Peptide Hydrolases in the Brush Border and Soluble Fractions of Small Intestinal Mucosa of Rat and Man

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A B S T R A C T Peptide hydrolases, catalyzing the hydrolysis of 13 dipeptides and 5 tripeptides into their respective amino acids, were studied in small intestinal mucosa and other tissues, in man and in the rat.

Studies on the subcellular distribution of these enzymes showed enzyme activities in both the soluble and brush border fractions of the rat small intestinal mucosa, the former constituting 80–90% and the latter 10–15% of the total activity. Zymogram studies of peptide hydrolases, in both fractions, yielded multiple bands indicating multiple zones of enzyme activity. With most substrates a rather broad range of enzyme activities was observed in the soluble fraction differing only slightly from substrate to substrate, the exception being when L-leucyl-L-proline was used: this latter led to a zymogram pattern which was quite distinct. The synthetic substrates, L-leucyl-β-naphthylamide and L-leucinamide appeared to be hydrolyzed by two electrophoretically distinct enzymes, different from those hydrolyzing other leucyl-containing peptide substrates.

Zymogram patterns of the brush border membrane fraction were quite different from those of the soluble fraction of rat small intestine indicating that enzymes from the two sources may be different. No comparable human data were obtained.

Peptide hydrolases in the soluble fractions of various organs from the same species gave similar zymogram patterns, while those from the plasma membrane-bound fractions of different organs in the same species were peculiar to each organ. From these data, it is suggested that peptide hydrolases in the brush border and the soluble fractions of small intestine are distinct enzymes and may play different roles in cellular function.

INTRODUCTION

Peptide hydrolases catalyzing the hydrolysis of di- or tripeptides into respective amino acids are present in the small intestinal mucosa. These enzymes have been reported to occur both in brush border membranes and in the soluble cytoplasmic fraction (1–6). Although data regarding the quantitative distribution of peptide hydrolases in these fractions are lacking, it has been reported that the brush border membrane contains less dipeptide hydrolase activity than the soluble cytosol fraction, while tripeptide hydrolase activity is much more active in the former (4, 5).

It is generally accepted that, in the adult, dietary protein appears in the portal circulation primarily as amino acids (6, 7). However it is not yet clear whether dietary protein is absorbed from intestinal lumen to mucosa primarily as amino acids or as small peptides. Several studies have recently shown that certain dipeptides and tripeptides may be absorbed as efficiently or even more efficiently than the constituent amino acids, suggesting that the dietary protein may be absorbed in both forms (8–11). Though peptide hydrolases of the small intestine are generally thought to play an important role in the final step of the digestion of dietary proteins, little is known of the subcellular fraction most concerned with this activity; furthermore the possible role of dipeptidases in other cellular functions has not been examined.

Peptide hydrolase activities have also been reported in many tissues other than small intestinal mucosa, such as red blood cells and mouse ascites tumor cells, kidney, liver, and connective tissue, but they have not been examined for structural and functional similarities (12–15).

The present studies were undertaken to investigate the substrate specificity of peptide hydrolases in the

small intestinal mucosa and to compare the electrophoretic mobilities of these enzymes with those of similar enzymes in other tissues. A study was also carried out to determine whether or not the enzyme activities in the soluble and brush border fractions of a particular tissue are due to the same or to different enzymes.

METHODS

All of the amino acids and dipeptides except for the following compounds were purchased from a single commercial source:[1] glycyl-L-leucine and glycyl-L-valine were supplied from another source[2] and L-leucyl-L-proline, L-prolyl-L-leucine, and all the tripeptides were supplied by a third one.[3] Other substrates used were L-leucyl-β-naphthylamide hydrochloride[2] and L-leucinamide hydrochloride.[3] Trypsin,[4] chymotrypsin,[4] papain,[5] Triton X-100,[6] Na deoxycholate,[7] Na glycodeoxycholate,[7] Na glycocholate,[7] and Enzite-papain[8] were used to release peptide hydrolases from the brush border membrane. Hydrolyzed starch was used for the preparation of the gel used in electrophoresis. Snake (*Crotalus adamanteus*) venom[1] was used as a source of L-amino acid oxidase. Horseradish peroxidase[1] and *o*-dianisidine[1] were used in zymogram studies. Neuraminidases from two sources (*Vibrio cholerae*[5] and *Clostridium perfringens*[1]) were used to treat soluble peptide hydrolases under conditions described by Ghosh, Goldman, and Fishman (16).

Tissue preparation. After an overnight fast albino male rats of the Sprague-Dawley strain (150–200 g) were killed by decapitation. All subsequent steps were carried out in the cold room. The small intestinal segments were quickly removed, rinsed in ice-cold isotonic saline, and everted over a glass rod. The everted gut was gently blotted, placed on a chilled glass plate, and the mucosa was scraped with a glass slide. Human small intestine was obtained at autopsy from patients without evidence of gastrointestinal diseases, within 12 hr of death, and the mucosa scraped as above.

Subcellular fractionation. Mucosal scrapings were homogenized in 14% glycerol (4 ml/g) in a Potter-Elvejhem tissue homogenizer with eight strokes of Teflon pestle with a 0.004–0.006 cm clearance driven by a Con-Torque (Eberbach Corp., Ann Arbor, Mich.) stirrer at medium speed, passed through two layers of cheesecloth, centrifuged at 3,000 g for 10 min to obtain a pellet containing the brush border and nuclear fraction. The supernate was then centrifuged at 16,500 g for 20 min to yield the mitochondrial pellet. The microsomal pellet was obtained by centrifuging the resultant supernate at 105,000 g for 2 hr and the postmicrosomal supernatant fraction was designated as the soluble fraction. These methods yielded morphologically and chemically homogeneous microsomal and mitochondrial fractions, as described in a previous study (17). All pellets and the final supernate were retained for assay of enzyme activity and protein content.

Solubilization of brush border enzymes. The following methods were used to release peptide hydrolases from the brush border membranes of rat small intestine: sonication, or treatment with proteolytic enzymes or detergents. "Partially purified brush border" from rat small intestine was prepared as described below and suspended in 14% glycerol. For extraction with proteases, 1 ml of each brush border preparation containing 3.5 mg protein was added to 1 ml of buffer, containing 2 mg of proteolytic enzyme and was incubated for 1 hr. The buffers, 0.046 M Tris-maleate with 0.0115 M CaCl₂, pH 8.1, 0.08 M Tris-maleate with 0.1 M CaCl₂, pH 7.8, and 0.1 M Na phosphate, pH 7.0 containing 1 mg of L-cysteine hydrochloride hydrate, were used for incubation with trypsin, chymotrypsin, and papain, respectively. For extraction with detergents, 20 μmoles of Na deoxycholate or Na glycodeoxycholate or Na glycocholate was added to 1 ml of 0.1 M Na phosphate buffer at pH 7.0. In other experiments Triton X-100 was added to make a final concentration of 0.1% (w/v) in buffer. Sonication was carried out for 15 sec at 80 w with a probe of terminal diameter, 3 mm. All the extraction mixtures were then centrifuged at 50,000 g for 30 min and the peptide hydrolase activity was determined in the supernate and in the pellet.

Zymogram method. For the zymogram studies, the mucosal scrapings were homogenized in 14% glycerol (4 ml/g) with eight strokes of a motor-driven Teflon pestle using a Potter-Elvejhem glass homogenizer, passed through two layers of cheesecloth, and centrifuged at 20,000 g for 15 min in a Sorvall[9] centrifuge. The supernate was then centrifuged at 105,000 g and the resultant supernate was kept frozen at −20°C. This fraction was called the "soluble enzyme fraction."

Skeletal muscle from the left gluteus maximus, pancreas, brain, and cardiac muscle were obtained from each rat. Liver and kidney specimens were obtained both from man, at autopsy, and from rat; these were minced in small pieces, homogenized in 14% glycerol (4 ml/g), and centrifuged as described above.

Brush border preparation was prepared from mucosal scrapings according to the methods of Eichholz and Crane (18). The fractions obtained after three EDTA washings were called "partially purified enzyme" and the finally purified material of Eichholz and Crane was designated as the "purified brush border." Both preparations were suspended in 14% glycerol and stored at −20°C overnight before use. Purified plasma membranes were prepared respectively from liver and kidney according to the methods of Neville (19) and Wilfong and Neville (20).

Vertical starch gel electrophoresis was performed with the Buchler[10] gel electrophoresis apparatus by the method of Lewis and Harris (14) at pH 7.4 (bridge buffer, 0.1 M Tris-maleate; gel buffer, 0.005 M Tris-maleate). A volume of 0.04 ml of enzyme was applied to each slot in the starch gel and electrophoresis performed in a cold room at 4°C for 20 hr at a potential of 5 v/cm. The starch gel was sliced into two layers after electrophoresis. The reaction mixture, containing the peptide under examination, L-amino acid oxidase, horseradish peroxidase, *o*-dianisidine dihydrochloride, and MnCl₂, was mixed with an equal volume of 2% aqueous agar (14), and poured over the sliced surface of the gel. The starch gel with its agar overlay was then incubated for 1 or 2 hr at 37°C.

Enzyme assay: peptide hydrolase. The method of Matheson and Tattrie, using a modified Yemm and Cocking ninhydrin reagent (21), was used to assay hydrolase activity in the subcellular as well as in the brush border fractions, against the dipeptides, L-leucyl-L-alanine, glycyl-L-valine, and the tripeptide, L-leucyl-L-leucyl-L-leucine. The standard assay mixture contained 0.1 ml of peptide substrate (0.25 μmoles of L-leucyl-L-alanine or 0.1 μmoles glycyl-L-valine or 0.05 μmoles of L-leucyl-L-leucyl-L-leucine), 0.02 ml of CaCl₂ (0.6 μmoles), 0.48 ml of 0.05 M borate buffer at pH 7.6 and 0.02 ml of enzyme preparation (3 μg protein from soluble fraction or 20 μg protein from particulate fractions) with a final volume of 0.62 ml. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by quickly placing the tubes in ice and adding 0.4 ml of 0.1 N acetic acid to each tube. For the control tube the reaction mixture was added after prior addition of 0.4 ml of 0.1 N acetic acid. The colorimetric reactions with ninhydrin reagents were carried out according to the method of Matheson and Tattrie (21), slightly modified; 0.5 ml of 0.2 M citrate buffer was added to 1.002 ml of the reaction mixture on ice and mixed well. Then 1.2 ml of KCN-methyl cellosolve-ninhydrin mixture was added and mixed slowly. The reaction mixture was placed in the boiling water for 11 min and cooled rapidly in ice for 5 min. Approximately 1 ml of each reaction mixture was transferred to a quartz cuvette (1.0 cm light path) and the absorbance at 570 mμ was read against the corresponding blank mixture, using a Gilford [11] spectrophotometer. In order to assay the rate of appearance of free amino acids in the presence of dipeptide, a series of standards was prepared, containing varying proportions of peptide and component amino acids. For the assay of peptide hydrolase activity after column chromatographic fractionation (see below), the method of Josefsson and Lindberg (22) was used. The standard assay mixture, containing 0.1 ml of peptide substrate (30 μmoles each of glycyl-L-leucine, glycyl-L-valine, L-leucyl-L-alanine, L-leucyl-L-leucine, L-leucyl-L-leucyl-L-leucine, L-prolyl-L-leucine, L-leucyl-L-proline, and L-leucinamide hydrochloride) and 0.9 ml of 0.05 M borate buffer, pH 7.4, was added to a quartz cuvette. Next, 50–100 μl of enzyme was pipetted in

and mixed quickly with a Teflon stirrer. The rate of change in absorbance at 220 mμ was recorded on a Gilford [11] recording spectrophotometer. The blank tube contained 1 ml of buffer and 50–100 μl of enzyme. Kinetic studies were first performed to establish optimum conditions for quantitative assays of peptide hydrolases and the assays were linear with respect to protein concentration and time.

Enzyme assays: sucrase, glutamic dehydrogenase, NADPH-cytochrome c reductase, and galactosyltransferase. Sucrase activity (EC 5.2.1.26) was determined as described by Dahlqvist (23). An equal portion of the subcellular fractions of small intestinal homogenate prepared in 14% glycerol was extensively dialyzed against water at 4°C for the assay of sucrase activity, since the presence of glycerol interfered with the assay for this enzyme. The method of Beaufay, Bendall, Baudhuin, and DeDuve (24) was used to assay glutamic dehydrogenase. NADPH-cytochrome c reductase activity was assayed according to the method of Phillips and Langdon (25). For the galactosyltransferase assay, a method previously described from this laboratory was used (26). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (27).

Gel filtration column chromatography. The soluble and the extracted partially purified brush border fractions were examined chromatographically. Gel filtration was carried out at 4°C on a 1.2 × 65 cm column of agar gel (Biogel A 0.5 M, Bio-Rad Labs, Richmond, Calif.) with 0.02 M borate buffer, pH 7.4 in 14% glycerol. Flow rates were maintained at 7.5 ml/hr and the effluent was collected in 1.25-ml fractions. For molecular size calibrations of the column, 1 ml of standard mixture was applied containing 1 mg each of chymotrypsinogen A, bovine serum albumin, human gamma globulin, and horse apoferritin obtained from "Non-enzyme protein molecular weight marker kit." [2] Molecular weight of peptide hydrolases were estimated by reference to a plot of elution volume against log molecular weight of the standard proteins.

RESULTS

Subcellular distribution of enzymes. In order to assess the efficacy of the subcellular fractionation procedure, the distribution of a number of known marker

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[11] Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

TABLE I

Glutamic Dehydrogenase, NADPH Cytochrome c Reductase, and Galactosyltransferase in Subcellular Fractions of Rat Intestinal Mucosa

Subcellular fractions	Glutamic dehydrogenase		NADPH-cytochrome c reductase		Galactosyltransferase	
	Enzyme activity*	%‡	Enzyme activity*	%‡	Enzyme activity§	%‡
Homogenate	6.9±0.6		26.2±3.5		4625±520	
Brush borders and nuclei	6.2±0.7	15.6	8.2±1.2	5.6	4232±646	20.4
Mitochondria	32.8±2.6	47.3	22.4±3.1	8.5	5284±524	10.4
Microsomes	5.0±0.4	13.0	82.8±6.4	56.9	12680±787	59.2
Cytosol	1.6±0.2	13.8	10.6±0.9	22.0	1460±115	14.7
Recovery		89.7		93.0		104.7

* Expressed as micromoles of substrate hydrolyzed per minute per gram of protein. Each number represents the mean enzyme activity ±SEM obtained from four experiments.

‡ Enzyme activity expressed as a per cent of the activity in the homogenate.

§ Expressed as disintegrations/minute per milligram protein per hour. Each number represents the mean enzyme activity ±SEM obtained from four experiments.

TABLE II

Subcellular Localization of Peptide Hydrolases and Sucrase

Subcellular fractions	L-leucyl-L-alanine		L-leucyl-L-leucyl-L-leucine		Sucrose	
	Enzyme activity*	%‡	Enzyme activity*	%‡	Enzyme activity*	%‡
Homogenate	179.9±19.8		81.0± 7.4		13.6±1.5	
Brush borders and nuclei	107.0±12.7	11.4	28.9± 3.8	10.1	31.1±4.2	56.1
Mitochondria	182.8±24.9	3.4	23.6± 1.7	1.2	24.3±2.1	12.4
Microsomes	229.1±15.8	5.4	34.7± 5.8	1.8	27.6±3.2	18.5
Cytosol	400.7±36.8	78.8	116.4±19.5	93.6	4.3±0.3	11.9
Recovery		99.0		106.7		98.9

* Expressed as micromoles of substrate hydrolyzed per minute per gram of protein. Each number represents the mean enzyme activity ±SEM, obtained from four experiments.

‡ Enzyme activity expressed as a per cent of the activity in the homogenate.

enzymes were studied. As shown in Table I, glutamic dehydrogenase was concentrated over fourfold in mitochondrial fraction while both NADPH-cytochrome c reductase and galactosyltransferase were concentrated about threefold in the microsomal fraction. Over 50% of sucrase activity, a marker for brush border, was present in the brush border and nuclear fraction with the remaining activity distributed throughout the rest of the fractions.
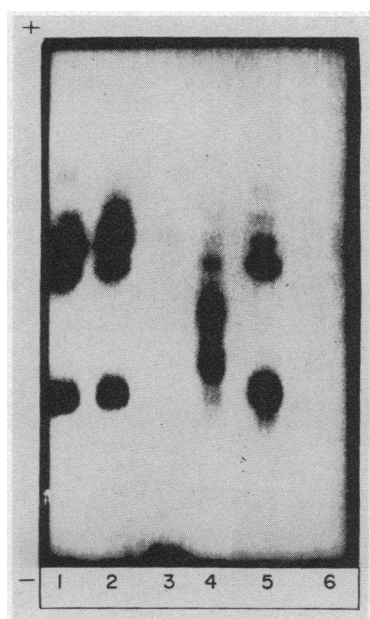

FIGURE 1 Typical zymograms of peptide hydrolases from mucosa of the rat small intestine with L-leucyl-L-leucine as a substrate. The samples tested were: 1. "soluble fraction"; 2. soluble fraction partially purified on Biogel A 0.5 M column; 3. purified brush border membranes; 4. papain-treated purified brush border membranes; 5. papain-treated soluble fraction; and 6. papain alone.

The peptide hydrolase activity was present in two major subcellular fractions, the soluble and the nuclear fractions. About 80–90% of the total enzyme activity was present in the soluble fraction while about 10% of the total activity was present in the brush border fraction. All three substrates, L-leucyl-L-alanine, glycyl-L-leucine, and L-leucyl-L-leucyl-L-leucine gave similar results (Table II). Addition of EDTA (0.1 mM) in the incubation mixture did not affect the percentage distribution of the peptide hydrolase L-leucyl-L-leucyl-L-leucine in various cellular fractions.

Zymogram studies. As shown in Fig. 1, multiple zones of enzyme activity with anodic mobility are seen in both soluble and brush border fractions. When control zymograms were developed using all the reagents except peptide, no bands were observed excluding the possibility that free amino acids present in the soluble fractions were responsible for the bands observed. Zymograms of crude soluble fraction showed several bands. These bands were shown more clearly when the soluble fraction had been partially purified on a Biogel column. With brush border fraction in the absence of papain treatment, virtually all the enzyme activity remained at the origin. However, with papain treatment there appeared two major bands having electrophoretic mobilities quite distinct from those of the soluble fraction. Two minor bands, one fast and another slow, were also present solely in the brush border preparation.

When the soluble enzyme was similarly treated with papain some of the bands disappeared, but the migration of the major bands remained unaltered. When papain alone was applied, no band appeared. The treatment of the soluble enzyme with neuraminidase did not alter the electrophoretic mobilities of the various bands.

Fig. 2 illustrates diagrammatically the representative

results of a series of zymograms from soluble and brush border fractions of rat small intestine using 13 dipeptides and 5 tripeptides as substrates.

Turning first to the soluble fraction, the zymogram patterns obtained with most dipeptides resembled those obtained with L-leucyl-L-leucine or L-prolyl-L-leucine. The main difference between these two was the absence of the slowest and the fastest migrating bands with L-prolyl-L-leucine. The following dipeptides gave zymogram patterns similar to those shown by L-leucyl-L-leucine: these were L-phenylalanyl-L-tyrosine, L-phenyl-alanyl-L-leucine, L-methionyl-L-leucine, L-leucyl-L-alanine, and L-leucylglycine. A zymogram similar to that of L-prolyl-L-leucine was shown by the following dipeptides: L-prolyl-L-phenylalanine, glycyl-L-leucine, glycyl-L-phenylalanine, glycyl-L-tyrosine, and L-valyl-L-leucine. There was one exception to these trends. When L-leucyl-L-proline was used as a substrate, four new distinct fast moving bands appeared with the loss of the bands seen with other dipeptide substrates. With tripeptides, all showed a pattern similar to that seen with L-leucyl-L-leucyl-L-leucine. The main difference from dipeptides was the absence of one major and one minor band in the middle zone.

With the synthetic substrates, L-leucyl-β-naphthylamide and L-leucinamide, only a single band of enzyme activity was seen for each substrate; these were distinct from each other.

In marked contrast to the zymograms obtained with soluble fractions, the brush border yielded uniform zymograms, almost irrespective of the peptide substrate, the exception being when proline-containing substrates were used. In this latter situation, no activity could be detected.

In Fig. 3, a series of zymograms obtained from human soluble fraction is presented: these data summarize the overall pattern of the activities diagrammatically. The electrophoretic mobilities of the various

FIGURE 3 A summary diagram of zymograms of peptide hydrolases from soluble and brush border membrane fractions of rat and human jejunal mucosa using several substrates.

bands obtained from soluble fractions of human jejunum differed from those obtained from rat small intestine. As with the rat small intestine, the soluble fraction of human postmortem jejunal mucosa yielded zymogram patterns which could be broadly classified into five different types. Most dipeptides gave patterns similar to those obtained respectively with L-leucyl-L-leucine and L-prolyl-L-leucine. Dipeptides such as L-phenylalanyl-L-tyrosine, L-phenylalanyl-L-leucine, L-methionyl-L-leucine, and L-leucyl-L-alanine gave patterns similar to those shown by L-leucyl-L-leucine, while dipeptides such as L-prolyl-L-phenylalanine, glycyl-L-leucine, L-valyl-L-leucine, glycyl-L-tyrosine, and glycyl-L-phenylalanine gave patterns similar to that shown by L-prolyl-L-leucine.

When L-leucyl-L-proline was used as a substrate, distinct bands appeared. Characteristic patterns were also seen with L-leucyl-β-naphthylamide and L-leucinamide. Tripeptides gave patterns similar to that observed with L-leucyl-L-leucyl-L-leucine. The zymograms obtained from human postmortem specimens were very similar to those observed from fresh human jejunal biopsy specimens.

Fig. 4 depicts the species and organ specificities of the peptide hydrolases of the soluble fraction. The zymograms obtained from human postmortem material were distinctly different from those obtained from the rat. However, within each species, the zymogram obtained from the soluble fraction of various organs varied little. In particular, that obtained from small intestine was no different from that obtained from other organs in the same species. Although not shown in Fig. 4, the soluble fractions from brain, cardiac muscle, and pancreas of the rat gave zymogram patterns similar to those obtained from the small intestine.
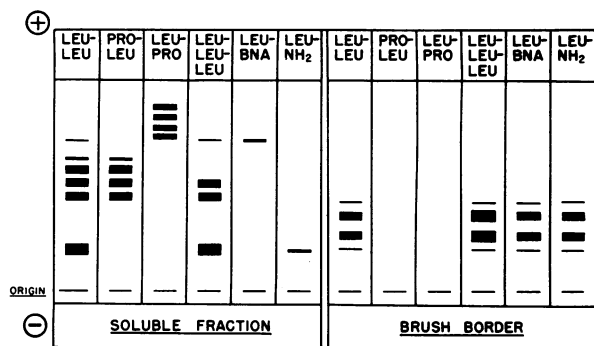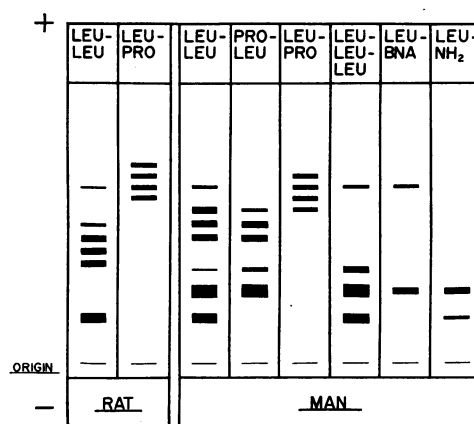

FIGURE 2 A summary diagram of zymograms of peptide hydrolases from soluble and brush border membrane fractions of rat small intestinal mucosa using several substrates.
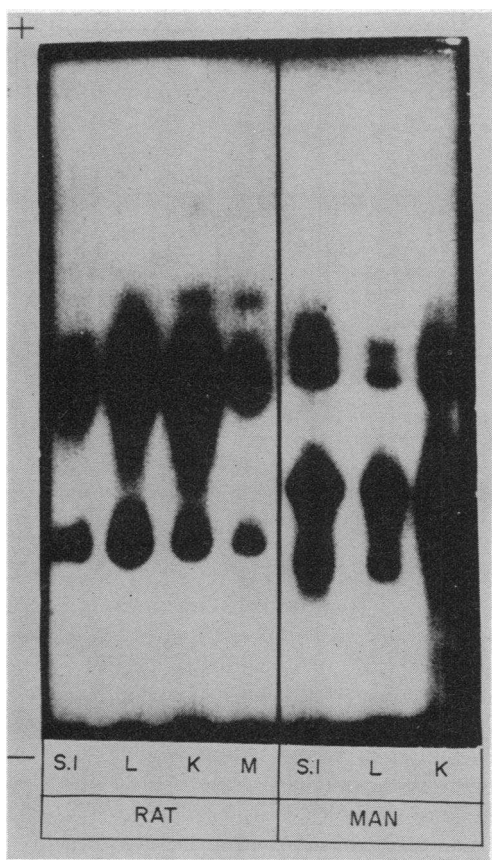
FIGURE 4 Zymograms of soluble peptide hydrolases from various organs of rat and man. The organs examined were: S.I., small intestinal mucosa; L, liver; K, kidney; and M, muscle. The substrate used was L-leucyl-L-leucine.

As a possible analogy to the brush border fractions of the intestinal mucosa, papain-treated plasma membranes of various organs were used as a source of membrane-bound peptidases with L-leucyl-L-leucine as a substrate for the zymogram studies. As shown in Fig. 5, plasma membranes of rat small intestine, liver, and kidney gave distinctive patterns, but no activity could be demonstrated with red cell membrane as a source. Thus, while the zymograms obtained from the soluble fractions of various organs all remained similar, those from the membrane-bound fractions varied with the organ. No band with cathodic electrophoretic mobility could be seen with any of the fractions studied.

Solubilization of brush border peptide hydrolases. The results of the various methods of solubilization are shown in Table III. Sonication and treatment of the "partially purified brush border" fraction with either trypsin, chymotrypsin, or glycodeoxycholate were relatively ineffective in solubilizing peptide hydrolases, while treatment with papain, Triton X-100, Na deoxycholate, and glycocholate was quite effective. Pa-

pain treatment consistently yielded the greatest peptide hydrolase activity, as evidenced by ninhydrin assay and by the intensity of color of bands in the zymograms. After treatment, more enzyme activity was measured than when the original pellet was used as a source of enzyme. None of the proteolytic enzymes used in extraction had peptide hydrolase activity against L-leucyl-L-alanine, glycyl-L-leucine, and L-leucyl-L-leucyl-L-leucine.

The zymogram pattern of the brush border fraction was found to be unaffected by the method of solubilization used (Fig. 6).

Effect of temperature on enzyme preparation. As shown in Fig. 7, peptide hydrolase activity of the soluble fraction of rat small intestinal mucosa was found to be heat labile while that of the brush border membrane was heat stable. The bands of the soluble fraction showed varying degrees of heat lability, the slowest migrating band being the most heat labile while the bands in the middle zones were relatively heat stable.

In order to rule out the possibility that the apparent heat stability of the brush border enzyme might be due to the continuous release of enzyme by papain from the small fragments of brush border membranes remaining in the supernate during heat treatment, a method of solubilization of peptide hydrolase from the brush border was used which allowed removal of the solubilizing enzyme from the supernate. Enzite-papain, which consists of papain bonded to insoluble durable cellulose support, was used for this purpose. No mea-
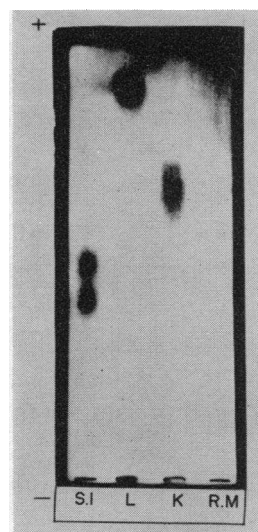

FIGURE 5 Zymograms of peptide hydrolases from plasma membranes of various organs of rat. The organs examined were: S.I., small intestinal mucosa, L, liver, K, kidney, and R. M., red cell membrane. The substrate employed was L-leucyl-L-leucine.

surable papain activity remained in the supernate after the centrifugation at 50,000 g for 30 min. Even with this method, peptide hydrolase activity of the brush border membrane was found to be heat stable under the conditions described.

Gel filtration chromatography. Fig. 8 shows the elution profile of proteins and peptide hydrolases of soluble fraction of rat small intestine. As judged by activities, the enzymes hydrolyzing L-leucyl-L-leucine, L-leucyl-L-alanine, L-leucyl-L-leucyl-L-leucine, glycyl-L-leucine, glycyl-L-valine, L-leucyl-L-proline, L-prolyl-L-leucine, and L-leucinamide hydrochloride were all eluted in approximately the same tubes. The zymogram studies of each eluent fraction, using L-leucyl-L-leucine as a substrate, revealed a very close correspondence of zymograms with the results of chemical assay. The slowest moving band was associated with the early enzyme peak and the faster migrating bands with the retarded fraction. Molecular weights of the first and the second peaks of the soluble enzyme were estimated to be 407,000 and 282,000, respectively, while that of brush border enzyme was 292,000. Papain treatment of the soluble
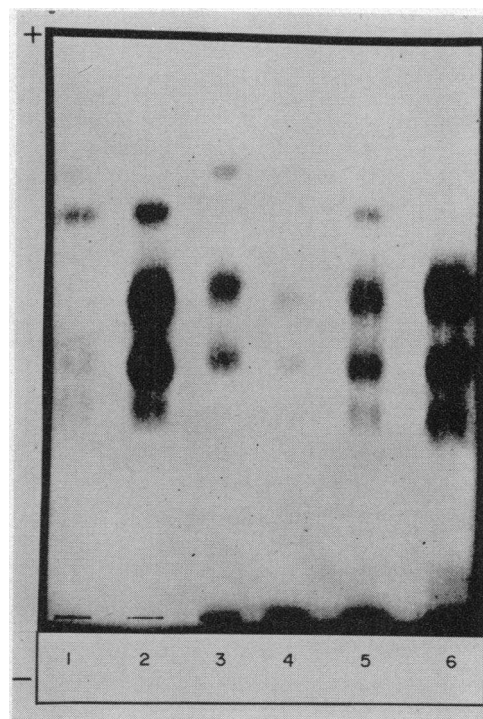

FIGURE 6 Zymograms of peptide hydrolases from brush border membranes of rat small intestinal mucosa solubilized by various methods. The methods of solubilization are as described in the text and are: 1. no treatment; 2. papain; 3. trypsin; 4. chymotrypsin; 5. Na deoxycholate; and 6. Triton X-100.

TABLE III

Solubilization of a Typical Peptide Hydrolase from the Rat Small Intestinal Brush Border Membranes

Effect of solubilizing agents	Fraction*	Enzyme activity‡	Ratio of enzyme activity (P/S)§	Total enzyme activity (P + S)	Per cent enzyme activity‖
No treatment¶	P	1.03			
	S	0.36	2.86	1.39	100
Papain	P	0.34			
	S	1.83	0.19	2.17	156
Na deoxycholate	P	0.30			
	S	1.35	0.22	1.65	119
Na deoxyglyco-cholate	P	0.25			
	S	1.04	0.24	1.29	93
Triton X-100	P	0.32			
	S	1.34	0.24	1.66	119
Trypsin	P	0.70			
	S	0.76	0.93	1.46	105
Na glycocholate	P	0.61			
	S	0.63	0.97	1.24	89
Chymotrypsin	P	0.85			
	S	0.72	1.18	1.57	113
Sonication	P	0.98			
	S	0.35	2.80	1.33	96

These numbers are the means of three experiments when L-leucyl-L-alanine was used as the substrate. Portions of the "partially purified brush border" preparation, each containing 9 mg of protein were used for each solubilization. The conditions of treatment with enzymes and detergents are as described in Methods.

* P, pellet; S, supernate.

‡ Enzyme activity expressed as micromoles of L-leucyl-L-alanine hydrolyzed per minute.

§ Ratio of enzyme activity in the pellet to that in the supernate.

‖ Enzyme activity of the treated brush border membranes expressed as a percentage of the activity in the starting material.

¶ "Partially purified brush border" was incubated in 0.1 M Na phosphate buffer at pH 7.0 at 37°C for 1 hr.

enzyme did not alter the molecular weights. With the soluble fraction of human small intestine, a similar profile of enzyme distribution was observed (Fig. 9). Molecular weights of the two peaks were estimated to be 300,000 and 150,000, respectively.

DISCUSSION

Several studies regarding the subcellular distribution of peptide hydrolases have been reported previously (4, 5, 28, 29). Robinson (28) found most of the enzyme activity to be located in the soluble fraction, while the remaining activity was found in the microsomal fraction. A subsequent study by Josefsson and Sjostrom (29) showed that dipeptidases are released quickly from the mucosal cells of the pig small intestine when mucosal scrapings are placed into aqueous solution. They therefore concluded that intracellular localization of dipeptidases could not be studied because of their rapid release into aqueous solvents. Since then it was reported that brush border membranes isolated from hamster small intestinal epithelial cells contained an appreciable amount of peptide hydrolase activity (4). Recently, using two different subcellular fraction-
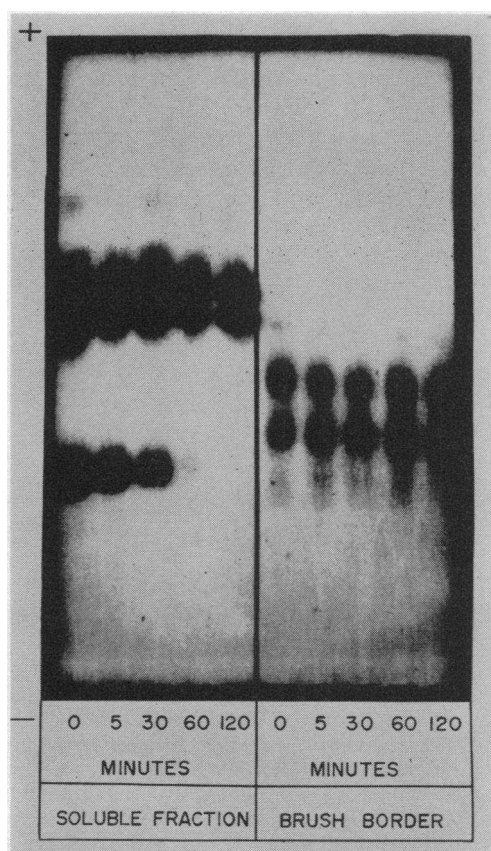
FIGURE 7 Effects of heat on activities of peptide hydrolases in soluble fraction and brush border membranes isolated from mucosa of the rat small intestine. The substrate used was L-leucyl-L-leucine. The time represents the number of minutes the fractions were incubated at 40°C.

ation techniques, Peters localized both di- and tripeptide hydrolase activity in brush border membrane and soluble fraction of small intestinal mucosa (5).

Since 14% glycerol was shown to prevent the rapid inactivation of peptide hydrolase activities, we homogenized the tissue in 14% glycerol and carried out the subcellular fractionation studies in this media.

Glutamic dehydrogenase is generally considered to be a mitochondrial enzyme (24) while NADPH-cytochrome c reductase a microsomal enzyme (25). Galactosyltransferase has also been shown to be associated with microsomes (17). Sucrase is considered to be a brush border membrane marker (23).

As shown in Tables I and II, respective marker enzyme in each fraction were concentrated three- to fourfold in mitochondrial and microsomal fractions and over twofold in brush border fractions.

Although the subcellular fractionation technique employed in the current study yielded fractions rich in respective subcellular organelle as judged by the specific activity of the marker enzymes, considerable cross contamination of each fraction occurred.

Despite this limitation, our fractionation studies show that the two major subcellular loci for peptide hydrolase are brush border membranes and soluble fraction. About 80–90% of di- and tripeptide hydrolase activity was localized in the soluble fraction while the brush border membranes contained about 10% of the total activity. Incomplete recovery of brush border, as indicated by the 54% recovery of sucrase would suggest that the percentage of peptide hydrolase activity really present in brush borders might be considerably higher, but is at least 10% of the total.

Our data on the subcellular distribution of a dipeptide hydrolase is similar to those reported by Peters. However our data on tripeptide hydrolase differ from those of Peters, which show that 19–63% of total tripeptide hydrolase activity was located in the brush borders while the soluble fraction contained 30–79% of the total enzyme activity (5).

This difference may be due either to loss of brush border fragments to other subcellular fractions or to differences in the tripeptide substrates used; as is evident from his data, the percentage distribution of activity varies widely with the substrate used.

The appearance of multiple bands on zymograms, using di- and tripeptide substrates and soluble or brush border fractions as a source of enzyme, indicated multiple zones of enzyme activity. Leucine aminopeptidase, purified from connective tissue was previously shown to occur in five electrophoretically different forms which all display identical enzymatic activity (13). Two kinds of subunits were suggested as being responsible for these forms. Dolly and Fottrell (30, 31) similarly reported that three electrophoretically distinct bands hydrolyzed di- and tripeptides in the human small intestine. These findings are not unique since intestinal disaccharidases have also been shown to occur in multiple forms: two lactases and five maltases have been reported (32, 33). Whether or not these multiple bands of peptide hydrolase activity represent structurally similar related molecules or different molecules having catalytic activity toward the same substrate must await further purification of these enzymes. Problems in the classification and nomenclature of isoenzymes are reviewed elsewhere (34, 35).

The varying electrophoretic mobilities of enzymes indicated by the various bands may depend either on molecular charge or size or on both. However, zymograms of the fractions obtained from gel filtration in the present study as shown in Figs. 7 and 8 suggest that variations in the molecular size may play a role in the electrophoretic mobility of these enzymes. The possibility that varying N-acetylneuraminic acid con-
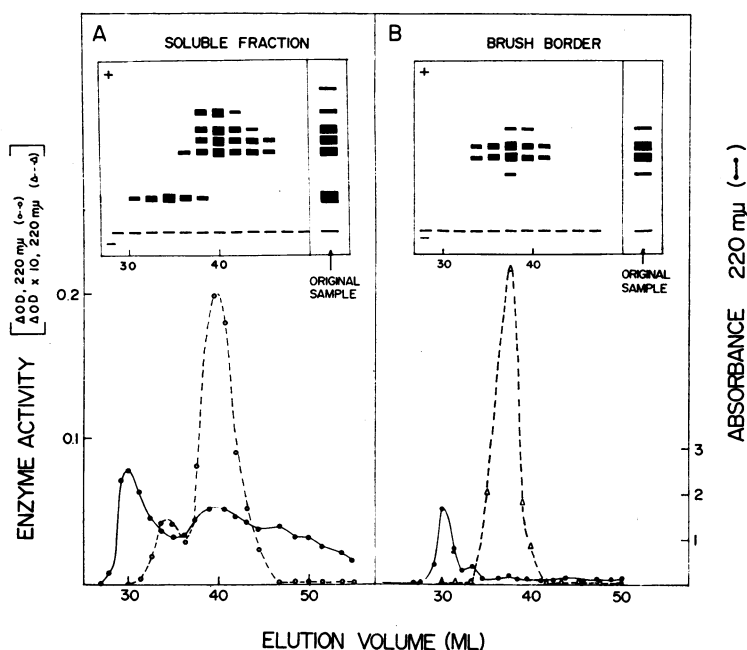
FIGURE 8 Chromatographic separation of a peptide hydrolases from soluble (A) and brush border (B) fractions of rat small intestinal mucosa, using a column containing Biogel A 0.5 M. The samples used in the zymogram (insert) correspond to the fractions of the column effluent. The zymogram pattern was drawn to match the relative intensity as closely as possible. Original sample represents the sample before it was applied to the column. The enzyme activity in all cases was measured using L-leucyl-L-leucine as the substrate.

tent of the enzymes was responsible for multiple bands, as in the case of human placental alkaline phosphatase (16), was ruled out by the observation that the zymograms were the same both with and without neuraminidase treatment.

A rather broad substrate specificity was observed with the soluble fraction although some hydrolases appear to be quite specific for certain substrates. For instance, zymogram pattern observed with L-leucyl-L-proline as substrate was quite distinct from those observed with other di- and tripeptides. Likewise, L-leucyl-β-naphthylamide and L-leucinamide appear to be hydrolyzed by two distinct enzymes, a slowly migrating band with L-leucyl-β-naphthylamide and a rapidly migrating band with L-leucinamide. These did not correspond in position with other bands of hydrolase activity against leucine-containing peptides. This is of interest since these two synthetic substrates have in the past been used as specific substrates in the assay of leucine aminopeptidase (36–38) regarded as a single enzyme. In the present study, these compounds appeared to reflect the activities of two different enzymes. Patterson, Hsiano, and Keppel (38) reported that in both mouse ascites cells and liver, the kinetic properties of

the enzymes hydrolyzing L-leucinamide ("leucine aminopeptidase") and those hydrolyzing the chromogenic substrate, L-leucyl-β-naphthylamide were different indicating that these were two distinct enzymes. Fleisher, Panko, and Warmka (39) compared the hydrolytic properties of the enzymes in serum on L-leucyl-β-naphthylamide and L-leucyl-glycine and concluded that these substrates were hydrolyzed by different enzymes. Our zymogram data clearly indicated the presence of at least four enzymes capable of hydrolyzing these leucine-containing substrates (L-leucyl-β-naphthylamide, L-leucinamide, L-leucylglycine, and L-leucyl-L-proline). These observations help to clarify some discrepancies between the data on histochemical localization of "leucine aminopeptidase" using L-leucyl-β-naphthylamide to the brush border membrane (40) and those on biochemical localization of enzyme activity in the soluble fraction which used different leucine-containing peptide substrates (28, 29). Although the precise classification of these enzymes must await further purification and studies on their chemical and kinetic properties, leucine aminopeptidase, leucinamidase, and leucyl-β-naphthylamidase hydrolyzing peptides with L-leucine at the amino
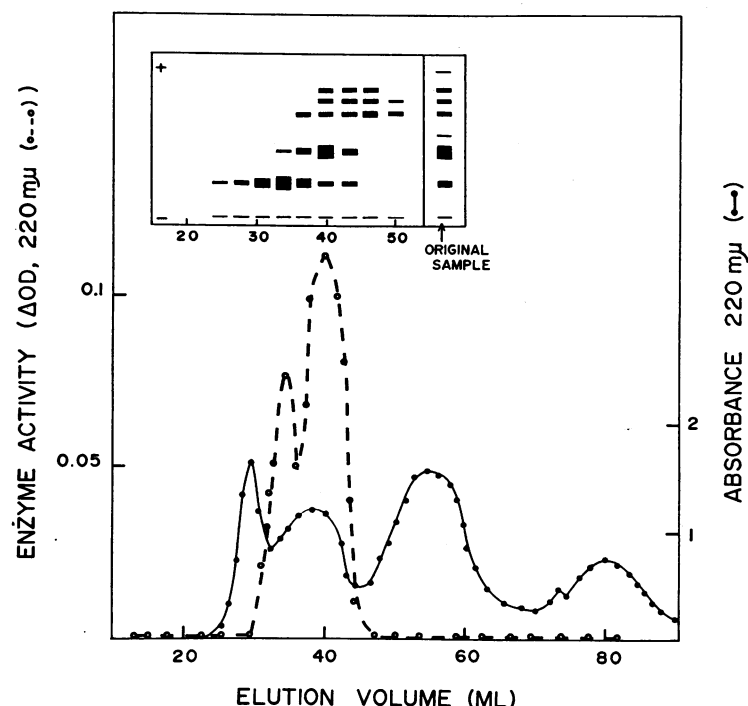
FIGURE 9 Chromatographic separation of a peptide hydrolase from soluble fraction of human jejunal mucosa using a column containing Biogel A 0.5 M. The insert depicts the zymogram pattern of the corresponding fractions of the column effluent. The same substrate was used in both cases, L-leucyl-L-leucine.

terminal, L-leucinamide and L-leucyl-β-naphthylamide, respectively, appear to be three distinct enzymes.

The zymogram patterns of the brush border membranes were quite different from those of the soluble fraction in rat small intestine indicating that enzymes from the two sources may represent different families of enzymes. This possibility was further supported by our observation as well as that by Heizer and Isselbacher (3) that the heat stability of the enzymes from two sources differed markedly. To our knowledge, zymogram studies on peptide hydrolases in the brush border membrane of small intestinal mucosa have not been carried out before. The distinct zymogram pattern shown by the brush border fraction might have been a result of the proteolytic action of papain on the enzyme molecules, but this seemed unlikely, since papain treatment of the soluble enzyme fraction failed to produce the typical zymogram pattern of the brush border enzyme fraction. Furthermore, irrespective of the method of solubilization of enzymes from the brush border, i.e., treatment with various proteases or detergents, the zymogram pattern remained similar. Untreated partially "purified brush border fraction" showed some enzyme activity in the supernatant fraction even in the absence of solubilizing agents. This is probably

due to incomplete removal of the brush borders from the supernate by the centrifugation method used and to some release of enzymes from the brush border under the conditions used. Since sonication alone did not effectively solubilize the enzymes, peptide hydrolases in the brush border fraction appear to be tightly bound to the membrane, as are the disaccharidases (32). With papain treatment, the peptide hydrolase activity, in the supernatant fraction alone, exceeded the original total activity of the untreated brush border. This is similar to recent data observed with enterokinase (41, 42) and suggests that some brush border membrane-bound enzymes are much more active in the soluble than in the membrane-bound form. The kinetics of this phenomenon needs further clarification. Papain treatment of the brush border membranes of hamster intestine was shown by Eichholtz to cause release of L-leucylglycine hydrolase and L-leucyl-β-naphthylamidase at different rates (43) suggesting that these are different enzymes. However, identical zymogram patterns obtained with solubilized brush border enzymes against both L-leucylglycine and L-leucyl-β-naphthylamide suggest that in the rat the same enzyme or enzymes may be responsible for the hydrolysis of these two sub-

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strates. In the present paper, the possibility of differential rates of solubilization was not studied.

The peptide hydrolase activity in the brush border fraction appeared to possess activity against a much broader range of substrates than that in the soluble fraction, showing uniform zymogram patterns with all of the peptide substrates studied, including both L-leucinamide and L-leucyl-β-naphthylamide. However, using this technique no enzyme activity could be detected against proline-containing dipeptides, imidopeptides, or iminopeptides. This is consistent with the recent observation of Gray and Cooper (44) who also failed to detect peptide hydrolase activity against proline-containing peptides in solubilized brush borders on chemical assay.

The electrophoretic mobilities of bands produced by the soluble fraction of human jejunal mucosa were distinct from those of the rat, suggesting a species difference. However, the zymogram patterns for soluble fractions, studied with various peptide and synthetic substrates, could be grouped in a strikingly similar manner in both species. Since human postmortem material was used, it is possible that autolytic release of brush border enzymes to the supernate might have occurred contributing spurious bands to the zymograms of soluble fraction. No comparable data on human brush borders were available. The treatment of the human mucosal homogenate with papain or trypsin did not result in the appearance of new bands, suggesting that all of the brush border enzymes had been released into the supernate, though it has been shown that there may be species differences in the release of brush border enzymes (disaccharidases) by proteolytic enzymes (45).

In addition to those in small intestinal mucosa, peptide hydrolases have been observed in many mammalian tissues such as kidney, liver, skeletal muscle, spleen, erythrocyte, connective tissue, mouse ascites cells, and other tissues and in serum (12–15). The functional role of these enzymes in tissues is not well understood. In this regard, our observation that the zymograms of peptide hydrolases obtained from soluble fractions of various organs from the same species were similar, i.e., non-organ-specific, while those from the plasma membrane-bound fractions varied with the organ, is of considerable interest. These observations, although not conclusive suggest that while the soluble enzymes may play a more general role in cellular protein metabolism, the brush border enzymes may be involved in functions related to the particular tissue.

The peptide hydrolases are extremely labile and, despite the use of glycerol which was found to stabilize these enzymes, attempts at further fractionation and purification of these enzymes did not succeed. Additional clarification of their molecular structure, chemical, and immunochemical properties may help to establish their functional roles in the digestion of small peptides or in intracellular protein metabolism. Studies of the changes in enzyme activity in various disease states, as recently initiated by Sadikali (10) and others (46), should also shed valuable light on their biological importance in humans.

ACKNOWLEDGMENTS

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