gly hydrolase and L-phenylalanine amidase activities was recovered in the soluble supernatant We did not study intracellular localization any further and have no proven explanation for the observed differences Possibly the large soluble fraction of peptide hydrolase activity in mucosal cells originates outside the mucosa, in the pancreas for example, and L-Phe–Gly hydrolase and L-phenylalanine amidase activities are either not produced by the external source or are poorly absorbed by the mucosal cells Such an hypothesis is not without precedent[26], and it could explain the rapid loss *in vitro* of certain peptide hydrolases from intact pig intestinal mucosa[12] If the origins of the peptide hydrolase activities in the soluble and particulate portions of mucosal epithelial cell preparations differ, then their functions may differ too It seems likely that the peptide hydrolases attached to the microvillus membrane, like the disaccharidases, are responsible for cleavage of dietary dipeptides If so, then, as has been pointed out[11], when homogenates are assayed, changes in the microvillus peptide hydrolases may be masked by the much larger amount of soluble enzyme

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