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INTESTINAL SURFACE PEPTIDE HYDROLASES: IDENTIFICATION AND CHARACTERIZATION OF THREE ENZYMES FROM RAT BRUSH BORDER

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Summary

Peptide hydrolases were solubilized from rat small intestinal brush border by papain and separated by Sephadex G-200 chromatography, velocity gradient ultracentrifugation and polyacrylamide disc electrophoresis and designated according to approximate molecular size from sedimentation studies. Peptidases I (apparent M_r 230 000) and II (apparent M_r 160 000) are oligopeptidases with maximum specificity for tripeptides with identical pH optima (7.5) and similar apparent K_m with L-Leu-Gly (I, 0.60 mM; II, 0.76 mM). L-Leucyl-β-naphthylamide is a competitive inhibitor of both enzymes. Concentration of peptidase II produced partial conversion to peptidase I on polyacrylamide disc electrophoresis. The third peptide hydrolase (III, M_r 120 000) is a dipeptidase with pH optimum 8.5 and apparent K_m for L-Leu-Gly of 0.65 mM.

These peptide hydrolases were inhibited appreciably (37—59%) by 0.2 M glycine/NaOH, Tris · HCl or Tris · glycine buffers. EDTA (5 mM) completely inhibited these enzymes but all activity was restored by dialysis against buffer without divalent ions. Subsequent addition of Mg^{2+}, Mn^{2+}, Co^{2+} or Zn^{2+} (1—2 mM) inhibited peptidases I and II variably (4—81%) depending upon the substrate and buffer used. In contrast peptidase III was activated slightly by metal ions (5—20%).

These peptide hydrolases are strategically located at the intestinal lumen-cell interface and possess biochemical characteristics making them ideally suited to play a pivotal role in the final stage of protein digestion.

Introduction

The small intestinal surface cell constitutes a protective barrier that permits entry only of substances for which it has specific transport mechanisms.

Although certain compact oligopeptide products of pancreatic protease action are transported intact into the intestinal cell [1,2] where intracellular peptidases [2—6] can hydrolyze them, isolated brush border membranes are highly active against small peptides [7,8] with specificity for many dipeptides and tripeptides [9,10]. The strategic location of the intestinal brush border surface for oligopeptide hydrolysis has prompted comparative studies of cell sap and brush border preparations demonstrating differences both of biochemical characteristics [11,12] and also in the level of peptide hydrolase activity in response to starvation [13] or high protein diet [14]. However, such studies have been carried out on intestinal preparations that probably contain several peptidases and delineation of a precise physiologic role for the brush border in protein digestion depends upon individual study of each component peptide hydrolase.

Since there is extensive evidence that dietary carbohydrates are hydrolyzed at the intestinal lumen-cell interface by surface oligosaccharidases [15,16], we were encouraged to separate and characterize the peptide hydrolases (EC 3.4.—.—) of the brush border. Our experiments identify three enzymes of the brush border which possess biochemical characteristics favoring hydrolysis of small peptides containing bulky aliphatic or aromatic residues. These peptidases are ideally suited to perform surface digestion of the peptide products of pancreatic protease action.

Materials and Methods

Animals. Sprague-Dawley rats of either sex (200—400 g) were allowed Purina rat chow ad libitum prior to sacrifice.

Peptides. Peptides composed of L-form amino acids* were obtained commercially. Leucyl-β-naphthylamide, and the natural peptides were obtained from Sigma Chemical Company, St. Louis, U.S.A., Cyclo Chemical Company, Los Angeles, U.S.A. and Fox Chemical Company, Los Angeles, U.S.A. Only peptides which had no detectable impurity by thin-layer chromatography [17] were used as substrates.

Isolation of brush borders. Brush borders were prepared from intestinal homogenates by use of controlled osmotic lysis to release the various cell organelles [18]. Mucosal scrapings from jejunum and ileum of rats were added to 200 ml of 300 mM sucrose/5 mM EDTA, pH 7.4 and homogenized for 30 s (Hamilton Beach, Model 8, Speed 4, Racine, Wisconsin, U.S.A.). The homogenate was filtered through silk gauze and the filtrate centrifuged at 1500 g for 10 min. The precipitate contained intestinal cell fragments, brush borders and other cell organelles. Pure brush borders were obtained by sequential 10 min exposure to increasingly hypotonic solutions of sucrose/5 mM EDTA, in which the sucrose was decreased from 300 mM in 75 mM increments with a final wash with 37.5 mM sucrose/5 mM EDTA. The process was monitored at each step with phase contrast microscopy. Resuspension in the two final solutions was repeated if the precipitate was observed to contain contaminants. The final

* Only peptides containing L-amino acids were used as substrates. The L designation has been omitted in this paper.

1500 × *g* precipitate containing only brush borders was taken up and dialyzed against 0.05 M Tris buffer pH 7.5—8.0. The entire isolation procedure was carried out at 4°C. Jejunal and ileal brush borders yielded similar assay results, and therefore they were combined for subsequent studies. Final preparations were free of cell sap peptidase activity (Pro-Leu as substrate), lysosomal enzyme activity (acid β-galactosidase) [19], and microsomal and mitochondrial contamination [12,18]. Specific activity of sucrase, a known brush border enzyme, increased 10—15-fold over the crude homogenate.

Solubilization of brush border peptidases. Papain (30 mg/ml crystalline suspension in acetate buffer pH 4.5 Sigma Chemical Co.), was used to release the peptidases from the brush borders. After activation of 20 mg of papain in 33 mM cysteine/2 mM EDTA, pH 7.4 for 15 min at 37°C, the activated enzyme solution was added to the brush border suspension to give a ratio of brush border protein to papain of 2 : 1. The brush border-papain mixture was incubated for 30 min at 37°C and the reaction terminated by cooling rapidly in a salt-ice bath. The solubilized brush border peptidases were then recovered in the high speed (100 000 × *g*, 60 min) supernatant and dialyzed against 0.05 M Tris pH 7.5—8.0.

Enzyme Assays

β-Naphthylamide hydrolase activity. β-Naphthylamide hydrolase activity was determined by scaling down the method of Goldbarg and Rutenberg [20] by a factor of 5 with use of leucyl-β-naphthylamide at 0.17 mM in the reaction mixture. The absorbance at 560 nm in 0.9 ml cuvettes with a 1 cm light path was linear over the range of 0—40 μg of released β-naphthylamine.

Peptidase activity. 1/10 ml of suitably diluted enzyme preparation was incubated for 10—60 min at 37°C with 0.1 ml peptide (1—60 mM) in 0.05 M Tris buffer at pH 7.5—8.5, depending on the experiment. The reaction was terminated by placing the tube in a 100°C water bath for 3 min. The amino acids released were estimated by a modification of the method described by Fujita et al. [10] using the L-amino acid oxidase-peroxidase coupled system. Leucine, phenylalanine and methionine reacted with the reagent when present as free amino acids but showed the same absorbance as the buffer blank when present in a peptide. The quantity of peptide hydrolyzed was determined from the amount of amino acid released and activity was expressed as μmol/min = I.U. (International Units).

When tri- or tetrapeptides containing more than one residue capable of reacting with L-amino acid oxidase were used as substrates, conditions were established to insure release of a single amino acid residue. Since the oligopeptides were hydrolyzed solely by removal of an N-terminal amino acid, this was accomplished by monitoring the reaction with thin-layer chromatography [17].

Protein. Protein was determined by the method of Lowry et al. [21]. Tris buffer in the same concentration as in the enzyme solutions was present in all standards.

Gel filtration chromatography. Cross-linked dextran (Sephadex G-200, Pharmacia) was placed in 0.05 M Tris buffer, pH 7.5—8.0 for 72 h and then packed at 25°C in a 2.5 cm × 45 cm column at a pressure of 10 cm of water

and allowed to equilibrate for 24 h at 4°C. The sample (0.3—1 ml) of solubilized brush border containing 60 mg protein in 0.05 M Tris/0.14 M NaCl was layered under the buffer at the top of the column and eluted at a rate of 4—5 ml/h.

Density gradient ultracentrifugation. Samples of 0.1 ml were layered onto 5.0 ml linear gradients of 2.5—10, 4—16, or 5—20% D-mannitol/0.05 M Tris · HCl, pH 7.5—8.0, and then centrifuged for 4.5 or 6.5 h at 4°C at 300 000 × *g* in the SW 65 Ti rotor. A slight precipitation of mannitol in the 4—16 and 5—20% solutions neither affected the linear sedimentation of the enzymes nor the collection of the gradient. After ultracentrifugation, the bottom of the tube was punctured with a hollow needle, and the fluid collected in 0.1 ml (7 drop) fractions by increasing air pressure at the top of the tube.

Results

Solubilization of brush border peptidases with papain. The brush border peptidases could not be solubilized by repeated washings, but removal from the membrane was accomplished by papain digestion. It was necessary to compromise between incomplete release of the peptidases or loss of the enzymes by inactivation. Papain treatment of the brush borders released 40—80% of the peptidase and leucyl-β-naphthylamide hydrolase activities (Table I). These specific activities of the solubilized peptidases were similar to those previously observed for intact brush borders [8,9,12], and, depending upon the peptide substrate, were 4—23 times those found for the cell sap (Table I).

Gel filtration chromatography of solubilized peptidases. Passage of the solubilized brush border material through a Sephadex G 200 column yielded 2 peaks of peptidase activity that were partially resolved (Fig. 1). The central portion of each peak was concentrated by vacuum dialysis at 4°C and analyzed for activity against a wide range of substrates, as shown in Table II. The leading peak (A-A′) was active against leucyl-β-naphthylamide and the natural peptides Gly-Leu, Gly-Phe, Leu-Gly and Phe-Val and showed maximal activity against tripeptides. In contrast the trailing peak (B) lacked significant activity toward leucyl-β-naphthylamide or Phe-Val and tripeptides, yet hydrolyzed Gly-Leu, Gly-Phe and Leu-Gly very rapidly. Hence it appeared to be primarily a dipeptidase.

TABLE I

SOLUBILIZED PEPTIDASE ACTIVITY FROM BRUSH BORDERS*

Brush border membranes were prepared and solubilized with papain-cysteine as described under methods.

Substrate	Specific activity (I.U./g protein)	% activity solubilized	Sp. act. ratio Brush border/Cell sap
LNA	90	55	23
Gly-Leu	240	81	4
Gly-Phe	280	79	10
Leu-Gly	250	43	5
Phe-Val	600	60	11

* LNA, Leucyl-β-naphthylamide.

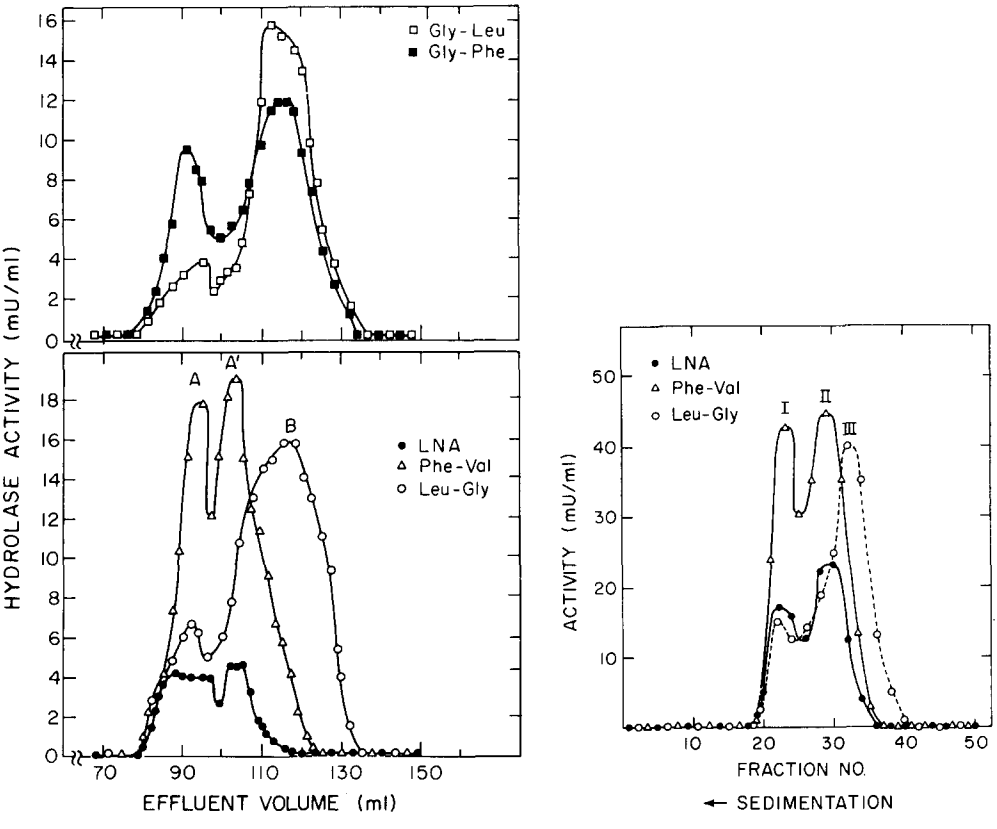


Fig. 1. Sephadex G-200 gel filtration chromatography of papain-solubilized rat brush border. A 2.5 cm × 45 cm column (V_0 = 62 ml) was equilibrated and eluted with 0.05 M Tris · HCl pH 7.5 as described in methods. LNA = leucyl-β-naphthylamide; the dipeptides were 10 mM in the reaction mixture. Recovery of peptidase activity was 82—90%.

Fig. 2. Velocity gradient ultracentrifugation of papain-solubilized brush border material. Gradients were 5—20% D-mannitol/0.05 M Tris · HCl pH 7.5 and centrifugation was carried out for 4.5 h at 300 000 × *g* in the SW65 Ti rotor in a Beckman L-265 instrument. Fractions (0.1 ml) were collected from the bottom of the tube and analyzed for hydrolase activity as described under methods. Peaks were numbered according to their relative rates of sedimentation. Recovery of added activity was 75—90%. LNA, leucyl-β-naphthylamide.

Density gradient centrifugation. When the solubilized brush border material was centrifuged in 5—20% D-mannitol at 300 000 × *g* for 4.5 h, three peaks of peptidase activity were identified (Fig. 2), the most rapidly moving enzyme (I) being followed closely by another (II) having very similar substrate specificity. The use of a longer centrifugation time in a less concentrated gradient improved the separation of the enzymes (Fig. 3) and showed that the tripeptide Leu-Gly-Gly was hydrolyzed more rapidly than dipeptides by enzymes I and II. The third major peptidase peak (III) found on density gradient centrifugation migrated much more slowly (Figs 2,3) and possessed similar substrate specificity to that of the trailing enzyme (B) on Sephadex G 200 (Table II).

Identification of Sephadex G 200 enzymes by density gradient centrifugation. After isolation and concentration by vacuum dialysis, the broad leading

TABLE II

SUBSTRATE SPECIFICITY OF BRUSH BORDER PEPTIDASE PEAKS FROM SEPHADEX G 200

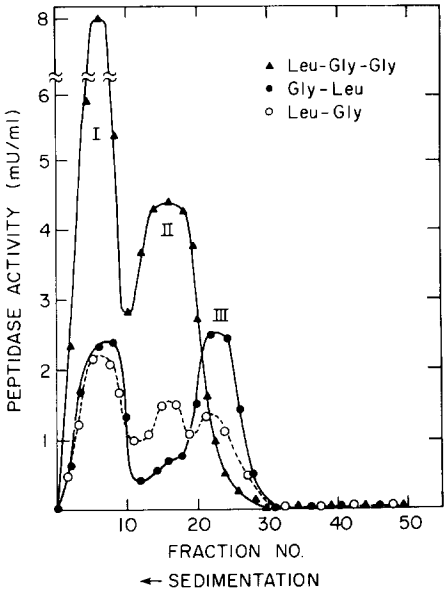
The center portion of the enzyme peaks were collected from gel filtration experiments (Cf. Fig. 1).

Substrate**	Substrate Concentration (mM)	Peak A, A' Relative activity*	Peak B Relative activity*
Gly-Leu	30	100	100
LNA	0.17	80	10
Gly-Phe	30	90	60
Leu-Gly	30	100	75
Leu-Gly-Gly	30	280	10
Leu-Phe	15	180	20
Phe-Gly	30	280	30
Phe-Gly-Gly	30	1030	40
Phe-Leu	30	120	20
Phe-Phe	20	90	10
Phe-Phe-Phe***	0.2	140	10
Phe-Phe-Phe-Phe***	0.2	86	10
Phe-Val	30	286	20
Pro-Leu	30	<1	<1
Leu-Pro	30	<1	<1

* Gly-Leu hydrolase activity = 100.

** LNA = Leucyl-β-naphthylamide.

*** Since removal of an N-terminal Phe residue yields a smaller peptide substrate from which Phe can also be hydrolyzed, short-term incubations were carried out with monitoring of reaction products by thin layer chromatography. Cleavage of the secondary substrate did occur but was minor compared to the primary reaction; however, values given must be considered approximate.



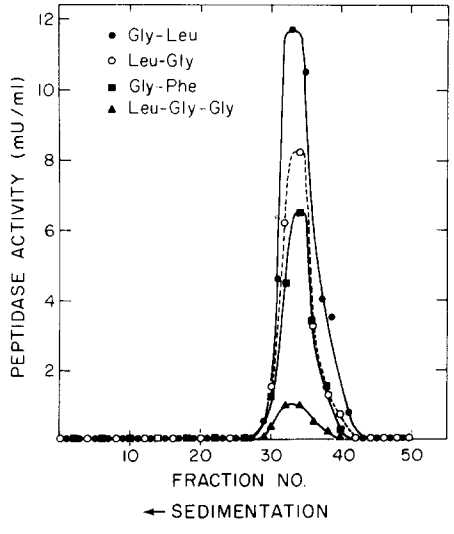


Fig. 3. Prolonged velocity ultracentrifugation of papain-solubilized brush border in dilute gradients. Conditions are as described for Fig. 2 except that gradients were 2.5—10% D-mannitol and centrifugation time was 6.5 h. Recovery of added activity was 80—94%.

Fig. 4. Velocity ultracentrifugation of Peak B (120—130 ml effluent) isolated from Sephadex G-200 gel filtration (Fig. 1). The material was concentrated 50-fold by vacuum dialysis and 0.1 ml layered over the gradient. Other conditions are described in Fig. 2. Recovery of activity added was 92%.

peptidase peak (A-A′) from the Sephadex G 200 column (Fig. 1) was found to consist of peptidases I and II when examined by gradient centrifugation. As expected, the trailing peptidase peak from Sephadex G-200 (peak B, Fig. 1) was found to sediment as peak III with specificity for dipeptides but minimal activity against tripeptides (Fig. 4). A longer centrifugation time (6.5 h) caused this trailing dipeptidase to migrate further, but it showed no tendency to become polydisperse.

Characteristics of the three peptidases. Further purification by use of DEAE-Sephadex or polyacrylamide disc electrophoresis produced appreciable losses of activity so the partially purified enzymes were used for comparison of biochemical characteristics. The center portion of each peptidase peak recovered from Sephadex gel filtration experiments was concentrated by vacuum dialysis against 0.05 M Tris pH 8.0 and further purified by velocity centrifugation on D-mannitol gradients as described under Methods. The sequential use of these two separation techniques yielded each peptidase with <1% contamination from another peptidase (cf. Fig. 4 for peptidase III). Specific activities of the peptidases isolated in this manner were approximately 10 times higher than that found for solubilized brush border (Table I). Acrylamide gel electrophoresis of the three peptidase peaks verified that each enzyme was a distinct entity and identified only one to three other protein bands all of which were devoid of any peptide hydrolase activity (Fig. 5).

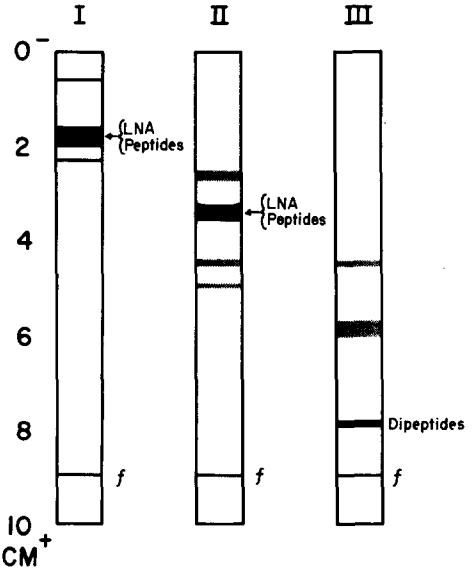


Fig. 5. Acrylamide gel electrophoresis of peptidase peaks I, II and III isolated from sequential Sephadex G-200 gel filtration and velocity gradient ultracentrifugation. System No. 2515 of Jovin, Dante and Crambach [26–28] was used with 4.25% total acrylamide/11.75% cross-linking/0.5% agarose in the stacking gel and 7% total acrylamide/2% cross-linking in the separation gel. The pH values of buffers were upper reservoir, 7.7; stacking gel, 6.1; separation gel, 7.2; and lower reservoir, 6.9. Electrophoresis was carried out at 7°C for 3 h at a current of 2 mA per gel. Staining was with coomassie blue. O, origin; f, position of bromphenol blue. Leucyl-β-naphthylamide hydrolyse (LNA) and peptide hydrolase activities were analyzed in 2 mm sections of duplicate gels and found to be confined to distinct protein bands as indicated.

TABLE III

SPECIFICITY OF THE BRUSH BORDER PEPTIDASES

The enzymes were isolated from sequential gel filtration and velocity gradient centrifugation as described in the text. Substrate concentrations are given in Table II. (+), appreciable activity present; (±), activity 10—20% of maximal with Gly-Leu as substrate; (—), activity <10% of maximal with Gly-Leu or not detectable.

	Peak I	Peak II	Peak III
LNA	+	+	—
Gly-Leu	+	—	+
Gly-Phe	+	—	+
Leu-Gly	+	+	+
Leu-Gly-Gly	+	+	—
Leu-Phe	+	—	±
Phe-Gly	+	+	—
Phe-Gly-Gly	+	+	—
Phe-Leu	+	+	—
Phe-Phe	+	±	—
Phe-Phe-Phe	+	+	—
Phe-Val	+	+	—
Pro-Leu	—	—	—
Leu-Pro	—	—	—

Substrate specificity of the peptidases. The substrate specificities of the three enzymes are compared in Table III. Peaks I and II both appear to be oligopeptidases with broad specificity for di- and tripeptides. Peak I showed specificity for all peptides tested except Pro-Leu and Leu-Pro and was particularly active against Phe-Val and tripeptides (Table III, Figs 2,3). Peak II also was active against most oligopeptides but possessed a narrower range of specificity for dipeptides with little or no capacity to hydrolyze Gly-Leu, Gly-Phe or Leu-Phe. The smallest peptidase (III) was maximally active against the dipeptides Gly-Leu, Leu-Gly and Gly-Phe but showed little or minimal activity against Phe-Val and other N-terminal phenylalanine peptides. In contrast to the findings for peptidases I and II, peptidase III hydrolyzed oligopeptides at only a fraction of the rates of dipeptides.

In other experiments not detailed here, blocking of the free N-terminus with a CBz group completely inhibited activity. In contrast peptide amides were readily hydrolyzed by peptidases I and II but were poor substrates for peptidase III. No carboxypeptidase or endopeptidase activities [22] were found for any of the enzymes.

pH activity studies. Peptidases I and II with leucyl-β-naphthylamide as substrate both showed a broad plateau of optimal activity at pH 6.5—7.5 and the pH-activity curves were indistinguishable for these two enzymes (Fig. 6). A narrower and slightly higher pH-optimum (7.5—8.0) was found for enzymes with the natural substrate Leu-Gly (Fig. 6) and varied somewhat depending on the buffer used. The dipeptidase peak (III) showed a higher optimal pH (8.5) and narrower pH-activity curve (not shown) than the other peptidases so that at pH 7 and 10, activity was only 10% of maximal.

Kinetic and molecular weight studies. All three enzymes obeyed Michaelis-Menten kinetics when studied by reciprocal plotting of substrate concentration

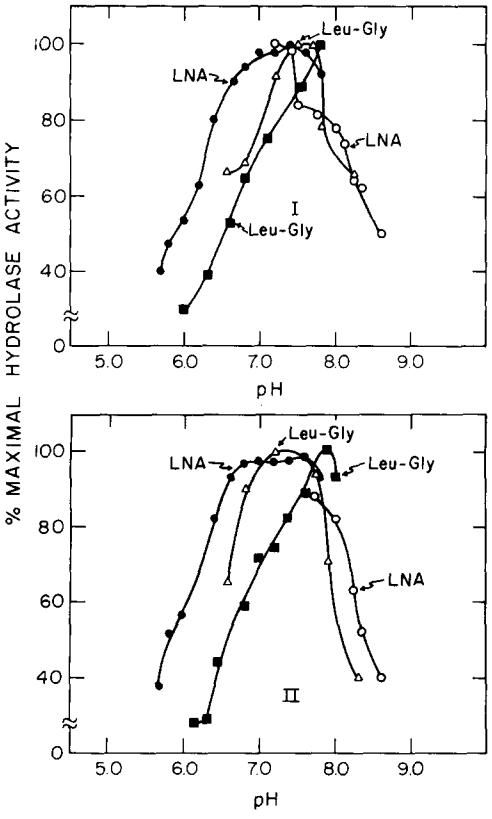


Fig. 6. pH-activity curves for peptidases I and II with leucyl-β-naphthylamide (LNA) (0.17 mM) and Leu-Gly (10 mM) as substrates. Buffers used were: ○———○, 0.05 M Tris · HCl; ●———●, 0.05 M sodium-potassium phosphate; ■———■, 0.05 M Tris · maleate; △———△, 0.1 M potassium phosphate.

and reaction rate. Competitive inhibition was demonstrated between leucyl-β-naphthylamide and Leu-Gly for both peptidases I and II by Lineweaver-Burk plots (not shown) verifying that the naphthylamide is hydrolyzed by the same active site as the natural substrates.

As shown in Table IV, peptidases I and II have very similar kinetic require-

TABLE IV

CHARACTERISTICS OF BRUSH BORDER PEPTIDASES

ISOLATED AS DESCRIBED IN TABLE III

No.	Principal Substrate	Mol. Wt. Approx.*	pH Opt.**	K_m (app.)	
				LNA (mM)	Leu-Gly (mM)
I	Oligopeptides	230 000	7.5	0.41	0.60
II	Oligopeptides	160 000	7.5	0.79	0.76
III	Dipeptides	120 000	8.5	—	0.65

* Estimated from density gradient centrifugation [29] at protein concentration of 5 μg/ml with alcohol dehydrogenase (Mol. Wt. 150 000) as the marker protein.

** With Leu-Gly as substrate in 0.05 M Tris · HCl.

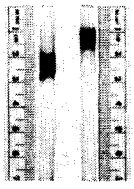


Fig. 7. Acrylamide gel electrophoresis of peptidases I and II recovered from a previous electrophoresis run (cf. Fig. 5). Conditions are the same as for Fig. 5 except that running time was 2.3 h and gels were stained histochemically for leucyl-β-naphthylamide hydrolase [34] activity rather than for protein. The tracking dye position is at the bottom of the figure. Peptidase I (right) migrates as a single band (1.0—1.5 cm); peptidase II (left) shows both the expected band (2.0—2.8 cm) and a new activity band (arrowed) at the position of peptidase I.

ments for the natural peptide Leu-Gly but their apparent K_m values with leucyl-β-naphthylamide differ by a factor of two and estimates of their molecular weights do not suggest a simple monomer-dimer relationship.

Conversion of peptidase II to peptidase I. Because of the similar characteristics found for peptidases I and II, the enzymes were studied for polydispersity by re-electrophoresis in polyacrylamide gels. The active enzymes were recovered from acrylamide electrophoresis (cf. Fig. 5) concentrated by vacuum dialysis and re-applied to acrylamide gels. As shown in Fig. 7, peptidase I migrated as a single bank in the expected position, but peptidase II showed an activity band both for II and for I. Hence peptidase II appears to aggregate partially to a form of the enzyme indistinguishable from peptidase I. Presumably this aggregation is favored by the preparative concentration prior to electrophoresis.

Effects of buffers and divalent ions on activity. Some buffers inhibited the peptidases appreciably (Table V). Glycine-containing buffers consistently depressed activity perhaps because the amino acid may compete for the active site when present at high concentrations (0.2 M). However, Tris, which allowed maximal activity at 0.05 M, interfered considerably with activity of peptidase III when buffer concentrations were higher (0.2 M).

Because previous studies with peptidases have emphasized stimulatory or inhibitory effects of divalent ions [23–25], we dialyzed 1 ml of each peptidase against 1 l of 5 mM EDTA in a non-inhibitory buffer at the optimal pH of the

TABLE V

% INHIBITION OF PEPTIDASES BY BUFFERS

Assays of the isolated enzymes (Cf. Table III) at pH optimum and 2 times K_m (app.) with Leu-Gly as substrate.

M/Buffer	Peptidase		
	I	II	III
0.05 Tris · HCl	0	0	0
0.1 $NaPO_4$	0	0	—
0.2 Tris · HCl	—	—	46
0.2 Tris · Glycine	—	—	62
0.2 Gly-NaOH	37	48	59

TABLE VI

EFFECT OF DIVALENT CATIONS ON INTESTINAL BRUSH BORDER PEPTIDASES

Each enzyme was dialyzed in sequence against 5 mM EDTA/buffer and buffer alone as described in text. Water used was twice de-ionized. EDTA completely inhibited activity of each peptidase but dialysis against buffer alone (no cation) restored activity (100%). Cations were preincubated with enzyme for 20 min at 37°C, prior to addition of substrate.

Cation*	Peptidase I			Peptidase II			Peptidase III
	LNA** (Tris) (%)	Leu-Gly (Tris) (%)	Leu-Gly** (NaKP) (%)	LNA (Tris) (%)	Leu-Gly (Tris) (%)	Leu-Gly (NaKP) (%)	Gly-Leu (Tris) (%)
None	100	100	100	100	100	100	100
Mg^{2+}	89	73	89	63	85	83	120
Mn^{2+}	96	41	56	84	57	50	108
Co^{2+}	84	62	81	59	66	77	105
Zn^{2+}	82	19	94	73	38	94	116

* As the chloride salt except for Zn^{2+} (Sulfate); at 1 mM except for Mg^{2+} (2 mM).

** Substrates and buffers (0.05 M Tris · HCl and 0.05 M Sodium-Potassium Phosphate pH 7.5).

enzyme at 4°C for 24 h with dialysis fluid changes every 6 h followed by the same dialysis procedure against the same buffer without EDTA. As can be seen from Table VI, peptidases I and II were inhibited by divalent ions as compared to the control enzyme in cation-free buffer alone but this inhibition varied not only with the divalent ion but also with the dialysis buffer. No activation was elicited by any cation. Zn^{2+} combined with Tris buffer produced the greatest inhibition when the natural substrate (Leu-Gly) was used. Peptidase III was activated slightly by all of the divalent ions. Increasing ionic concentration from 1 to 2 mM did not produce any additional activation or inhibition of any of the enzymes.

Discussion

Although the location in the cell was unknown, an intestinal aminopeptidase was identified more than 40 years ago by Linderstrøm-Lang [30] and a partially purified preparation was later studied extensively by Smith and his colleagues [31—33] with demonstration of maximal specificity for di- and tripeptides having an N-terminal aliphatic residue. Since an N-terminal leucine seemed to confer the most rapid hydrolytic rates, the enzyme was called leucine aminopeptidase.

Nachlas et al. [34], localized leucyl-β-naphthylamide hydrolase activity to the intestinal surface histochemically and Eichholz [35] was able to solubilize and separate hamster brush border leucyl-β-naphthylamide hydrolase activity from disaccharidases. Kim et al. [12] identified at least two peptidase fractions in rat brush border by qualitative starch gel electrophoresis; these appeared to hydrolyze both leucyl peptides and leucyl-β-naphthylamide, but kinetic studies were not carried out. In preliminary reports on solubilized rat brush border

peptide hydrolases studied by DEAE-cellulose chromatography, Heizer and Shoaf [36,37] identified two or three peptide hydrolases that appear to have similar substrate specificity to those described herein, but comparison is difficult since other biochemical parameters and data on relative molecular size were not provided. No other study on the separation and characterization of the intestinal brush border peptidases has been reported, although Maroux et al. [38] have recently isolated a single enzyme from hog brush border that appears to be similar in specificity and size to one of the rat enzymes (peptidase I). We were successful in solubilizing and separating three enzymes from one another with specific activities 50—100 times those of the crude intestinal preparation. Each of these hydrolases has suitable biochemical characteristics required for a digestive role at the intestinal lumen-cell interface.

The use of papain treatment to release brush border membrane enzymes has been validated for the study of disaccharidases [39] since it produces enzymes having indistinguishable properties from those found when spontaneous autolysis of intestine occurs in vitro at 37°C [40]. Kim et al. [12] found identical migration of rat brush border peptidases on starch gel electrophoresis whether solubilization was accomplished by papain, sodium deoxycholate or Triton X-100. Hence it appears that papain treatment is a reasonable method of obtaining these membrane hydrolases in soluble form.

Two aminopeptidases, a γ-glutamyltranspeptidase and a neutral endopeptidase were recently solubilized from kidney tubule brush border [22,41,42]. Although we have not found any hydrolytic activity in intestinal brush border against pentagastrin or dipeptides blocked at both the amino- and carboxy-terminus, intestinal peptidases I and II have similar specificity to kidney aminopeptidase M [22]. However, our experiments demonstrate that the aminopeptidases of intestinal brush border possess maximal specificity for natural peptides with specific activities higher than those of kidney aminopeptidase M, an enzyme with more affinity for naphthylamides. Hence, it seems unlikely that the kidney and intestinal aminopeptidases are related enzymes despite their comparable location in the brush border membrane. This implies that the intestinal aminopeptidases may be organ-specific, something that is not surprising when the specialized digestive role of the intestinal surface membrane is considered.

Comparison of the three brush border peptidases reveals that peptidases I and II are primarily oligopeptidases and that III is a dipeptidase (Tables III, IV; Fig. 3). That peptidase I and II are capable of hydrolyzing leucyl-β-naphthylamide was established by kinetic studies. In contrast, peptidase III is highly active only against dipeptides containing glycine or leucine.

Because of indistinguishable pH-activity curves (Fig. 6), very similar substrate specificity and conversion of peptidase II to I upon concentration (Fig. 7), it is very likely that peptidases I and II are related enzymes. Certainly, it is tempting to speculate that peptidase II is composed of two subunits of molecular weight 80 000; peptidase I would then constitute a trimeric form of the enzyme. However, the exact relationship of the enzymes will have to await purification of sufficient quantities to allow systematic study of their amino acid and carbohydrate compositions.

Divalent cations have been found to be activators of leucine aminopep-

tidases [7,24,43], but experimental conditions have varied and EDTA inhibition rather than direct demonstration of activation after removal of the chelator has often been interpreted as evidence of metal ion requirement.

Although activity has been found to be reduced to less than 10% of control after temporary exposure to EDTA, removal was accomplished by dialysis against unbuffered water [7,43], a procedure that may have denaturing effects independent of metal ion requirements. We attempted to remove divalent ions by exhaustive dialysis against buffered EDTA followed by dialysis against buffer alone. Whereas concentrated Tris or glycine buffers inhibited activity of all three peptidases independent of metal ion effects (Table V) as previously noted for connective tissue peptidases [44], a 24-h exposure to EDTA followed by dialysis against metal ion-free buffer did not reduce activity. Furthermore, subsequent additions of divalent ions reduced activity against peptidases I and II to a variable degree and stimulated peptidase III activity only slightly (Table VI). Although unexpected for aminopeptidases, this minimal effect of divalent ions on the peptide hydrolases supports the recent work of Kim et al. [45] using lower cation concentrations (0.05—0.5 mM) on a solubilized rat brush border preparation. Furthermore, our finding of reversible inhibitors of the brush border peptidases by EDTA is consistent with recent reports of spontaneous restoration of aminotripeptidase [25] and liver aminopeptidase [46] upon removal of the chelator. Although intestinal surface aminopeptidases appear to be metalloenzymes [38], the cations may be tightly bound so that EDTA treatment at 4 °C does not remove them [46].

Leucine-β-naphthylamide has been widely used, particularly in analysis of serum in clinical laboratories, as a substrate for assay of leucine aminopeptidase because of the simplicity of the assay [20]. Unfortunately, other enzymes incapable of hydrolyzing natural peptides may split the chromogenic substrate [47,48] and use of the amino acid β-naphthylamide has fallen into disrepute. Our kinetic experiments now establish that two of the brush border oligopeptidases hydrolyze leucyl-β-naphthylamide with similar affinity to that for natural N-terminal leucyl peptides. No "arylamidase" having specificity principally or solely for amino acid naphthylamides was found in the brush border preparations, and there appears to be insignificant leucyl-β-naphthylamide-splitting activity in the cell cytoplasm [12,34]. Hence, assay of crude intestinal homogenates for leucyl-β-naphthylamide hydrolase activity should provide an accurate estimate of brush border aminopeptidase activity. If similar results are found for human intestine, analysis of small intestinal biopsy specimens for amino acid naphthylamidase activity may provide the basis for accurate assessment of intestinal surface peptide hydrolase activity in intestinal disease.

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References

1 Newey, H. and Smyth, D.H. (1960) J. Physiol. 152, 367—380
2 Matthews, D.M., Lis, M.T., Cheng, B. and Crampton, R.F. (1969) Clin. Sci. 37, 751—764
3 Robinson, G.B. (1963) Biochem. J. 88, 162—168
4 Josefsson, L. et al. (1965) Biochim. Biophys. Acta 105, 149—166
5 Heizer, W.D. and Laster, L. (1969a) Biochim. Biophys. Acta 185, 409—423
6 Heizer, W.D. and Laster, L. (1969b) J. Clin. Invest. 48, 210—228
7 Rhodes, J.B., Eichholz, A. and Crane, R.K. (1967) Biochim. Biophys. Acta 135, 959—965
8 Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968) Biochem. J. 106, 381—390
9 Peters, T.J. (1970) Biochem. J. 120, 195—203
10 Fujita, M., Parsons, D.S. and Wojnarowska, F. (1972) J. Physiol. 227, 377—394
11 Heizer, W.D., Kerley, R.L. and Isselbacher, K.J. (1972) Biochem. Biophys. Acta 264, 450—461
12 Kim, Y.S., Birtwhistle, W. and Kim, Y.W. (1972) J. Clin. Invest. 51, 1419—1430
13 Kim, Y.S., McCarthy, D.M., Lane, W. and Fong, W. (1973) Biochim. Biophys. Acta 321, 262—273
14 Nicholson, J.A., McCarthy, D.M. and Young, S.K. (1974) J. Clin. Invest. 54, 890—898
15 Miller, D. and Crane, R.K. (1961) Biochim. Biophys. Acta 52, 293—298
16 Ugolev, A.M., Jesuitova, N.M. and de Laey, P. (1964) Nature 203, 879—880
17 Randerath, K. (1968) Thin-Layer Chromatography, 2nd edn, p. 110, New York Academic Press
18 Boyd, C.A.R., Parsons, D.S. and Thomas, A.V. (1968) Biochim. Biophys. Acta 150, 723—726
19 Alpers, D.H. (1969) J. Biol. Chem. 244, 1238—1246
20 Goldbarg, J.A. and Rutenberg, A.M. (1958) Cancer, 283—290
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
22 Kerr, M.A. and Kenny, A.J. (1974) Biochem. J. 137, 477—488
23 Smith, E.L. (1946) J. Biol. Chem. 163, 15—27
24 Behal, F.J., Asserson, B., Dawson, F. and Hardman, J. (1965) Arch. Bioch. Biophys, 111, 335—344
25 Chenoweth, D., Mitchel, R.E.J. and Smith, E.L. (1973) J. Biol. Chem. 248, 1672—1683
26 Jovin, T.M., Dante, M.L. and Crambach, A. (1970) Multiphasic Buffer Systems Catalogue, PB 196090, National Technical Information Service, Springfield, Va.
27 Jovin, T.M., Dante, M.L. and Crambach, A. (1970) Multiphasic Buffer Systems, Instructions for use of the systems catalogue, PB, National Technical Information Service, Springfield, Va.
28 Jovin, T.M., Dante, M.L. and Crambach, A. (1970) Multiphasic Buffer Systems, Systems output tape no. 3. PB 196087, National Technical Information Service, Springfield, Va.
29 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
30 Linderstrøm-Lang, K. (1929) Z. Physiol. 182, 151—174
31 Smith, E.L. and Bergmann, M. (1944) J. Biol. Chem. 153, 627—651
32 Smith, E.L. and Polglase, W.J. (1949) J. Biol. Chem. 180, 1209—1223
33 Smith, E.L., Spackman, D.H. and Polglase, W.J. (1952) J. Biol. Chem. 199, 801—817
34 Nachlas, M.M., Monis, B., Rosenblatt, D. and Seligman, A.M. (1960) J. Biophys. Biochem. Cytol. 7, 261—264
35 Eichholz, A. (1968) Biochim. Biophys. Acta 163, 101—107
36 Heizer, W.D. and Shoaf, C.R. (1972) Gastroenterology 62, 762 (Abstract)
37 Heizer, W.D. and Shoaf, C.R. (1973) Clin. Res. 21, 515 (Abstract)
38 Maroux, S., Louvard, D. and Baratti, J. (1973) Biochim. Biophys. Acta 321, 282—295
39 Aurrichio, S., Dahlqvist, A. and Semenza, G. (1963) Biochim. Biophys. Acta 73, 582—587
40 Gray, G.M. and Santiago, N.A. (1969) J. Clin. Invest. 48, 716—735
41 George, S.G. and Kenny, A.J. (1973) Biochem. J. 134, 43—57
42 Kerr, M.A. and Kenny, A.J. (1974) Biochem. J. 137, 489—495
43 Smith, E.L. and Spackman, D.H. (1955) J. Biol. Chem. 212, 271—299
44 Schwabe, C. (1969) Biochemistry 8, 771—781
45 Kim, Y.S., Kim, Y.W. and Sleisenger, M.H. (1974) Biochim. Biophys. Acta 370, 283—296
46 Garner, Jr., C.W. and Behal, F.J. (1974) Biochemistry 13, 3227—3233
47 Patterson, E.K., Hsiao, S.-H. and Ke-pel, A. (1963) J. Biol. Chem. 238, 3611—3620
48 Fleischer, G.A., Pankow, M. and Warmka, C. (1964) Clin. Chim. Acta 9, 259—268