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ISOLATION AND CHARACTERIZATION OF FOUR PEPTIDE HYDROLASES FROM THE BRUSH BORDER OF RAT INTESTINAL MUCOSA

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Summary

Peptide hydrolases (EC 3.4.—.—) were solubilized from purified brush borders of rat intestinal mucosa by papain digestion. Three peptide hydrolases, I, II, and III, with different substrate specificities were isolated by means of DEAE-cellulose chromatography and preparative acrylamide gel electrophoresis. On repeat preparative acrylamide gel electrophoresis under slightly different conditions, enzyme II was resolved into two proteins, IIa and IIb, with very similar, possibly identical, substrate specificities. Efforts to discover additional brush border peptide hydrolases revealed none. Studies using more than 50 substrates showed that enzyme I was most active against Met-Met, Met-Ala, and Met-Phe while enzyme II was most active against Phe-Gly, Phe-Ser, and Leu-Gly-Gly, and enzyme III most rapidly hydrolyzed Gly-Leu, Leu-Gly, and Met-Gly. Efforts to discover substrates which are highly discriminating for each enzyme were partly successful. Thus, a number of substrates including leucine amide, leucyl-β-naphthylamide and Phe-Asp were hydrolyzed almost exclusively (95% or more) by enzyme II while Gly-Leu was similarly specific for enzyme III. No substrate highly discriminating for enzyme I was discovered. Ion-exchange chromatography resulted in increases in specific activity of 10- and 120-fold for enzymes II and III, respectively. By sequential use of ion-exchange chromatography and preparative acrylamide gel electrophoresis, each of the three enzymes was partially purified to the point that they were free of contaminating disaccharidases and enzymes I and II gave single dense bands on analytical acrylamide gel electrophoresis while enzyme III gave a single dense band plus one additional faint protein band. Under appropriate conditions, analytical gel electrophoresis also resolved enzyme II into two bands with enzyme activity. The three enzymes were isolated from intestinal brush borders of germ-free rats indicating that none of the enzymes is of bacterial origin. With Phe-Gly as substrate, pH optima for enzymes I, II, and III were 8.0, 8.0, and 8.5, respectively. Molecular weights determined by gel filtration were 283 000,

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284 000, and 134 000, respectively. Studies of activation by metal ions and inhibition by metal ion chelators suggested that the activity of each of the enzymes is dependent on a relatively tightly bound metal cofactor.

Peptide hydrolases of the intestinal mucosa play an essential role in protein digestion. The studies presented here help to clarify the total number and substrate specificities of these enzymes in the rat brush border.

Introduction

Many small peptides are formed in the intestinal lumen by the action of proteases and are hydrolyzed to free amino acids during the process of absorption [1—3]. Thus, peptide-hydrolyzing enzymes (EC 3.4.—.—) of the intestinal mucosa play an essential role in protein digestion analogous to the role of disaccharidases in carbohydrate digestion. Unlike disaccharidases which are found almost exclusively in the brush border, peptide hydrolase activities are present in the cell cytosol as well as in the brush border [4—7]. It has been shown that cytosol and brush border peptide hydrolase activities are attributable to different enzymes [8,9] and it is not yet clear which set of enzymes is most important for digestion of peptides derived from intraluminal proteins. However, it seems likely that brush border peptide hydrolases play a significant role in this process.

Both direct and indirect evidence indicates that the intestinal brush border contains more than one peptide-hydrolyzing enzyme [9—15]. Based on kinetic studies of whole brush borders, Fujita et al. [10] concluded that the rat brush border had three peptide hydrolases with distinct substrate specificities. Kim et al. [9] detected four peptide-hydrolyzing bands on starch gel electrophoresis of rat brush border enzymes solubilized by several different methods but they detected no difference in substrate specificities by this method. Very recently, Wojnarowska and Gray [15] reported separation and partial characterization of three peptide hydrolases from rat brush borders. Investigations of the development, control, and function of these enzymes is hampered by inability to measure the activity of individual enzymes in whole brush borders or partially purified preparations. Activities can be, and have been, measured by means of substrates chosen at random from the more than 500 dipeptides and thousands of tripeptides comprising the commonly occurring amino acids. However, results of such measurements are inadequate for many purposes because the number of enzymes hydrolyzing each substrate is unknown.

The present study, in which four rat brush border peptide hydrolases are separated and partially characterized, clarifies the discrepancies in the reported number of these enzymes and provides additional data on the substrate specificities of individual enzymes. Portions of this work have appeared previously in abstract form [16,17].

Materials and Methods

Peptides were obtained from Biosynthetika (Oberdorf, Switzerland), Bachem, or Sigma Chemical Co. Lyophilized *Crotalus adamanteus* venom and *o*-

dianisidine dihydrochloride were obtained from Sigma Chemical Co., free amino acids from Mann Research Laboratories, 1,10-phenanthroline from Fisher Scientific Co., dithioerythritol from Cyclo Chemical Co., and horseradish peroxidase and crystallized papain from Worthington Biochemical Corp. All of the chemicals used were of reagent grade. Amino acids and peptides used for quantitative assays gave single ninhydrin-reactive spots on high-voltage paper electrophoresis by the method previously reported [18]. All free and peptide-bound amino acids were L-configuration unless otherwise stated.

Tissue preparation. Sprague Dawley rats (175—400 g males) were fasted overnight, killed by a blow on the head and mucosa from the entire small intestine was harvested by scraping. Purified brush borders were prepared by the method of Forstner et al. [19] with simple modifications [20] except that the glass wool filtration step was not eliminated. Based on sucrase as a marker, brush border recovery ranged from 5 to 26%, mean 15.7%. Specific activity of sucrase in purified brush borders averaged 7-fold greater than that of whole mucosal homogenates.

Enzyme solubilization. Purified brush borders were washed three times in 100 mM potassium phosphate, pH 7.0, and suspended in 0.1—0.4 volume (based on original weight of mucosa) of 10 mM potassium phosphate, pH 7.0. Crystallized papain, 0.5 mg per mg of brush border protein, and cysteine, 0.5 mM final concentration were added and the mixture incubated at 37°C. After 1 h, digestion was stopped by addition of *p*-hydroxymercuribenzoate, 0.5 mM final concentration, and the mixture was cooled to 4°C and centrifuged for 1 h at 86 000 × *g*. Because of the presence of large amounts of papain, specific activity of sucrase in the supernatant averaged only 1.6-fold greater than that of purified brush borders.

Quantitative peptide hydrolase assay. The previously described colorimetric assay [8,21] was used. The standard incubation mixture contained 5 μmol of substrate, 50 μmol of Tris · HCl buffer, pH 8.0 and enzyme in a total volume of 0.5 ml. Units are μmol of substrate hydrolyzed per min. Both the composition of the peptide substrates and their reaction products were documented by use of high-voltage paper electrophoresis following exhaustive hydrolysis of individual peptides by purified brush borders. Each dipeptide yielded only the expected free amino acids while tri- and tetrapeptides yielded the expected free amino acids plus di- and tripeptides consistent with the presence of aminopeptidase activity in the brush borders.

Semi-quantitative peptide hydrolase assay. 1 μmol of Tris buffer, pH 8.0, 0.8 μmol of substrate, and enzyme all in 0.075 ml were incubated 30 min at 37°C and the reaction terminated by placing the tubes in boiling water for 3 min. The solutions were cooled, applied to high-voltage electrophoresis paper, and the reaction products and degree of hydrolysis were determined as previously described [18].

Peptide hydrolase spot test. To quickly localize enzyme activity in column effluents or gel slices, 50—300 μl of enzyme solution or 1 gel slice were added to 50 μl of 0.05 M substrate in 0.25 M Tris · HCl, pH 8.0, in depressions of a disposable plastic spot tray (Linbro Chemical Co.). After incubation for 15—60 min at 37°C, 300 μl of phosphate-amino acid oxidase-phenanthroline reagent (see ref. 21) were added. After an additional 45 min incubation, intensity of

the brown color was recorded on a 0 to 4 scale.

Other enzyme assays. Disaccharidase activity was determined by the method of Dahlqvist [22] and leucyl-β-naphthylamidase by the method of Martinek et al. [23]. The enzymatic release of *p*-nitroaniline from L-phenylalanine *p*-nitroanilide was followed at 410 nm in a Gilford-240 automatic recording spectrophotometer. The substrate was prepared according to the method of Erlanger et al. [24] and recrystallized twice from an ethanol/water mixture before use.

Protein assays. The method of Lowry et al. [25] was used for most assays. Purified enzyme preparations containing too little protein for Lowry determination were assayed by determining absorbance at 220 nm in quartz cuvettes with a Beckman DU spectrophotometer. Crystalline bovine serum albumin was used as standard for both methods.

DEAE-cellulose chromatography. Standard type 70 DEAE-cellulose (S and S, approx. 0.90 mequiv./g) equilibrated with 5 mM sodium phosphate, pH 7.0, saturated with toluene to retard bacterial growth, was prepared and packed by air pressure in 2.5 × 30 cm columns as described by Peterson [26]. Columns were eluted at 4°C by upward flow maintained with a peristaltic pump at 70 ml/h. Fractions were collected automatically for 18-min intervals. Fresh columns were prepared for each new sample and charged with 1—4 ml (1.5— 6 mg protein) of solubilized brush border material obtained from the mucosa of one or two rats. Linear NaCl gradients were formed by the method of Parr [27] using two identical vessels, one containing 600 ml of 5 mM sodium phosphate buffer and the other 600 ml of 0.25 M NaCl in the same buffer.

Gel filtration chromatography. Molecular weights were estimated by gel filtration on Sephadex G-200 (Pharmacia) prepared according to the manufactuerer, equilibrated with 0.2 M sodium phosphate, pH 7.0, and packed in a 1.5 × 60 cm column. Samples of 0.36 ml followed by 0.36 ml of 10% sucrose were applied through a rubber diaphragm at the bottom of the column and flow in the upward direction was maintained at 3.1 ml/cm^2 per h at 4°C by a peristaltic pump. Total bed volume (V_t) was 79.9 ml and void volume (V_0) determined with Blue Dextran 2000 was 33.5 ml. 17-min fractions were collected in tared tubes and volumes of each fraction were calculated from net weights and measured specific gravity of the elution buffer. The column was calibrated by use of apo-ferritin, aldolase, bovine albumin, ovalbumin, and ribonuclease.

Analytical disc electrophoresis. Disc acrylamide electrophoresis [28,29] in 6 or 7% gels, 0.5 × 4.5 cm, was carried out as described by the Canalco Co. [30]. Gels were photopolymerized and samples of 50 μl (10—100 μg protein) were applied in 3% sucrose. Electrophoresis at 2.5 mA per gel was continued until the tracking dye disc was 4.5 cm from the surface of the separating gel. Immediately after electrophoresis, the gels were either cut into approx. 1.5-mm segments with a gel slicer for enzyme assays or fixed in 12% trichloroacetic acid, stained with 1% Coomassie blue, and destained overnight in 7% acetic acid.

Preparative disc electrophoresis. Preparative acrylamide gel electrophoresis was carried out in 6 or 7% gels, 2.5 cm in diameter and 3—8 cm long in the apparatus (Uniphor column electrophoresis apparatus, LKB) described by Bergrahm and Harlestam [31]. Preparative grade reagents were used. Electrophore-

sis was contineud for 30—60 h at 800—1000 V. The bottom of the gel column was eluted with buffer at 7—21 ml/h by peristaltic pump and 15—30-min fractions were collected.

Starch gel electrophoresis. Vertical starch gel electrophoresis was performed by the method of Lewis and Harris [32] and Kim et al. [9] by use of the Buchler gel electrophoresis apparatus. Initially, the gel and bridge buffers were 5 mM and 100 mM Tris/maleate, pH 7.4, respectively. Identical zymograms were obtained when 10 mM and 100 mM sodium phosphate, pH 7.4, respectively, were used for the gel and bridge buffers in order to eliminate possible interactions between the enzymes and maleate ion. Loading, electrophoresis, and development of the gel were as described by Kim et al. [9] except that only 35 μl of enzyme solution was applied to each slot, $MnCl_2$ was omitted from the agar overlay solution, and incubation was at room temperature after addition of the agar overlay.

Concentration of protein solutions. Enzyme solutions were concentrated at 4°C by ultrafiltration on Amicon UM-20E or UM-10 membranes in 10- and 50-ml cells pressurized with nitrogen at 40 lb/inch^2. Purified enzymes were not adequately retained by UM-20E membranes.

Results

Enzyme solubilization. Several methods were used in attempts to solubilize phenylalanylglycine (Phe-Gly) and glycylphenylalanine (Gly-Phe) hydrolase activities of intestinal brush borders. Treatment of purified brush borders with chelating agents (EDTA and *o*-phenanthroline), 1 M KCl, distilled water, saline at 54°C, citrate/phosphate buffer at pH 6.0, Tris · HCl buffer at pH 8.5, and carbonate/bicarbonate buffer at pH 10 each yielded less than 10% solubilization. Extraction of brush borders with various concentrations of butanol (1 and 2 phases) yielded up to 50% solubilization of Gly-Phe hydrolase but total recovery of Phe-Gly hydrolase activity was poor. Treatment of brush borders with 1% Triton X-100, a non-ionic detergent, gave 70—80% solubilization with 90—100% total recovery, but efforts to separate the solubilized proteins by chromatography and electrophoresis failed and multiple efforts to separate Triton from the solubilized protein either failed or resulted in destruction of enzyme activity. A dialyzable, low molecular weight fraction of the detergent was isolated from commercially available Triton X-100 by gel filtration on Sephadex G-200. This fraction gave excellent solubilization of brush border peptide hydrolases and disaccharidases but when solubilized protein was exhaustively dialyzed against buffer to remove the low molecular weight Triton, the enzymes precipitated and could not be resolubilized with buffer.

When purified brush borders were treated with papain for 60 min, up to 80% of the Phe-Gly and 120% of the Gly-Phe hydrolase activities remained in the supernatant after centrifugation for 1 h at 87 000 × *g*. Total recovery in supernatant and pellet ranged from 111 to 161%.

Isolation of brush border peptide hydrolases. Chromatography of papain solubilized brush border enzymes on DEAE-cellulose by elution, first with 5 mM phosphate buffer, then with a linear NaCl gradient, gave four well-separated peaks when the effluent was assayed for both Phe-Gly and Gly-Phe hy-

drolase activity (Fig. 1). By use of the semi-quantitative high-voltage paper electrophoresis assay, fractions comprising each of the four peaks, the area between peaks 1 and 2, the area between peaks 3 and 4, and the area beyond peak 4 were tested for their ability to hydrolyze 49 dipeptides, six tripeptides, and three tetrapeptides. The results indicated that peak 1 has little activity, that peaks 2, 3, and 4 each have distinct substrate specificities, and that there are no additional peptide hydrolase peaks.

On the basis of the semi-quantitative assay results, Met-Phe was chosen as a substrate for further investigation of peaks 1 and 2. Use of this substrate to assay the column fractions yielded a proportionately much greater activity in these peaks compared to peaks 3 and 4 than was obtained with Gly-Phe or Phe-Gly as substrate, see Fig. 2. Peak 1 enzyme activity eluted in the break-through volume for the column, along with several free amino acids which produced a high assay blank. When peak 1 was concentrated and reapplied to a fresh column, the activity eluted in the peak 2 position indicating that this activity results from excess peak 2 enzyme which did not bind to the column. Thus, these studies indicate the presence of at least three rat brush border peptide hydrolases which are separated by ion-exchange chromatography.

Preliminary quantitative substrate studies were done with peaks 3 and 4 to confirm some of the semi-quantitative findings and to discover which sub-

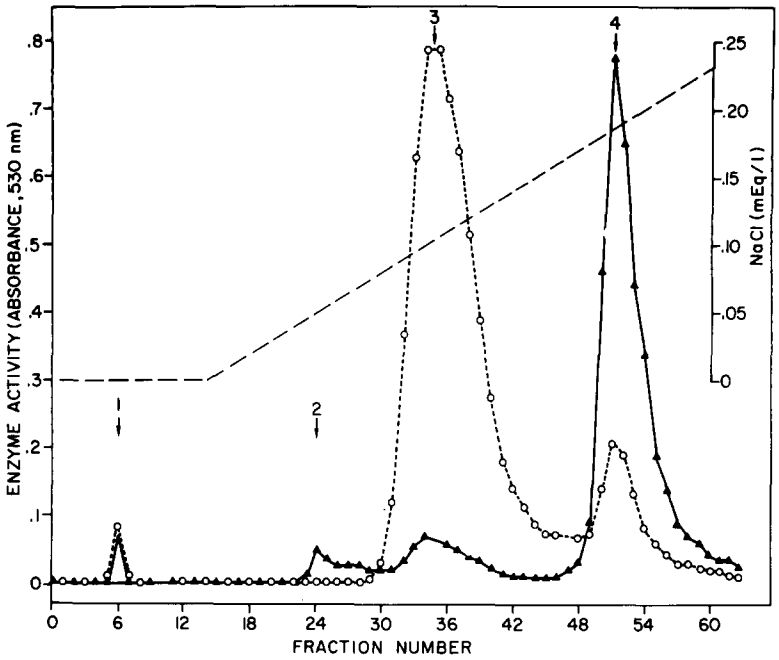

Fig. 1. Separation of brush border peptide hydrolases on DEAE-cellulose. Papain-solubilized brush border proteins from one rat were applied to a 2.5 X 30 cm column of DEAE-cellulose equilibrated with 5 mM sodium phosphate buffer and eluted with a linear NaCl gradient (— — —). Column fractions were assayed for Phe-Gly (o - - - - - - o) and Gly-Phe (▲———▲) hydrolase activities.

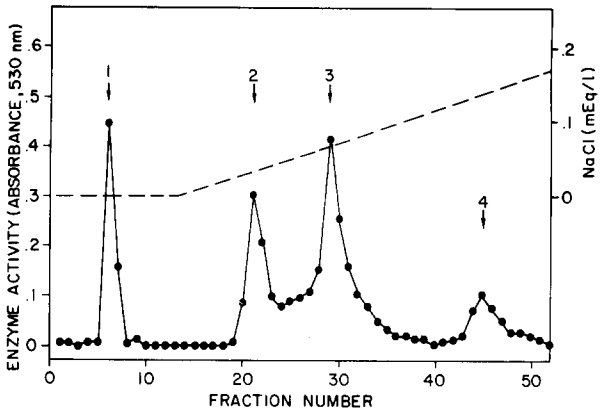

Fig. 2. Met-Phe hydrolase activity of brush border protein fractions separated by DEAE-cellulose chromatography. Solubilized brush border protein was chromatographed as described in the legend to Fig. 1 and column fractions assayed for Met-Phe hydrolase activity.

strates were split most specifically by each enzyme. As shown in Table I, the specific activities of peak 3 for release of phenylalanine from Phe-Gly, Phe-Gly-Gly, and Phe-Gly-Phe-Gly were several-fold greater than the specific activities of peak 4 for these substrates. In contrast, Gly-Phe and Gly-Leu were hydrolyzed by peak 4 much more rapidly than by peak 3. Similar differences between the two peaks were apparent when results for each peak were expressed as units per animal rather than units per mg protein.

The three brush border enzymes were designated BB-I, BB-II, and BB-III based on the order of their elution from DEAE-cellulose. Column peaks 1 and 2 are both enzyme I. Throughout this study, enzyme I has usually been identified by its activity against Met-Phe, Ser-Met, or Met-Met although these substrates are rapidly split by the other two enzymes as well. Enzyme II has been identified by its aminopeptidase or Phe-Gly hydrolase activity and enzyme III by its activity against Gly-Phe or Gly-Leu.

In order to confirm the results obtained by ion-exchange chromatography and to look for additional peptide hydrolases, papain solubilized brush border proteins were also separated by disc acrylamide gel electrophoresis. By use of

TABLE I

PEPTIDE HYDROLASE ACTIVITIES OF DEAE PEAKS 3 AND 4

Papain-solubilized brush border enzymes separated on DEAE-cellulose were assayed following concentration by membrane filtration.

Substrate	Specific activity (μmol/min per mg protein)		Ratio peak 3/peak 4
	Peak 3	Peak 4	
Phe-Gly-Gly	23.9	1.8	13
Phe-Gly-Phe-Gly	37.3	2.8	13
Phe-Gly	14.0	4.7	3
Gly-Phe	4.0	41.4	0.1
Gly-Leu	1.3	32.4	0.04

analytical disc techniques, as many as 14 protein bands, some very faint, were visible and three peptide hydrolase bands were detected by testing gel slices for hydrolysis of Phe-Gly and Gly-Phe (Fig. 3). Under the conditions used, papain was shown to migrate toward the anode, thus, it was removed from the solubilized brush border proteins during disc electrophoresis. No solubilized brush border proteins were noted to migrate toward the anode. When brush border proteins were subjected to preparative acrylamide gel electrophoresis on gels 4—8 cm long most of the protein and other material absorbing at 280 nm eluted before the peptide hydrolases. Assays with multiple substrates revealed only three peaks and appropriate substrates were used to identify the three enzymes. They migrated from the gel in reverse order from that in which they eluted from DEAE-cellulose (Fig. 4).

Substrate specificities. Solubilized brush border protein was subjected to DEAE-cellulose chromatography as described in the legend to Fig. 1 except that the amount of sample applied was reduced so that no unbound peptide hydrolase activity appeared in the break-through volume. The three peptide hydrolase peaks were detected by assaying each fraction for Met-Phe hydrolysis. The two most active fractions from each peak, representing between one-fourth and one-half of the total activity, were pooled and assayed for hydroly-

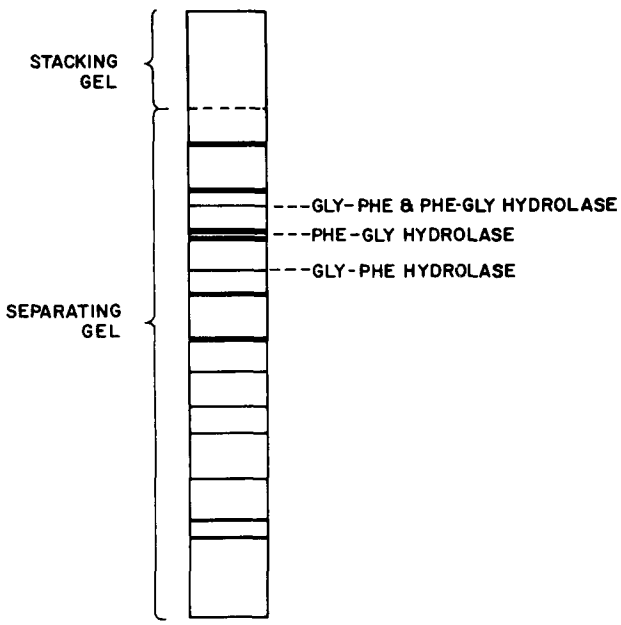

Fig. 3. Analytical acrylamide gel electrophoresis of papain solubilized brush border proteins. Solubilized brush border protein was applied to 0.5 × 4.5 cm light polymerized 7% gels and electrophoresed as described in Materials and Methods. The stacking gel buffer was Tris · HCl, pH 6.7; the separating gel buffer was Tris · HCl, pH 8.9; and the electrode buffer was Tris/glycine, pH 8.3. Protein bands were stained with Coomassie blue. An identically treated gel was cut into 1.5 mm slices, and the two halves of each slice were tested for Gly-Phe and Phe-Gly hydrolase activity. These results and subsequent testing of preparative gel fractions with more specific substrates confirm that the fastest migrating activity is enzyme III, the next enzyme II, and the slowest is enzyme I.

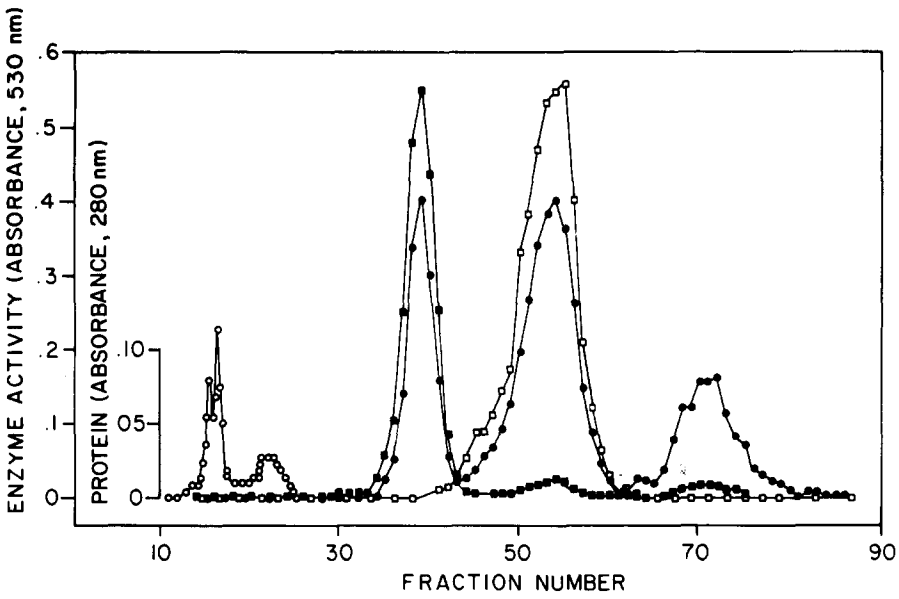

Fig. 4. Preparative acrylamide gel electrophoresis of papain-solubilized brush border proteins. Solubilized brush border material (1.2 ml) was applied to the surface of the gel as 3% sucrose solution. The column consisted of a 4 cm, 7% separating gel and 1.2 cm stacking gel photopolymerized with riboflavin as catalyst. The electrode buffer was Tris/glycine, pH 8.3; the stacking gel buffer was Tris · HCl, pH 6.7; and the separating gel buffer was Tris · HCl, pH 8.9. There was no electrophoresis prior to adding the sample. The sample was electrophoresed at 1000 V, 8—10 mA. Temperature at 4°C was maintained by constant circulation of refrigerated water through electrode and column jackets and the entire apparatus was kept in a cold room at 4°C. The bottom of the column was eluted at 20.4 ml/h with electrode buffer. Fractions were collected for 20-min intervals. Eluted fractions were assayed for hydrolysis of Gly-Phe (■———■), leucyl-β-naphthylamide (□———□), and Met-Phe (●———●). Substrate specificities clearly indicate that the three enzyme peaks centered at fractions 39, 54, and 72 are enzymes III, II, and I, respectively. Absorbance at 280 nm (○———○) is also shown up to fraction 25. Under these conditions, minimal absorbance at 280 nm was detected in fractions beyond 25, and it is not plotted. Much of the 280 nm-absorbing material may be impurities eluted from the gel even though preparative grade components were used.

sis of 35 peptide substrates comprising 15 different amino acids. For each substrate, the units of activity and the percentages of total recovered activity in each peak were determined (Table II). Marked differences in the substrate specificities of the three enzymes is apparent. The most rapidly hydrolyzed substrates for each enzyme are Met-Ala and Met-Phe for enzyme I, Phe-Ser and Phe-Val for enzyme II, and Gly-Leu and Gly-Met for enzyme III. It is noteworthy that, while certain substrates, such as Met-Ala, are hydrolyzed approximately equally well by all three enzymes, other substrates are very discriminating. Thus, 99% of the leucine amide hydrolase activity recovered is attributable to enzyme II while Gly-Leu and Arg-Leu are hydrolyzed almost exclusively by enzyme III (Table III). In contrast, all the substrates tested which are hydrolyzed by enzyme I are also hydrolyzed rapidly by enzyme II or III or both. The most discriminating substrates for enzyme I are Met-Phe and Sr-Met but no more than 50% of the total brush border activities against each of these peptides is attributable to this peptide hydrolase.

Proper interpretation of the above data depends on knowing the recovery

TABLE II

SUBSTRATE SPECIFICITIES OF THE THREE BRUSH BORDER PEPTIDE HYDROLASES ISOLATED BY DEAE-CELLULOSE CHROMATOGRAPHY

Units (μmol/min) have been rounded to one decimal place. Percentages were calculated prior to rounding.

Substrate	Enzyme I		Enzyme II		Enzyme III	
	Units	Percent of total	Units	Percent of total	Units	Percent of total
Met-Phe	11.1	48	9.8	43	2.1	9
Ser-Met	6.9	47	4.0	28	3.7	25
Val-Phe	0.5	46	0.5	43	0.1	11
Val-Met	1.5	42	1.8	50	0.3	8
Met-Ala	21.0	39	16.0	30	17.1	32
Pro-Met	0.3	39	0.0	3	0.5	58
Asp-Leu	0.3	37	0.1	17	0.3	45
Leu-Phe	5.5	37	8.2	54	1.4	9
Ser-Phe	2.7	34	4.0	50	1.3	16
Leucine amide	0	0	24.6	99	0.2	1
Phe-Glu	0	0	4.2	96	0.2	4
Phe-Val	1.5	6	25.2	93	0.2	1
Phe-Ser	0.9	3	32.6	93	1.5	4
Arg-Phe	0.3	4	8.1	89	0.7	7
Phe-Pro	0.1	10	0.7	82	0.1	8
His-Leu	0.1	8	0.9	75	0.2	17
Val-Leu	0.1	7	0.7	75	0.2	18
Met-Val	6.6	24	20.1	73	0.8	3
Phe-Met	6.3	24	18.4	72	1.1	4
Thr-Phe	0.3	15	1.7	71	0.3	14
Leu-Leu	2.0	7	18.8	70	6.2	23
Leu-His	0.5	6	6.4	67	2.7	27
Ala-Met	6.4	17	24.9	65	7.1	18
Gly-Leu	0.3	1	2.4	4	60.1	95
Pro-Leu	0.0	2	0.1	14	0.4	84
Arg-Leu	0.2	4	0.8	16	4.4	81
Gly-Met	3.2	10	4.8	15	25.0	75
Lys-Leu	0.2	2	1.6	23	5.2	75
Thr-Leu	0.1	6	0.3	23	0.9	71
Leu-Ser	2.4	11	5.4	23	15.1	66
Ala-Leu	0.9	3	18.3	57	12.9	40
Leu-Ala	6.6	18	17.7	49	12.2	33
Met-Asp	1.7	9	11.1	59	6.1	32

TABLE III

DISTRIBUTION OF RECOVERED PEPTIDE HYDROLASE ACTIVITIES AMONG THE ENZYMES ISOLATED FROM RAT INTESTINAL BRUSH BORDER

The three peptide hydrolases were isolated as described in the text under Substrate specificities. The figures below are mean percentages (±1 S.D.) of total recovered activity determined in three separate experiments.

Enzyme	Hydrolyse activity (%)		
	Ser-Met	Leucine amide	Gly-Leu
Enzyme I	50.3 (5.5)	0.2 (0.4)	0.9 (1.6)
Enzyme II	26.7 (2.1)	98.1 (2.4)	7.0 (1.7)
Enzyme III	23.0 (7.4)	1.7 (2.5)	92.1 (1.8)

of the three peptide hydrolases following papain solubilization and separation by DEAE-cellulose chromatography. Percentage recoveries were obtained from results of three separate experiments in which activity was determined using the most appropriate substrate for each enzyme. Following papain solubilization, mean recovery (±1 S.D.) of hydrolase activities in the soluble fraction was 56 (±4)% for sucrose, 134 (±8)% for Ser-Met, 75 (±8)% for leucine amide, and 247 (±14)% for Gly-Leu. Mean activity remaining in the pellet following papain digestion was 16% for Ser-Met, 13% for leucine amide, and 24% for Gly-Leu. When the solubilized material was chromatographed on DEAE-cellulose, the mean recovery of activity in all column fractions was 59 (±19)% for Ser-Met, 121 (±66)% for leucine amide and 65 (±25)% for Gly-Leu. The low recovery and large standard deviation for leucine amide recovery is probably due to technical error as recovery of activity against other substrates highly discriminating for enzyme II, Leu-Gly-Gly and leucyl-β-napthylamide, was 103 (±18)% and 117 (±14)% respectively. Distribution of the recovered activities among the three enzyme peaks confirms the results of the initial specificity tests (Table III).

The use of highly discriminating substrates permits meaningful estimation of the degree of contamination of one enzyme by another. Thus, based on the results shown in Table III, contamination of enzymes I and III by enzyme II could be no greater than approx. 0.2 and 1.7%, respectively. Contamination of enzymes I and II by enzyme III could be no greater than 0.9 and 1%, respectively. It seems likely that enzymes II and III are equally free of contamination by enzyme I but this remains uncertain in the absence of a highly discriminating substrate for enzyme I.

The three enzymes were also isolated by preparative acrylamide gel electrophoresis and tested for hydrolysis of a number of substrates. Results were similar to those obtained following separation by ion-exchange chromatography though recovery of enzyme activities was not as adequate nor as consistent. These experiments do provide data for additional substrates. As shown in Table IV, enzyme II contributes more than 90% of the recovered hydrolase activity against leucyl-β-naphthylamide, Phe-Asp, Phe-Arg, and Leu-Gly-Gly (amino terminal bond) while enzyme III contributes 85% or more of the total activity against Leu-Gly and Gly-Phe. Again, no substrate was hydrolyzed predominantly by enzyme I.

Additional studies revealed that enzyme II provides more than 90% of the recovered hydrolase activities for the *p*-nitroanilides of leucine, phenylalanine, alanine, glycine, and methionine.

Partial purification. The three brush border peptide hydrolases isolated have been partially purified. Availability of highly specific substrates for enzymes II and III permitted accurate determination of these two enzyme activities in whole purified brush borders and in subsequent partially purified preparations. The specific activities (units/mg protein) of enzymes II and III were increased 10-fold and 120-fold, respectively, following DEAE-cellulose chromatography and 4.7-fold and 90-fold, respectively, following preparative acrylamide gel electrophoresis.

Peptide hydrolases separated by either ion-exchange chromatography or preparative gel electrophoresis were contaminated with other brush border en-

TABLE IV

SUBSTRATE SPECIFICITIES OF THE THREE ISOLATED BRUSH BORDER PEPTIDE HYDROLASES

Solubilized brush border protein was electrophoresed on 2.5 × 4 cm acrylamide gel as described in the legend to Fig. 5. The three enzyme peaks localized by assaying fractions for Met-Phe hydrolase activity were concentrated 5-fold and assayed quantitatively for their ability to hydrolyze the substrates listed. For each enzyme, the column on the left gives the total activity in the peak and the column on the right gives percent of total recovered activity.

Substrate	Enzyme I		Enzyme II		Enzyme III	
	nmol/min	Percent of total	nmol/min	Percent of total	nmol/min	Percent of total
Met-Met	1040	23	1770	39	1760	38
Ser-Phe	112	21	255	48	163	31
Phe-Ala	153	20	344	45	267	35
Leucyl-β-naphthylamide	5	1	686	97	12	2
Phe-Asp	12	11	1395	95	61	4
Phe-Arg	60	3	1620	94	43	3
Leu-Gly-Gly	18	1	1850	94	108	5
Phe-Glu	25	4	525	88	49	8
Leu-Gly	4	0	424	15	2500	85
Gly-Phe	47	3	191	12	1300	85
Met-Ser	190	8	538	23	1630	69
Met-Gly	20	1	1380	36	2410	63
Met-Leu	145	5	1200	39	1720	56
Phe-Gly	42	1	3180	82	652	17
Ala-Phe	48	3	996	66	461	31
Arg-Phe	35	6	466	80	82	14
Lys-Phe	19	4	195	37	307	59
Asp-Phe	59	12	319	66	109	22
His-Phe	32	5	523	87	48	8
Pro-Phe	9	20	13	29	23	51

zymes as shown in Figs. 5 and 6. When the DEAE chromatography was repeated using a column equilibrated with 1 mM dithioerythritol, the pattern of elution of peptide hydrolases and disaccharidases was identical to that obtained in the absence of this agent suggesting that reversible disulfide bridging was not playing a role in the co-elution of peptide hydrolases I and II with disaccharidases A and B, see Fig. 5.

Although peptide hydrolases II and III were readily adsorbed by alumina and hydroxyapatite gels (less well by calcium phosphate gel) in 10 mM sodium acetate, pH 5.5, the peptide hydrolases were not separated from contaminating disaccharidases by adsorption or by selective de-sorption using dipeptide substrates or various concentrations of sodium phosphate buffer, pH 7.0.

In order to further purify the peptide hydrolases and separate them from the disaccharidases, each peptide hydrolase peak obtained by DEAE-cellulose chromatography was subjected to preparative gel electrophoresis. In this way, enzyme I was separated from the contaminating disaccharidase (Fig. 7). In contrast, activity against Met-Phe and Ser-Phe (Fig. 7) as well as Gly-Met and Phe-Met, not shown, eluted in a single peak. Similarly, enzyme II was well separated from contaminating disaccharidase while hydrolase activity against Phe-Gly,

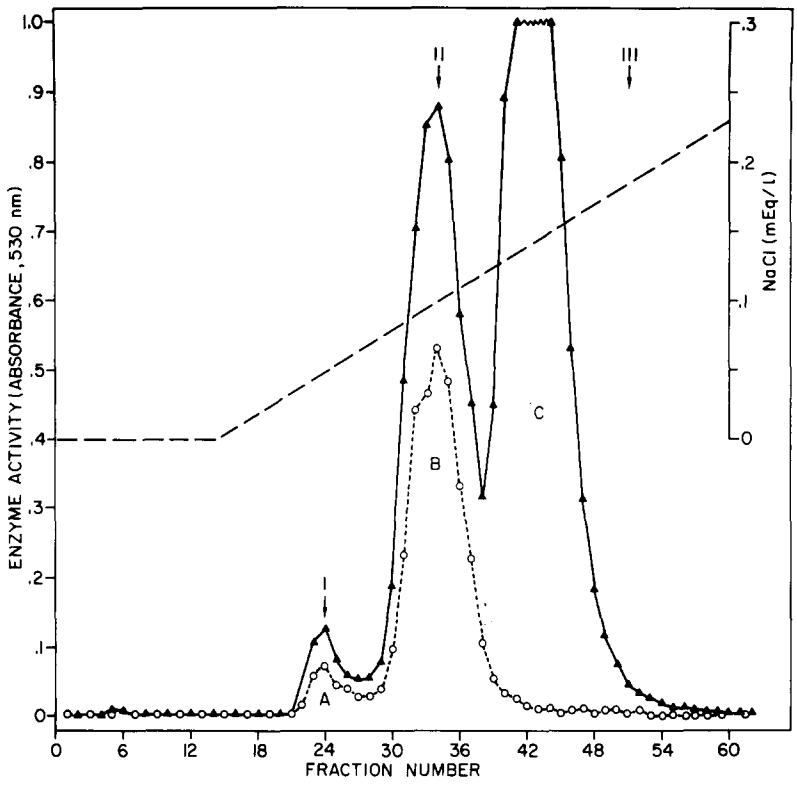

Fig. 5. Separation of brush border disaccharidases on DEAE-cellulose. Papain-solubilized brush border proteins were chromatographed as described in the legend to Fig. 1. Fractions were assayed for sucrase (○ - - - - - - ○) and maltase (▲———▲) activities. Location of peptide hydrolases I, II, and III, indicated by the arrows, was determined by assaying fractions for appropriate specific activities. Disaccharidase peaks are labeled A, B, and C for later identification.

Leu-Gly-Gly and leucyl-β-naphthylamide eluted in a single peak (Fig. 8). Enzyme III obtained by DEAE chromatography, although not significantly contaminated with disaccharidase, was subjected to acrylamide gel electrophoresis and yielded a single peak when column effluent was tested for hydrolysis of Gly-Phe, Phe-Gly, Leu-Gly, Gly-Leu, and Met-Gly. On a few occasions, the enzyme II peak showed evidence of a shoulder (see below). This was never observed for enzymes I or III.

When the three enzymes were partially purified by subjecting brush border proteins to DEAE-cellulose chromatography followed by preparative acrylamide gel electrophoresis, only 2.5—7.5 μg of protein per peak were obtained from each rat. To obtain sufficient protein for analytical acrylamide gel studies of homogeneity, individual enzymes were isolated by DEAE chromatography from the mucosa of nine rats by applying solubilized protein from three rats to a fresh column on each of three successive days. Fractions containing enzyme I were pooled, equilibrated with 5 mM phosphate, pH 7.0, by diafiltration, concentrated, and subjected to preparative gel electrophoresis. Specific activity of the pooled enzyme against Ser-Phe was 3.32 units/mg protein after DEAE chromatography and 1.66 units/mg protein (10% recovery of activity)

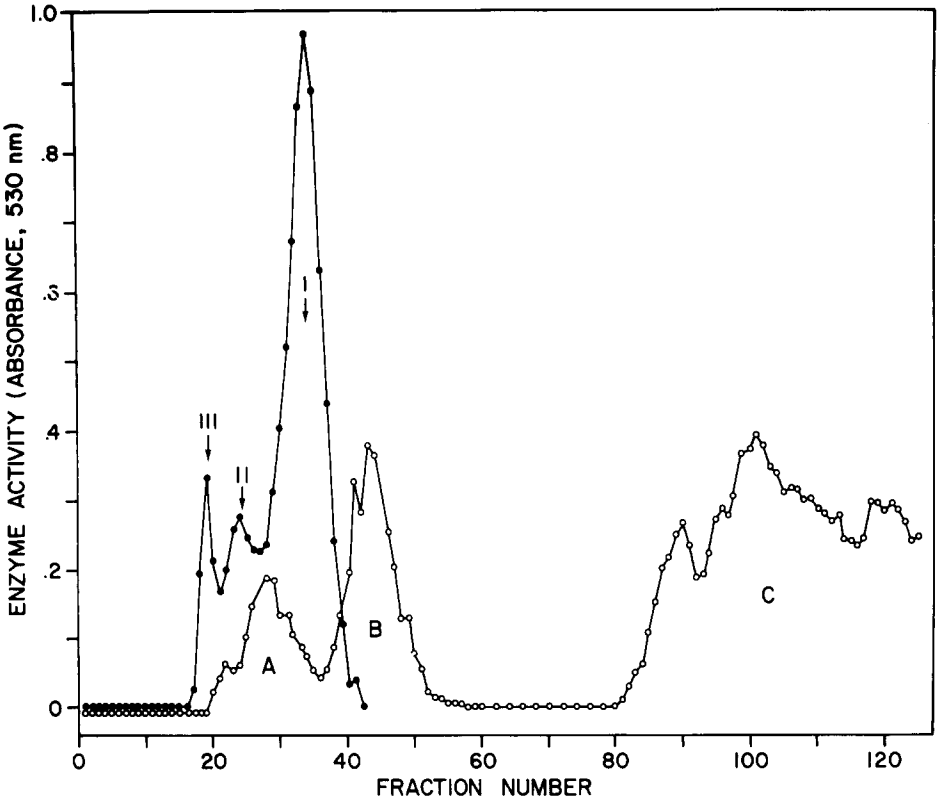

Fig. 6. Separation of brush border peptide hydrolases and disaccharidases by preparative acrylamide gel electrophoresis. Solubilized brush border protein (1.3 ml) was applied to the surface of the gel as a 5% sucrose solution. The 8 cm, 5% acrylamide gel was polymerized with persulfate and pre-electrophoresed for 18 h prior to adding sample. Electrode buffer was 5 mM Tris/glycine/HCl, pH 7.9, and gel buffer was 50 mM Tris/glycine, pH 8.1. Electrophoresis was carried out at 1000 V, 18 mA at 4° C. Elution was at 7.4 ml/h with 10 mM Tris/glycine/HCl, pH 7.9, and 30 min fractions were collected. Depicted are results of assaying fractions for Met-Phe hydrolase (●———●) and maltase (○———○) activity. Although maltase activity may be depressed by the presence of Tris (an inhibitor of disaccharidase activity) no addiitonal peaks appeared when each fraction was dailyzed to remove Tris. The identity of peptide hydrolases I, II, and III was determined by assay with specific substrates. Disaccharidase peaks are lettered as in Fig. 6. Identity of the three disaccharidases isolated by ion-exchange chromatography and acrylamide electrophoresis was established by assaying fractions from both procedures with additional disaccharides (sucrose and isomaltose) and by subjecting individual peaks from DEAE-cellulose chromatography to preparative acrylamide gel electrophoresis.

after preparative gel electrophoresis. The decrease in specific activity is probably the result of inactivation of the enzyme due to time required for purification and possibly due to the loss of a metal ion cofactor. Total purification obtained by the two-step procedure could not be determined due to lack of a suitably specific substrate with which to assay enzyme I in whole brush borders. Analytical disc electrophoresis of the final preparation produced a single protein band which coincided with enzyme activity (Fig. 9).

Enzymes II and III were also partially purified by use of both ion-exchange chromatography, and preparative disc electrophoresis. In the case of these enzymes, protein from two or three rats was first applied to fresh preparative

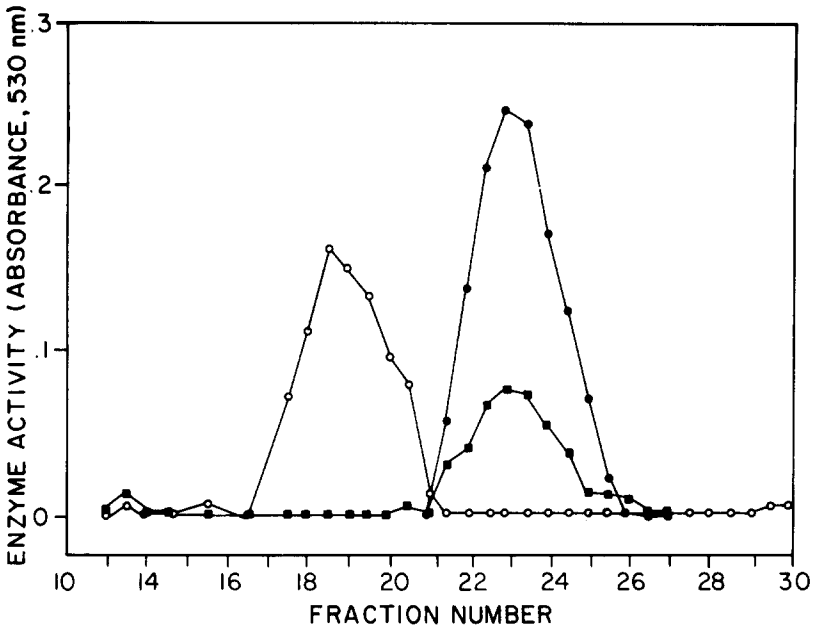

Fig. 7. Preparative acrylamide gel electrophoresis of the enzyme I peak isolated by DEAE chromatography. Fractions comprising brush border enzyme I isolated from two rats by DEAE-cellulose chromatography were pooled, concentrated 30-fold and equilibrated with 5 mM sodium phosphate (pH 7.0) by membrane filtration, and subjected to preparative electrophoresis. The conditions were identical to those described in the legend for Fig. 6. The sample volume was 1.0 ml in 5% sucrose. Results shown are for Met-Phe (●———●) and Ser-Phe (■———■) hydrolases and maltase (○———○).

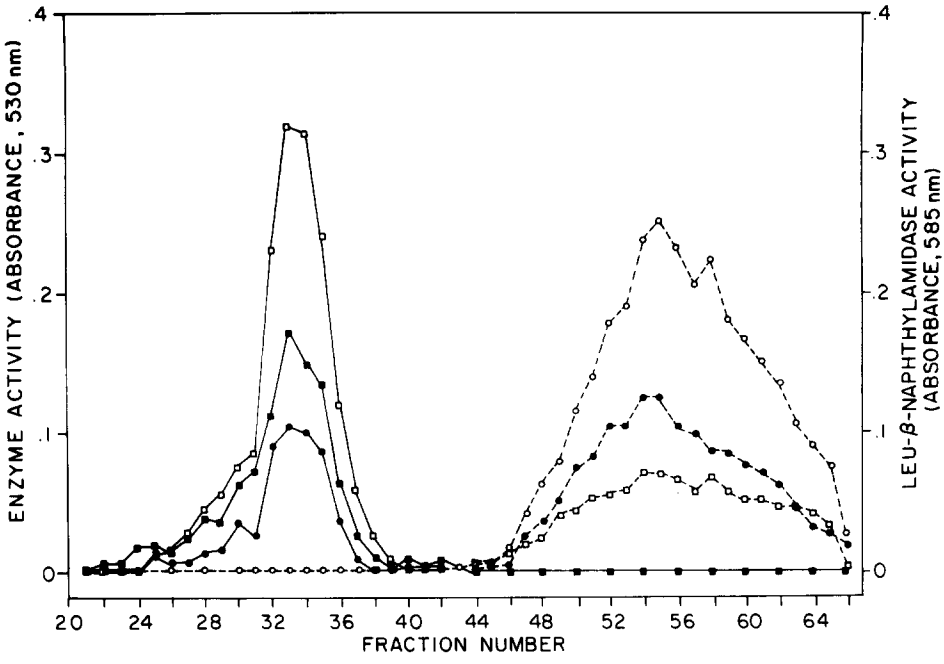

Fig. 8. Preparative acrylamide gel electrophoresis of enzyme II peak isolated by DEAE chromatography. Fractions from DEAE chromatography comprising brush border enzyme II were isolated, concentrated, and electrophoresed as described in the legend to Fig. 7. The sample volume was 1.2 ml in 5% sucrose. Results shown are for hydrolysis of Phe-Gly (■———■), Leu-Gly-Gly (●———●), leucyl-β-naphthylamide (□———□), sucrose (□ - - - - - - □), maltose (○ - - - - - - ○), and iso-maltose (● - - - - - - ●).

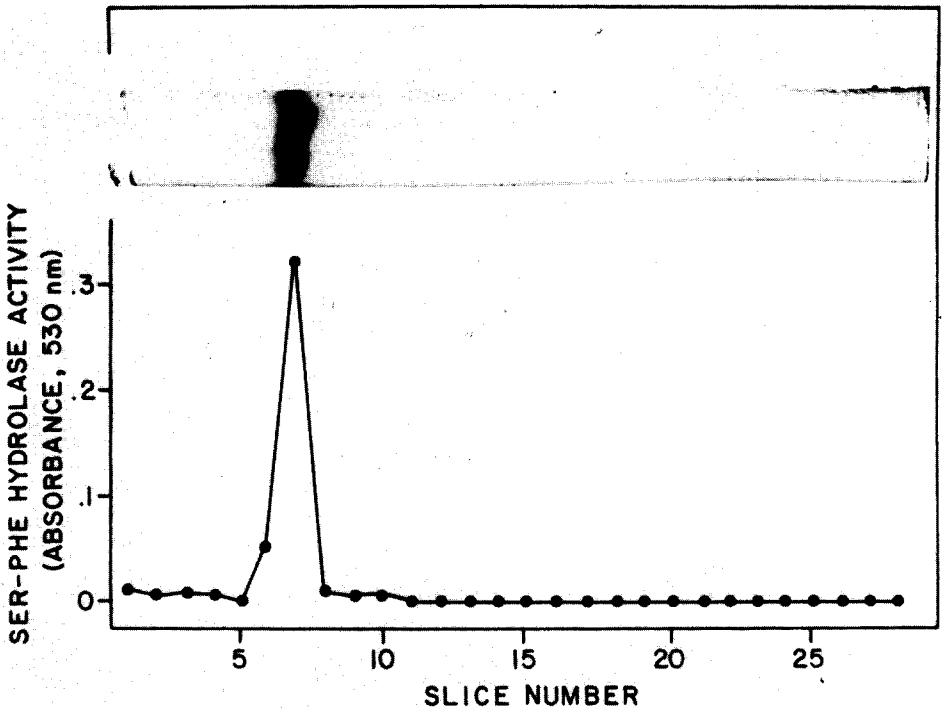

Fig. 9. Analytical acrylamide gel electrophoresis of purified, concentrated enzyme I. Enzyme I was isolated from the intestinal brush border of nine rats by means of DEAE chromatography of material from three rats on three successive days. Fractions comprising enzyme I were concentrated 186-fold, equilibrated with 5 mM Tris/glycine/HCl buffer, pH 7.9, and subjected to preparative acrylamide gel electrophoresis. Conditions for gel electrophoresis were the same as described in the legend to Fig. 7 except that the pre-electrophoresis time was only 3 h, 1.0 ml of sample was applied, 750 V, 20 mA, and the elution rate was 11.5 ml/h. Fractions comprising enzyme I were concentrated 45-fold and 0.05 ml of the concentrated material was subjected to analytical acrylamdie gel electrophoresis. Gels were electrophoresed until marker dye reached 4.5 cm from the separating gel surface. One gel was stained for protein with Coomassie blue and another was immediately sliced horizontally into 1.5-mm segments which were crushed and analyzed quantitatively for Ser-Phe hydrolase activity. In plotting the data correction was made for slight elongation of the gel produced by swelling during staining for protein.

acrylamide gel columns on each of three different days followed by DEAE-cellulose chromatography of the pooled, concentrated fractions for each enzyme. The final degree of purification of the two enzymes as evidenced by increase in specific activities was not great, 3-fold for enzyme II and 50-fold for enzyme III. Nevertheless, the preparations were free of disaccharidase activity and analytical gel electrophoresis of the final enzyme preparations yielded a single broad band which coincided with enzyme activity in the case of enzyme II (Fig. 10) and a heavy protein band coinciding with enzyme activity plus an additional faint protein band for enzyme III (Fig. 11).

Bacterial enzymes. To determine whether some of the enzymes isolated from purified brush borders could be of bacterial origin, germ-free rats (no growth on aerobic or anaerobic culture of intestinal contents) were used to prepare solubilized brush border enzymes. DEAE-cellulose chromatography of this material yielded results identical to those shown in Figs. 1 and 5. The sub-

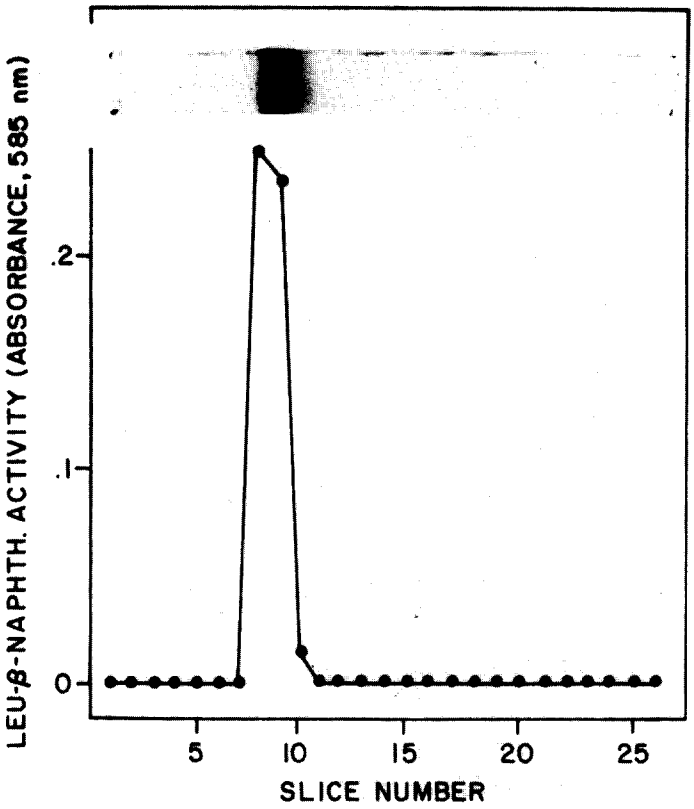

Fig. 10. Analytical acrylamide gel electrophoresis of purified enzyme II. Enzyme II was isolated from the intestinal brush border of seven rats by means of preparative acrylamide gel electrophoresis of material from two or three rats on three occasions. Gels were prepared and run as described in the legend for Fig. 5 except that 2.5—4.6 ml of solubilized brush border material was electrophoresed on the three occasions. Fractions comprising enzyme II were pooled, concentrated 72-fold, equilibrated with 5 mM sodium phosphate buffer, and chromatographed on DEAE-cellulose eluted with a 0—0.15 M NaCl gradient. Column fractions comprising enzyme II were concentrated 178-fold and 0.1 ml of the concentrated material was subjected to analytical acrylamide electrophoresis. In addition to leucyl-β-naphthylamidase activity shown Phe-Gly, Met-Phe, and Leu-Gly-Gly hydrolase activities were also present only at the location of the single band shown.

cellular distribution of Phe-Gly hydrolase (38% in the supernatant and 54% in the pellet) and of Gly-Phe hydrolase (90% in the supernatant and 2% in the pellet) in the germ-free rats was also similar to that for conventional animals.

pH optima. Enzymes I, II, and III isolated by DEAE chromatography had pH optima of 8.0, 8.0, and 8.5, respectively, when Phe-Gly was substrate and 8.5, 7.5, and 8.5, respectively, when Gly-Phe was substrate.

Molecular weights. Molecular weights determined by gel filtration chromatography on Sephadex G-200 were 275 000 and 290 000 (mean 283 000) for enzyme I, 252 000 and 316 000 (mean 284 000) for enzyme II, and 134 000 for enzyme III.

Metal activation. The three peptide hydrolases isolated by preparative acrylamide gel electrophoresis were tested for activation by 30-min preincubation in 0.125 M Tris buffer, pH 8.0, with 1, 0.1, and 0.01 mM final concentrations

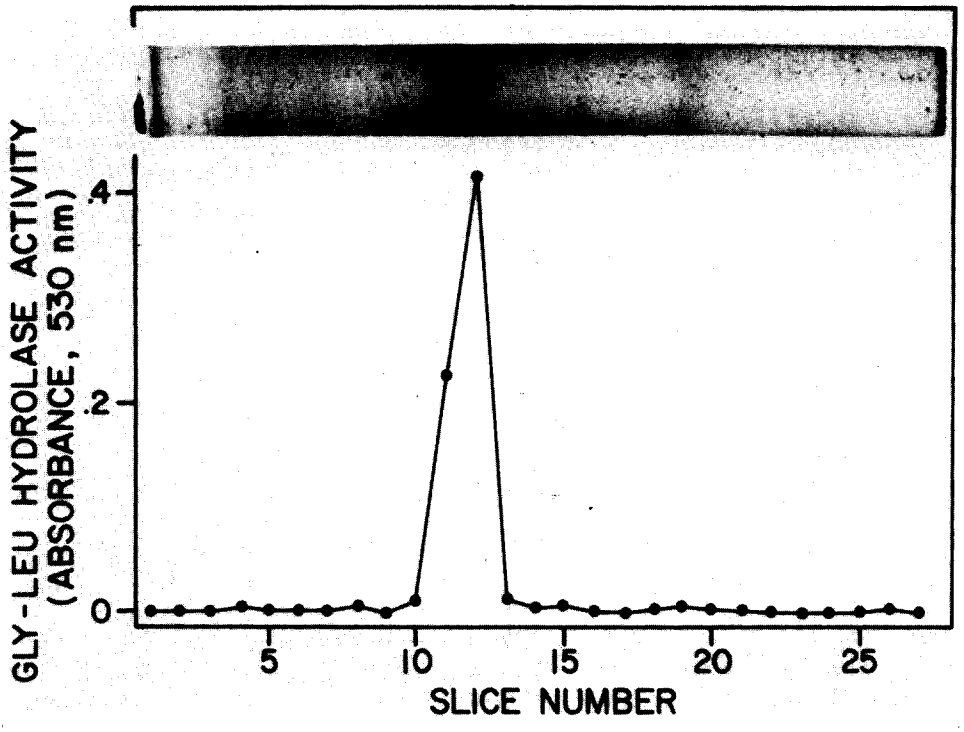

Fig. 11. Analytical acrylamide gel electrophoresis of purified enzyme III. Enzyme III was isolated from the intestinal brush border of seven rats by means of preparative acrylamide gel electrophoresis of material from two or three rats on three occasions. Gels were prepared and run as described in the legend for Fig. 5 and 2.5—4.6 ml of solubilized brush border material was electrophoresed on the three occasions. Fractions comprising enzyme III were pooled, concentrated 53-fold, equilibrated with 5 mM sodium phosphate buffer, pH 7.0, and chromatographed on DEAE-cellulose equilibrated with the same buffer eluted with a 0—0.25 M NaCl gradient. Column fractions comprising enzyme III were concentrated 194-fold, and 0.1 ml of concentrated material was applied to analytical acrylamide gels. Activity was limited to the dense band at slice 12 which had Met-Phe, Met-Gly, and Gly-Phe hydrolase activities as well as the Gly-Leu hydrolase activity shown.

of Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+}, and 0.01 mM concentrations of Co^{2+}. Met-Phe hydrolase activity of enzyme I was activated slightly by 0.01 and 0.1 mM Ca^{2+} (3—17%) and Mg^{2+} (11—14%) but was slightly inhibited by 1 mM concentrations of either. No activation of the Leu-Gly-Gly aminopeptidase activity of enzyme II was obtained with any metal tested. Gly-Leu hydrolase activity of enzyme III was activated by Co^{2+} (96%) and by 0.1 mM Zn^{2+} (52%). All enzymes were markedly inhibited by 1 mM Cu^{2+} and enzyme II was inhibited by 1 mM Ca^{2+}, Mn^{2+} and Zn^{2+}.

To further define the metal requirements of brush border peptide hydrolases, the three isolated enzymes were tested for inhibition by metal ion chelating agents. As shown in Table V, 1 mM EDTA markedly inhibited enzyme I, had no effect on enzyme II, and slightly inhibited enzyme III. The effect of 1,10-phenanthroline was similar to that of EDTA on enzyme I and enzyme III. However, unlike EDTA, this agent also markedly decreased the activity of enzyme II.

TABLE V

EFFECT OF METAL ION CHELATORS ON ACTIVITIES OF BRUSH BORDER PEPTIDE HYDROLASES

Brush border enzymes I, II, and III were isolated by preparative acrylamide gel electrophoresis. Each enzyme was assayed with two or more substrates in the absence of inhibitor and in the presence of 1 mM final concentrations of EDTA and 1,10-phenanthroline. The results are expressed in percentage of inhibition.

Enzyme	Substrate	1 mM EDTA	1 mM 1,10-phenanthroline
I	Ser-Met	78	79
	Ser-Phe	44	48
II	Phe-Gly	0	85
	Leu-Gly-Gly	0	24
	Leucyl-β-naphthylamide	5	97
III	Gly-Leu	23	32
	Ser-Met	16	18
	Gly-Phe	7	11
	Ser-Phe	5	5

Inhibitors. The effect of several agents on enzymes II and III were tested. Leucyl-β-naphthylamidase activity of enzyme II and Gly-Leu hydrolase activity of enzyme III were unaffected by 0.004% NaN_3 and saturating amounts of toluene. Saturating amounts of chloroform which had no effect on enzyme II decreased activity of enzyme III by 22%.

Recently, it has been shown that aminopeptidase activity from several different sources is inhibited by tertiary butyl threonylphenylalanylproline [33, 34]. In the present study, we tested both partially purified brush border enzyme II and cytosol activity (supernatant of intestinal mucosal homogenate) for inhibition by this peptide *. As shown in Table VI, the hydrolysis of leucyl-β-naphthylamide and the aminopeptide bond of Leu-Gly-Gly by brush border enzyme II were not affected by the inhibitor, while hydrolysis of Phe-Gly by this enzyme was inhibited 56%. This result probably reflects either a lower affinity of the dipeptide substrate for the enzyme or a different active site on the enzyme for the dipeptide. In contrast, hydrolysis of all three substrates, especially Leu-Gly-Gly, by the cytosol preparation was significantly inhibited by the tripeptide (Table VI). The effect of the peptide inhibitor on brush border enzymes I and III was also tested (results not shown). At an inhibitor to substrate mol ratio of 1.0, Met-Phe hydrolase activity of brush border enzyme I was unaffected by the tripeptide and Gly-Leu hydrolase activity of enzyme III was inhibited only 27%. Cytosol activity for both these substrates was inhibited less than 6%.

Stability. The effect of heat and freezing on brush border enzyme activities was investigated. When purified brush borders were kept at 53°C for 1 h at 1 M KCl, pH 7.4, recovery of Phe-Gly and Gly-Phe hydrolase activities was 105 and 103%, respectively, while only 9% of sucrase activity was recovered. When the brush borders were similarly heated in 0.15 M NaCl buffered at pH 7.4 with

* Kindly supplied by Dr. Rolf Jost, the Weizmann Institute, Rehovot, Israel.

TABLE VI

INHIBITION OF BRUSH BORDER ENZYME II AND CYTOPLASMIC PEPTIDE HYDROLASE ACTIVITIES BY TERTIARY BUTYL THEREONYLPHENYLALANYLPROLINE

Brush border enzyme II was isolated by preparative acrylamide gel electrophoresis. To prepare the cytosol, intestinal mucosa was homogenized in 10 volumes of 5 mM sodium phosphate buffer, pH 7.0, and centrifuged 1 h at 105 000 × *g*. Concentrations of Leu-Gly-Gly and Phe-Gly were reduced to one-fourth the amount present in the standard assay. Results are the means of three separate experiments performed with enzyme prepared from two different rats. The tripeptide inhibitor did not interfere with the enzyme assays and neither enzyme preparation released detectable amounts of free phenylalanine from the inhibitor.

Substrate	Inhibition (± 1 SD) (%)		Mol ratio inhibitor/substrate
	Brush border enzyme II	Cytosol	
Leucyl-β-naphthylamide	14 (12)	45 (5)	0.73
	3 (19)	57 (6)	1.83
Leu-Gly-Gly	6 (18)	95 (1)	1.0
Phe-Gly	56 (2)	50 (5)	1.0

1 mM $NaHCO_3$, recoveries were 100% for the Phe-Gly hydrolase, 75% for the Gly-Phe hydrolase, and 0% for sucrase. The solubilized and partially purified brush border peptide hydrolases were also very heat-stable. After 2 h at 50°C, solubilized enzyme II retained 100% of its leucyl-β-naphthylamidase and Phe-Gly-Gly aminopeptidase activity and 106% of its Phe-Gly hydrolase activity. Under the same conditions, enzyme III retained 117 and 114% of its Gly-Phe and Gly-Leu hydrolase activities, respectively. After 1 h at 60°C, 71% of the leucyl-β-naphthylamidase activity of enzyme II and 88% of the Gly-Leu hydrolase activity of enzyme III remained. When dilute solutions of these two partially purified enzymes were frozen for 24 h in buffered saline, recovery of leucyl-β-naphthylamidase, Phe-Gly-Gly aminopeptidase, and Phe-Gly hydrolase activities of enzyme II were 90, 94, and 96%, respectively, while only 34 and 31%, respectively, of the Gly-Phe and Gly-Leu hydrolase activities of enzyme III were recovered.

Isoelectric points. Although recovery of enzyme activity following isoelectric focusing of solubilized brush border proteins was too poor to permit use of this technique for purifying the enzymes, results of studies within several pH ranges showed the isoelectric point of enzyme II to lie between 4.3 and 4.6 (mean 4.5). One experiment with enzyme III revealed an isoelectric point of 3.2. Brush border peptide hydrolase activity was not instantaneously inhibited by the ampholytes used in these studies, but rather decreased slowly to very low levels after 18—24 h at 4°C. Activity lost by exposure to ampholytes could not be restored by addition of divalent metal ions.

Inhomogeneity of enzyme II. Despite the apparent homogeneity of purified enzyme II, evidence indicated that it was composed of two poorly separated enzymes with similar substrate specificities. As noted, preparative acrylamide gel electrophoresis occasionally revealed a shoulder on the enzyme II peak. Kim et al. [9] have previously reported that zymograms produced by means of

vertical starch gel electrophoresis of papain solubilized rat brush border protein reveal four peptide hydrolases. We obtained zymograms identical to theirs when papain solubilized brush border material was electrophoresed and zymograms developed for Met-Phe or Met-Met hydrolase activity. Results did not change when the starch gel buffer was changed from Tris/maleate to phosphate in order to avoid possible artifact due to binding of maleate to enzyme proteins (Fig. 12). The identity of each of the zymogram spots was established by use of specific substrates and by starch gel electrophoresis of individual enzymes isolated by DEAE-cellulose chromatography and preparative acrylamide gel electrophoresis. These studies showed that the most rapidly migrating activity on starch gel (position 4) is enzyme III, that the least rapidly migrating activity (position 1) is enzyme I, and that enzyme II yields the two incompletely separated activities with intermediate mobility (positions 2 and 3) (Fig. 12). The activities migrating in positions 2 and 3 have been designated enzymes IIa and IIb, respectively. The possibility that the two spots represented different forms of the same enzyme was considered. However, pretreatment of enzyme II with heat, aging at 4°C, dithioerythritol, *p*-hydroxymercuribenzoate, maleate, EDTA, 1,10-phenanthroline, and divalent metal ions failed to cause any visually detectable change in the relative intensity of the two spots following starch gel electrophoresis. Furthermore, gel filtration of enzyme II on Sephadex G-200 yielded only a single sharp peak indicating that IIa and IIb do not differ greatly in molecular weight.

To determine whether IIa and IIb could be separated by acrylamide gel electrophoresis, enzyme II isolated by ion-exchange chromatography was subjected to electrophoresis on analytical gels of various pore sizes using several different buffer systems. Enzyme activity was detected in situ in the gels by the method of Nachlas et al. [35] using L-leucyl-4-methoxy-2-naphthylamide as substrate. Conditions which gave adequate separation of enzyme IIa and IIb

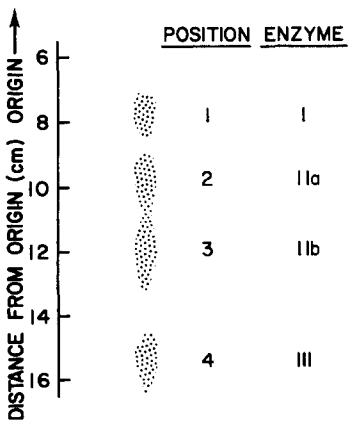

Fig. 12. Separation of brush border peptide hydrolases by vertical starch gel electrophoresis. Papain-solubilized brush border protein was electrophoresed for 20 h at 10 V/cm on a 12.5%, 20 cm long gel as described in Materials and Methods. The sliced gel was overlayed with 2% agar containing Met-Met substrate, *o*-dianisidine, horseradish peroxidase, and snake venom L-amino acid oxidase. The pattern was traced after development of color for 40 min at room temperature.

were determined. Preparative acrylamide gel electrophoresis using these same conditions also yielded excellent separation of the two enzymes (Fig. 13). In four experiments, the mean recovery of enzyme II, Leu-Gly-Gly hydrolase activity, following preparative acrylamide gel electrophoresis was 50.2% (±6.4, 1 S.D.). Of the activity recovered, 67% (±13%) was attributable to enzyme IIb and the remainder to enzyme IIa. Fractions comprising approx. 90% of each peak were pooled, concentrated, and assayed for hydrolysis of several substrates. Ratios of the rate of hydrolysis by enzyme IIb to the rate of hydrolysis by enzyme IIa for the substrates tested were 2.0 for Leu-Gly-Gly, 1.8 for Ala-Phe, 2.2 for Phe-Leu, 1.5 for Met-Phe, 2.1 for Phe-Gly, 1.9 for Phe-Glu, 1.3 for Gly-Leu, 1.6 for Met-Gly, 1.9 for Met-Leu, and 1.7 for phenylalanine-*p*-nitroanilide. Further evidence for the close similarity of substrate specificities of the two enzymes was obtained by determinations of the inhibition of phenylalanyl-*p*-nitroanilide hydrolysis in the presence of certain peptides. Inhibitory constants for enzymes IIb and IIa, K_i, calculated from these data were $3.48 \cdot 10^{-5}$ and $3.48 \cdot 10^{-5}$, respectively, for Met-Ser, $1.18 \cdot 10^{-4}$ and $1.10 \cdot 10^{-4}$ for Met-Leu, $7.10 \cdot 10^{-5}$ and $8.55 \cdot 10^{-5}$ for Met-Gly, and $12.73 \cdot 10^{-3}$ and $9.15 \cdot 10^{-3}$, respectively, for Gly-Leu. These results indicate that the substrate specificities for enzymes IIb and IIa are very similar or identical. Further study will be required to determine whether both enzymes are normally present in the intact brush border or whether they derive from a single enzyme during the processes of solubilization and isolation.

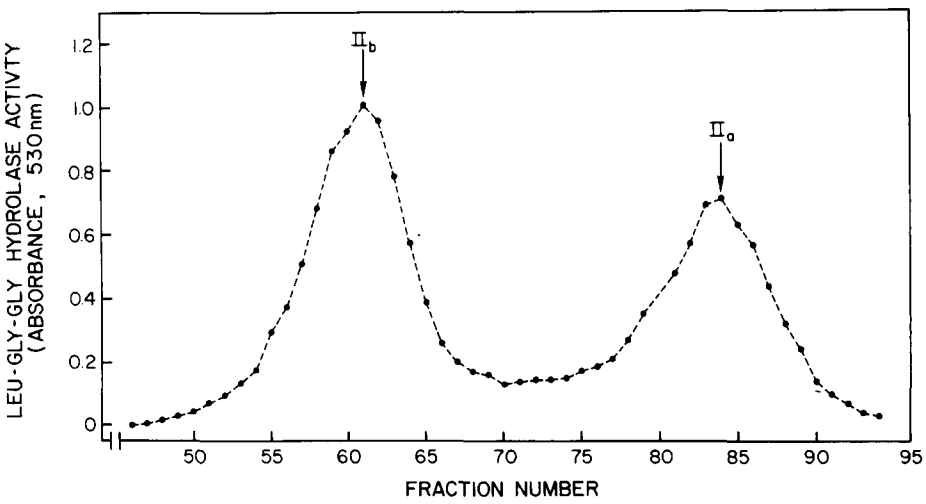

Fig. 13. Separation of rat brush border enzymes IIa and IIb. Papain-solubilized protein from intestinal brush border was subjected to DEAE-cellulose chromatography as previously described. Fractions comprising enzyme II were pooled, concentrated 125-fold, and 1.7 ml of the concentrate made 3% with sucrose was applied to the top of a preparative acrylamide gel. The 2.5% stacking gel, 0.7 cm long, was buffered with Tris · HCl, pH 6.7. The 6% separating gel, 3.5 cm long, was buffered with Tris · HCl at pH 8.9. The gels were polymerized by light overnight at 4°C. Tris/glycine, 5 mM, pH 8.3, was used as upper and lower electrode buffer and as elution buffer. Current was controlled at 4 mA for the first 2 h then at 7 mA for the remainder of the run. Elution was at 16 ml/h and 18-min fractions were collected.

Discussion

In 1968, Eicholz [13] reported that papain released leucyl-β-naphthylamidase and Leu-Gly hydrolase from hamster intestinal brush border membranes at different rates and that the former activity is much more resistant to inactivation by prolonged papain digestion than the latter. This suggested that at least two different peptide-hydrolyzing enzymes were present in hamster brush border. The present study demonstrates that leucyl-β-naphthylamide and Leu-Gly are also hydrolyzed by different rat brush border enzymes, namely enzymes II and III, respectively (see Table IV).

Fujita et al. [10] obtained evidence that both Leu-Gly and Phe-Gly are hydrolyzed by more than one rat brush border enzyme and on the basis of kinetic studies, 10 other peptides were divided into three groups: I, Gly-Leu, Gly-Met, and Gly-Phe; II, Gly-Tyr and Gly-Try; and III, Phe-Gly, Phe-Val, Val-Leu, and Met-Glu, Their results are, in large part, confirmed and explained by results of the present study. Enzymes II and III both contribute substantially to the brush border hydrolysis of Leu-Gly and Phe-Gly. Further, the group I peptides of Fujita et al. [10] appear to be substrates for enzyme III, and Phe-Gly in their group III is predominantly hydrolyzed by enzyme II (see Fig. 1).

Physical separations of intestinal peptide hydrolases have been reported by several groups. Dolly and Fottrell [36] detected several peptide-hydrolyzing enzymes in human intestinal mucosa by use of starch gel electrophoresis. They used a 12 000 × *g* supernatant of whole mucosal homogenate which probably included primarily cytosol peptide hydrolases and little if any brush border enzyme activity. More recently, Donlon and Fottrell [12] reported detecting five different peptide-hydrolyzing enzymes in brush border-nuclear fractions of guinea pig intestinal mucosa by starch gel electrophoresis of sonicated preparations. The three most active enzymes detected were designated "a" which hydrolyzed tripeptides, "α" which was most active against leucyl-β-naphthylamide, Leu-Leu-Leu, and Tyr-Tyr-Tyr, and "β" which was most active against Gly-Leu, Leu-Leu, Gly-Try, Gly-Met, leucine amide, and tetraleucine. Although the substrate specificity of their "α" enzyme resembles that of enzyme II and that of their "β" slightly resembles the substrate specificity of enzyme III, the correlation between the findings of Donlon and Fottrell [12] and those of the present study is poor. The discrepancies undoubtedly result from differences in animals (rat vs. guinea pig), tissue preparation (purified brush border vs. brush border-nuclear fraction), solubilization (papain digestion vs. sonication), and the fact that they were observing zymograms rather than quantitative enzyme assays.

Kim et al. [9] detected four peptide-hydrolyzing bands on starch gel electrophoresis of rat brush border enzymes solubilized by several different methods, including papain digestion. As outlined in Results, we obtained identical results following starch gel electrophoresis and have established the correct correspondence between the starch gel bands and enzymes isolated by DEAE chromatography and acrylamide gel electrophoresis. Kim et al. [9] reported no evidence that the different enzyme bands had different substrate specificities as the zymograms appeared identical when Leu-Leu, Leu-Leu-Leu, leucyl-β-naphthylamide, and leucine amide were used as substrates. These results are

undoubtedly due to the fact that the color intensity of zymogram bands are not necessarily proportional to enzyme activity so that equally dense bands can result from enzymes with very different activities against the substrates used.

Wojnarowska and Gray [15,1] isolated three peptide hydrolases from the brush border of rat small intestine by means of gel filtration and density gradient centrifugation. The enzyme which they designate peptidase III is clearly identical to our enzyme III. It is active against dipeptides, especially Gly-Leu, Leu-Gly, and Gly-Phe, with little aminopeptidase activity. The molecular weight for peptidase III, 120 000, compares to 134 000 obtained for our enzyme III and the pH optima are identical. The peptidases I and II isolated by Wojnarowska and Gray [15] both actively hydrolyze leucyl-β-naphthylamide and the amino-terminal bond of tripeptides and thus appear to correspond to enzymes IIa and IIb isolated in the present study. The molecular weight we obtained for enzyme II, 284 000, approximates that for peptidase I, 230 000, but not that for peptidase II, 160 000, and the pH optimum we obtained, 7.5—8.0, is slightly different from that obtained by Wojnarowska and Gray [15] for peptidases I and II, 6.5—7.5. Furthermore, they found peptidases I and II to have different substrate specificities while we discovered no such differences between enzymes IIa and IIb. Finally, they apparently did not detect on enzyme comparable to enzyme I described here. It is difficult to resolve these differences unequivocally with the data available, but the fact that their peptidase I has a broader substrate specificity than peptidase II and that the location of their peptidase I following acrylamide gel electrophoresis is comparable to the position occupied by enzyme I described here suggests the possibility that what Wojnarowska and Gray [15] term peptidase I is, in fact, a mixture of the enzymes we have designated I and IIb. Their peptidase II would then correspond to our IIa. This possibility is supported by the fact that the enzyme which we have designated I has a relatively high molecular weight, comparable to that for enzymes IIa and IIb, and would be expected to appear in the vicinity of enzymes IIa and IIb following the gel filtration chromatography or density gradient ultracentrifugation techniques used by Wojnarowska and Gray [15]. These authors obtained evidence which suggested that the two amino peptidase peaks may result from a minor chemical or physical change in a single enzyme.

The present study has demonstrated that rat intestinal brush border contains four, and probably only four, peptide hydrolases. If it is assumed that enzymes IIa and IIb are closely related forms of the same enzyme then the three enzymes isolated, I, II, and III have distinctly different though somewhat overlapping substrate specificities. Substrates hydrolyzed almost exclusively by two of the enzymes have been identified. Such knowledge of substrate specificities makes possible a more rational selection of substrates for assays of the brush border peptide hydrolases. For example, if one wished to study the response of rat brush border peptide hydrolase activity to any manipulation, certain substrates (Met-Met, Met-Phe, Ser-Phe, Phe-Ala, and undoubtedly many others) may be poor choices since they are hydrolyzed approximately equally well by the three different brush border enzymes. Thus, even marked changes in one of the enzymes might be overlooked if the others did not change or changed in the opposite direction. In contrast, leucyl-β-naphthylamide, Phe-Arg, and Phe-

Asp which are highly discriminating substrates for enzyme II and Gly-Leu which is appropriate for enzyme III would be more suitable substrates. At present, Ser-Mer, Met-Phe, and Met-Met are the most specific substrates detected for enzyme I, but they are all split at least equally rapidly by one or both of the other two enzymes. It may be possible to estimate enzyme I activity in a mixture of brush border peptide hydrolases by determining hydrolysis of a substrate such as Ser-Met then subtracting the contribution of enzymes II and III to this activity.

The patterns of substrate hydrolysis by the three enzymes which was observed in the semi-quantitative and quantitative experiments indicate that enzyme II has aminopeptidase activity in addition to a broad range of dipeptide hydrolase activity. Results suggest that enzymes I, II, and III preferentially hydrolyze dipeptides containing methionine, arginine or aspartic acid, and glycine, respectively. However, presence of these amino acids in the peptide substrate is not necessary for activity of the enzymes and, as yet, no pattern has been discovered which would permit one to predict with certainty which dipeptides, among the many not tested, are likely to be rapidly hydrolyzed by each enzyme.

Although it is clear that peptide hydrolases of the intestinal mucosa play an essential role in protein digestion, the relationship, if any, of these enzymes to disease is uncertain. Certain investigators have recently measured peptide hydrolase activity of human intestinal mucosa [18,37—40] and Sadikali [40] and Cornell and Townley [41] have reported patients with apparent deficiencies of these enzymes. In none of these studies was the choice of substrates based on knowledge of either the number or the substrate specificities of the enzymes being measured. Furthermore, the results of these studies reflect primarily cytoplasmic enzyme levels because measurements were made either on supernatant preparations or on whole homogenates. Most brush border dipeptide hydrolase activities studied to date comprise less than 10% of the total mucosal activity. Measurements of both brush border and cytoplasmic peptide hydrolases of human mucosal biopsy specimens are being carried out in this laboratory and preliminary results have been published [42]. However, the substrates for these studies were also not chosen on the basis of detailed knowledge of substrate specificities of human brush border and cytoplasmic peptide hydrolases as this information is not yet available.

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