Biochimica et Biophysica Acta, 433 (1976) 469–481

BBA 77305

ENZYMIC SOLUBILIZATION OF THE HUMAN INTESTINAL BRUSH BORDER MEMBRANE ENZYMES

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(Received September 12th, 1975)
(Revised manuscript received January 26th, 1976)

SUMMARY

The releases of proteins, maltase, lactase, sucrase, trehalase, alkaline phosphatase, γ-glutamyltransferase and leucylnaphthylamide-hydrolyzing activity from human intestinal brush border membrane vesicles by various enzymes (especially pancreatic proteases) have been studied.

The brush border membrane enzymes are not solubilized by digestion with trypsin and chymotrypsin but are largely released after treatment with papain or elastase. Most of the enzymes are fully active after the proteolytic treatment. All proteins released by papain and elastase have been identified by electrophoresis to already known intestinal hydrolases.

Electron microscopy of brush border membrane vesicles demonstrates "knob-like" structures (particles) attached to the external side of the membrane. During papain treatment, enzyme removal runs parallel with the disappearance of the particles. During elastase treatment it is not possible to correlate the release of the enzymic activities with the removal of the particles.

The results indicate that most of the intestinal hydrolases are surface components attached to the external side of the membrane. They are in accord with the concept that the brush border membrane enzymes are organized within the membrane in a mosaic-like pattern.

INTRODUCTION

We have previously described methods for the preparation of purified human intestinal brush border membrane vesicles [1], their solubilization and fractionation [2] and the identification of the brush border membrane enzymes after gel electrophoretic separation [3]. The current paper describes the solubilization of the brush border membrane enzymes from isolated human brush border membrane vesicles by

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various proteolytic enzymes. The activities studied include maltase (α-D-glucoside glucohydrolase, EC 3.2.1.20), lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23), sucrase (sucrose α-glucohydrolase, EC 3.2.1.48), trehalase (α, α-trehalase, EC 3.2.1.28), alkaline phosphatase (EC 3.1.3.1) γ-glutamyltransferase (EC 2.3.2.2.) and leucylnaphthylamide-hydrolyzing activity. Since electron microscopic studies have demonstrated knob-like structures on the outer surface of human intestinal brush border membranes [1] the release of these enzymes activities and the removal of the particles (knobs) external to the brush border membrane (as determined by electron microscopy) have been monitored simultaneously with time in order to clarify whether or not the enzymes and the knobs were removed at the same time. It was anticipated that such an enzymic digestion might contribute to our understanding of the morphochemical relationship between the various brush border enzymes, and between them and the membrane.

MATERIALS AND METHODS

Chemicals. α-chymotrypsin (EC 3.4.4.5) (3 ×crystallized, salt free) lyophilized from bovine pancreas was purchased from Grand Island Biological Company. Trypsin (EC 3.4.4.4.) (2 ×crystallized, dialyzed and lyophilized) from bovine pancreas, elastase (2 ×crystallined) from hog pancreas and papain (EC 3.4.4.10) (2 × crystallized) lyophilized powder or insoluble enzyme attached to carboxymethyl cellulose were obtained from Sigma. Cysteine free-base crystalline was from Sigma. Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine were from Eastman Kodack Co. Sodium dodecyl sulfate was from Mallinckrodt. The reagents and substrates used for the determination of the enzymic activities were from the same commercial sources previously noted [1–3]. Glucose oxidase was from Miles Laboratories. Other materials were reagent grade commercial preparations.

Intestinal samples. Full-thickness sections of macroscopically normal human small intestine were obtained from a total of 16 patients. Two duodenal, 7 jejunal and 7 ileal specimens are included in this study.

Membrane preparation. Brush border fragments (fraction P_2) and brush border membranes (fraction F_{II}) prepared and characterized as previously described [1] were suspended in cold distilled water.

Incubation procedure. Brush border fragments or microvillus membranes were incubated for 5 min in 14.3 mM potassium phosphate buffer (pH 7.1) with 1.8 mM EDTA and re-incubated subsequently for 60 min in the presence of trypsin (0.14 mg/ml), chymotrypsin (0.14 mg/ml), elastase (18 units/ml) or papain (10 μg/ml). The incubation was carried out at 37 °C in a capped tube which was shaken in a horizontal position by using a water bath shaker (model 2156, American Optical) with the shaker assembly set at 100 cycles per min. The concentration of the brush border protein was between 0.120 and 0.560 mg/ml incubation mixture in all experiments. 0.8 ml samples were removed from the incubation immediately after the addition of the proteolytic agent and after 15, 30, 45 and 60 min of digestion. Samples were cooled in ice water diluted 5-fold at 4 °C and the brush border fragments or membranes vesicles were sedimented by centrifugation at 30 000 ×g for 30 min at 4 °C [4]. The supernatant was decanted and the pellet was suspended in 1 ml of cold distilled

water. The supernatant and the resuspended sediment were analyzed for protein and the enzymic activities specified below.

When the effect of papain was tested, the enzyme was first activated by cysteine. Cysteine (2.5 mM) was also present in the incubation mixture. In the experiments in which insoluble papain was used, the papain was removed by centrifugation at $800 \times g$ for 10 min at 4 °C [5]. The supernatant was then re-centrifugated at $30\,000 \times g$ from 30 min to separate the unreacted membranes from the released particles.

Samples were taken for electron microscopy and gel electrophoresis.

Enzymic assays. Disaccharidase activities (maltase, lactase, sucrase, trehalase) were assayed according to a modification by Lloyd and Whelan [6] of Dahlqvist's method [7]. Leucylnaphthylamide-hydrolyzing activity and alkaline phosphatase activity were assayed according to Goldberg and Rutenburg [8] and Eichholz [9], respectively. γ-Glutamyltransferase activity was assayed according to Naftalin et al. [10]. Protein was assayed according to Lowry et al. [11] using cystalline bovine serum albumin as standard. The distribution of the brush border marker enzymes, and of the amount of protein between the supernatants and sediments of the treated brush border were calculated as per cent of the total amount or activity present at zero time. For correction of protein loss and enzymic release due to shaking, control experiments were done by incubating untreated brush border fragments (P_2) or brush border membranes (F_{11}) for 60 min at 37 °C.

Electron microscopy. Freshly prepared 2 % phosphotungstic acid adjusted to pH 7.1 with 1.0 M KOH, was used for negative staining [12]. All samples were prepared similarly. A drop of a sample suspension was placed on a carbon-coated specimen grid and excess fluid was removed with filter papers. The grid was then floated on the surface of a drop of 2 % phosphotungstic acid solution. After 10 s the grid was taken out. The excess fluid was removed with filter papers and the grid was air dried. Preparations were immediately examined with a Philipps EM300 electron microscope.

Polyacrylamide-gel electrophoresis. Electrophoresis was carried out as previously described [2, 3]. Acrylamide concentration of the standard gels was 10 %. 50–100 μg of brush border protein were layered into the gel column through the upper buffer. The identification of the electrophoretically separated bands was done by biochemical assay as previously described [3]. Molecular weights were calculated from the migration of markers of known molecular weights [2].

RESULTS

Each enzyme shows unique kinetics with respect to its solubilization. For each enzyme, the pattern of solubilization obtained from brush border fragments resembles that obtained with brush border membranes. For each enzyme the patterns of solubilization obtained from duodenal, jejunal and ileal brush border membranes were similar. Therefore, the independent assays performed were averaged in each case.

Release by trypsin

The release of protein and various enzymatic activities from isolated human brush border membranes by treatment with trypsin is seen in Fig. 1. 66 % and 80 % of the total protein were solubilized respectively after 15 and 60 min. The leucylnaphthylamide-hydrolyzing activity was released much more rapidly than any of the

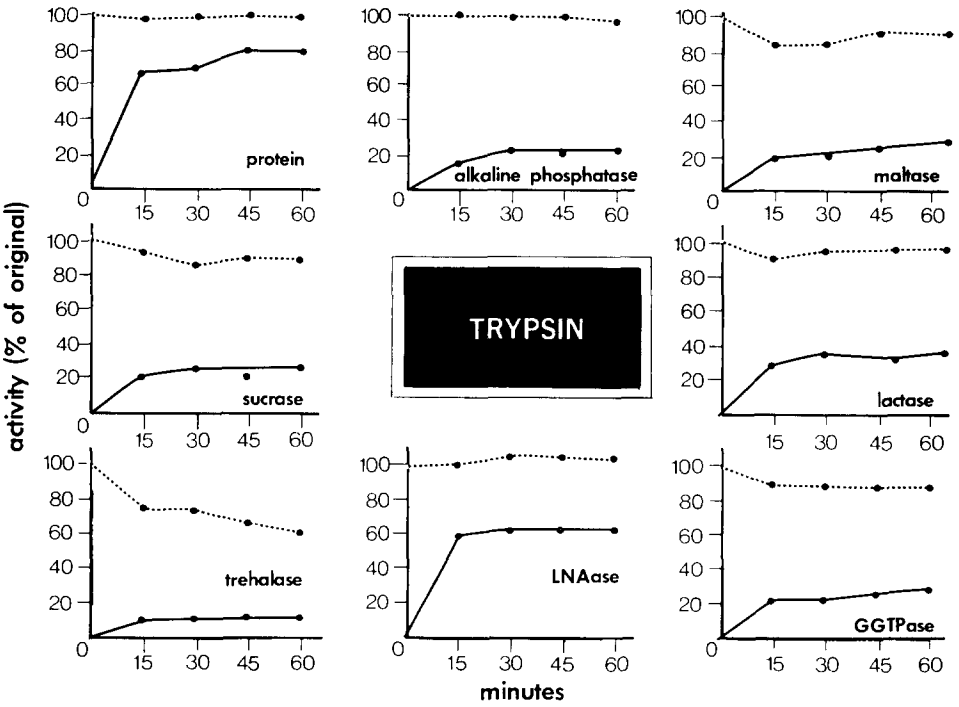

Fig. 1. Release of proteins and enzymic activities from isolated human brush border membranes with time of treatment with trypsin (0.14 mg/ml incubation). ● — ●, The released fractions at various times; ● · · · ● total activity (recovery) at the corresponding times. Six independent assays (2 duodenum, 2 jejunum, 2 ileum) were performed and averaged in each case. Values in the curves have in both cases been expressed as per cent of the total activity present at zero time. LNAase = leucylnaphthylamide-hydrolyzing activity. GGTPase = γ-glutamyltransferase.

other activities studied. About 60 % of the total leucylnaphthylamide-hydrolyzing activity was liberated during conditions which released only small amounts of the other brush border membrane enzymes (20–30 %, for alkaline phosphatase, maltase, sucrase, lactase, γ-glutamyltransferase and 10 % for trehalase). The solubilization by trypsin of most of the enzymic activities studied was not associated with significant decreases of the activities of the enzymes as judged from the recovery values (Fig. 1). However, trehalase is gradually inhibited by trypsin and only 60 % of this activity was recovered after 60 min of trypsin treatment.

Release by chymotrypsin

Four independent assays (2 jejunum, 2 ileum) were performed. Chymotrypsin gave a release pattern very similar to that caused by trypsin. About half of the total leucylnaphthylamide-hydrolyzing activity was released after 60 min of chymotrypsin treatment. The other enzymatic activities were released to a small extent.

40 % of the total maltase activity was inhibited by chymotrypsin treatment, while the other disaccharidases were slightly inhibited. Alkaline phosphatase, leucylnaphthylamide-hydrolyzing activity and γ-glutamyl transferase activities were not decreased by the chymotrypsin treatment, as judged from the recovery values.

Release by papain

The use of soluble papain with units of activity approximately equal to those of papain cellulose gave essentially the same results as complexed papain. The release of protein and various enzymatic activities from isolated human intestinal brush border membranes by treatment with papain is seen in Fig. 2. About 80 % of the total protein were solubilized after 15 min of papain treatment. During the same conditions some of the enzymic activities were very well solubilized (92, 82, 74, 94 and 68 % for maltase, sucrase, leucylnaphthylamide-hydrolyzing activity and γ-glutamyl transferase, respectively, while alkaline phosphatase and trehalase were solubilized only to a small extent (40 % and 12 %, respectively). The solubilization by papain of the various enzymatic activities studied was associated with a significant decrease of the activities of alkaline phosphatase and lactase.

Release by elastase

The results obtained by treatment with elastase are seen in Fig. 3. After 15 min of treatment, 84 % of the total proteins were released. Maltase, sucrase, lactase and leucylnaphthylamide hydrolyzing activities were easily released (86, 72, 54 and 84 % respectively) while alkaline phosphatase, trehalase and γ-glutamyl transferase were released to a small extent (14, 16 and 20 % respectively). As judged from the recovery values, the solubilization of the various enzyme activities by elastase, was associated

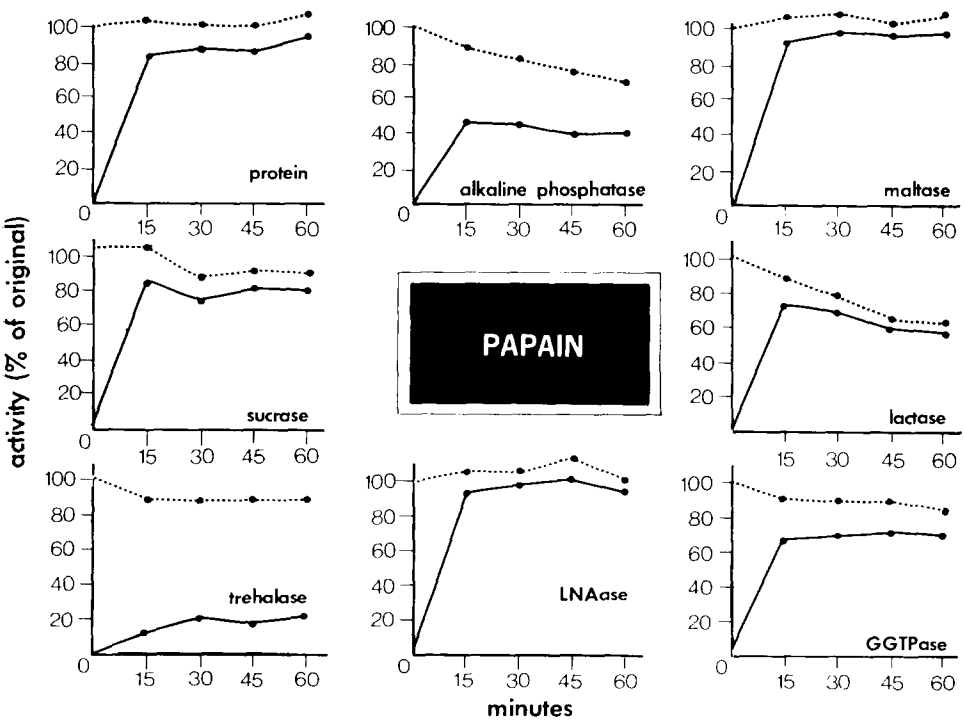

Fig. 2. Release of protein and enzymic activities from isolated human brush border membranes with time of treatment with papain (10 μg/ml incubation mixture). Five independent assays (1 duodenum, 2 jejunum, 2 ileum) were performed. For the explanation of the curve symbols and legends see Fig. 1.

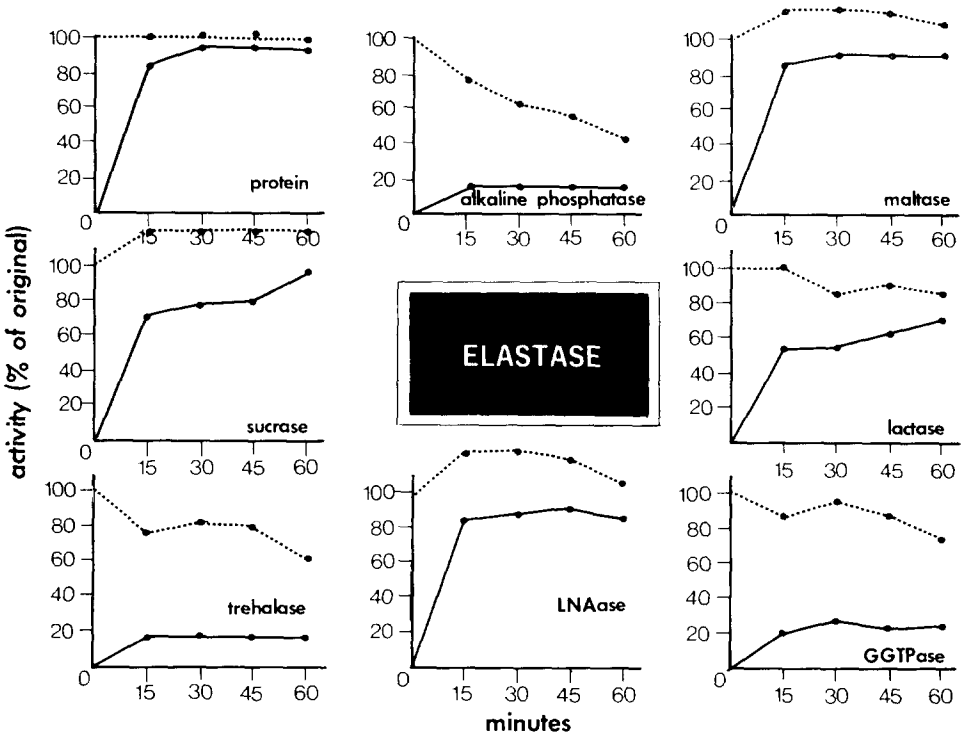

Fig. 3. Release of protein and enzymic activities from isolated human brush border membranes with time of treatment with elastase (18 units/ml incubation mixture). Five independent assays (1 duodenum, 2 jejunum, 2 ileum) were performed and averaged in each case. For the explanation of the curve symbols and legends see Fig. 1.

TABLE I

ENZYMES LIBERATED BY MECHANICAL SOLUBILIZATION

Release of protein and enzymic activities from isolated human brush border membrane vesicles with time of incubation. The undigested vesicles were incubated at 37 °C for the period of time indicated and the resulting mixture was centrifuged to give a supernatant (soluble) and a pellet (insoluble) which were analyzed separately. Results are expressed as per cent of the total amount or activity present at zero time. Three independent assays were performed and averaged in each case. LNAase = leucylnaphthylamide-hydrolyzing acitivity.

Incubation time (min)		Protein	Alkaline phosphatase	Maltase	Sucrase	Lactase	Trehalase	LNAase	γ-glutamyl transferase
15	scluble	30	16	16	15	22	18	24	21
	insoluble	68	84	84	85	78	82	76	79
30	soluble	36	19	17	16	25	21	26	22
	insoluble	64	81	83	84	75	79	74	78
45	soluble	30	17	16	17	20	16	22	19
	insoluble	70	83	84	83	80	84	78	81
60	soluble	30	19	15	16	22	17	22	16
	insoluble	70	81	85	84	78	83	78	84

with significant decreases of trehalase and γ-glutamyl transferase activities and significant increases of maltase, sucrase and leucylnaphthylamide-hydrolyzing activities.

Control experiments

The protein loss and enzymic releases due only to shaking (mechanical solubilization) are expressed in Table I. It can be seen that the shaking caused a mechanical solubilization of 15–26 % of all enzyme activities studied, and a 30–36 % solubilization of the total protein. No inactivation of enzymes was observed in the course of these assays. To determine the effect of the proteolytic treatment on the solubilization of the brush border membrane enzymes, the releases obtained at timed intervals were corrected for the mechanical solubilization observed at the corresponding times.

The true solubilization of the several enzymatic activities caused by either trypsin, chymotrypsin, papain or elastase, after 15 min of treatment is described in Table II. It is evident that all enzymes measured (except leucylnaphthylamide-hydrolyzing activity after 15 min of treatment with trypsin) are solubilized poorly or not at all by digestion with trypsin and chymotrypsin. In contrast, maltase, sucrase and leucylnaphthylamide-hydrolyzing activities are well recovered in soluble form after digestion with papain or elastase, but under these same conditions, alkaline phosphatase and trehalase are solubilized poorly by papain or elastase treatment. Elastase gave a release pattern very similar to that caused by papain, except that γ-glutamyltransferase activity is well solubilized by papain (44 %) and not at all by elastase. The disaccharidases are to some extent inhibited by trypsin and chymotrypsin while alkaline phosphatase, leucylnaphthylamide-hydrolyzing activity and γ-glutamyltransferase are not affected by these treatments. Alkaline phosphatase and trehalase are to some extent inactivated by papain and elastase.

TABLE II

RESULTS OF SOLUBILIZATION OF THE ENZYMES BY DIFFERENT PROTEOLYTIC TREATMENTS

Release of protein and enzymic activities from isolated human brush border membrane vesicles after 15 min of proteolytic treatment. The proportions of the protein and enzymes released in the supernatant were corrected for the mechanical solubilization (Table I). Recoveries (supernatant plus pellet) are given in parentheses. Each value represents the mean of six determinations. Abbreviations are the same as for Table I.

Protease	Amount or activity % of original							
	Protein	Alkaline phosphatase	Maltase	Sucrase	Lactase	Trehalase	LNAase	γ-Glutamyl transferase
Trypsin	34	0	4	6	8	0	36	0
	(98)	(100)	(84)	(94)	(92)	(74)	(100)	(90)
Chymotrypsin	28	0	0	4	0	0	6	4
	(100)	(100)	(60)	(70)	(90)	(84)	(96)	(100)
Papain	50	12	76	64	32	0	70	44
	(102)	(90)	(104)	(102)	(88)	(88)	(104)	(92)
Elastase	52	0	70	46	32	0	60	0
	(100)	(76)	(120)	(120)	(100)	(76)	(120)	88

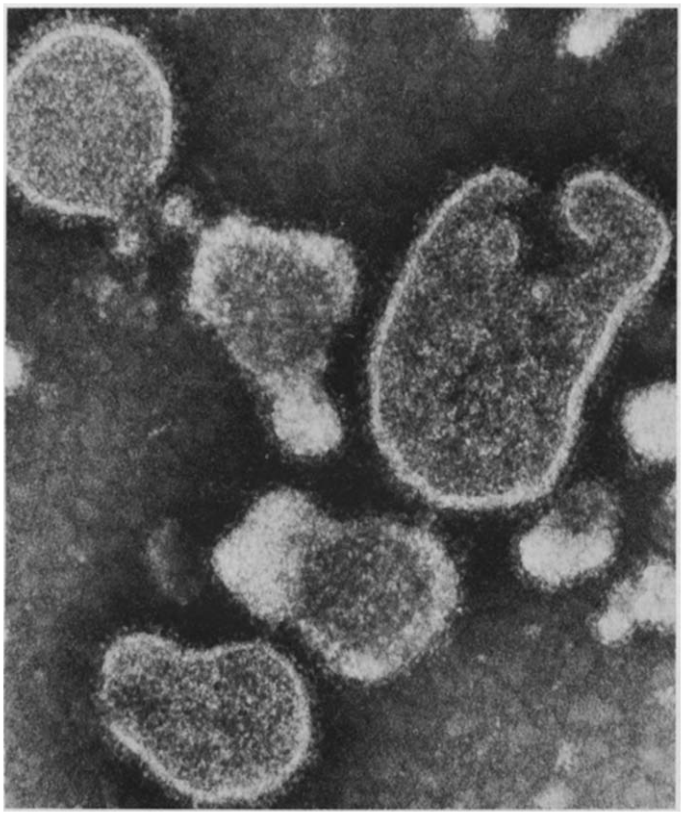

Fig. 4. Electron micrographs of negatively stained brush border membrane preparations. Stained with 2 % phosphotungstic acid pH 7.1. Control brush border membrane, freshly isolated, undigested. The specimen contained vesicles which varied in sizes and shapes. There are some membrane fragments which did not form any closed cavity. Note the uniform distribution of particles over the surface of the membrane vesicles (× 113 400).

Effects of the proteolytic agents on the morphology of isolated brush border membranes

The effects of trypsin, chymotrypsin, papain and elastase on the morphology of isolated human intestinal brush border membranes were examined concurrently with the kinetic studies (Figs. 4–7). Electron micrographs of negatively stained brush border membranes prior to their incubation with the proteolytic agent show (Fig. 4) "Knob"-like structures on the outer surface of the vesicle membrane. It was noted that the inner membrane surface is free of any material, whereas there are variable amounts of material on the outer surface. These knob-like structures (particles) probably correspond to those structures, 45 Å in diameter and 75 Å in height, observed during electron microscopic study of the microvillus membranes [1]. Vesicles show nearly the same morphology whether freshly prepared, preincubated 5 min or incubated for a prolonged period without enzymes. This would seem to indicate that mechanical solubilization causes no variation in the morphology of the vesicles. After digestion with trypsin or chymotrypsin, the treated brush border membrane vesicles resemble undigested (control) brush border membrane vesicles, since particles

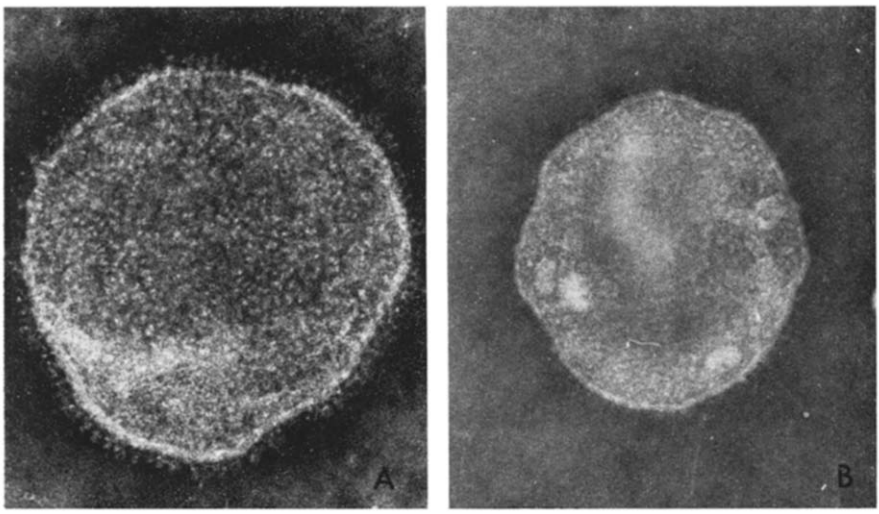

Fig. 5. Electron micrographs of negatively stained brush border membrane preparations. Stained with 2 % phosphotungstic acid pH 7.1. Control (A) and papain treated (B) brush border membrane vesicles. In the control (vesicles incubated for 30 min without papain) particles are clearly seen on the outer surface (× 135 000). After 30 min of digestion with papain (B) these particles are no longer visible (× 121 500).

are clearly seen on the outer surfaces of both. The distribution of these particles over the surface of digested and undigested membrane vesicles is uniform even after 60 min of trypsin or chymotrypsin treatment. After 30 min of digestion with papain, the digested vesicles had lost their granular appearance and the particles were no longer visible (Fig. 5B). At higher magnification it was noted that no particles remained attached to the membrane's outer surface (Fig. 6). After 30 and 60 min of digestion with elastase, vesicles were found to which some particles were still attached (Fig. 7).

DISCUSSION

The release patterns of enzymic activities from human duodenal, jejunal and ileal brush border fragments and membranes are not significantly different. However, the various enzymes studied, vary in the degree of their solubilization by proteolytic treatment and all show a distinct release pattern. Results obtained when solubilized enzyme activities are expressed as a per cent of total activity present at zero time (Figs. 1–3) correspond exactly to electron microscopy studies. They can be correlated to the removal of the particles external to the microvillus membrane since the solubilization of the enzyme (as determined by biochemical kinetic studies) and the removal of the particles (as determined by electron microscopy) were monitored simultaneously at 15-min intervals. However, it is possible that the results of earlier studies [4, 12–16] may have been overinterpreted since they were not corrected for solubilization due to shaking. The only previous investigation of solubilization of human intestinal enzymes was done using mucosal homogenates treated with a relatively high concentration of papain and trypsin [15]. In our study, brush border membrane vesicles were used and digestions were carried out in presence of trypsin and chymotrypsin at

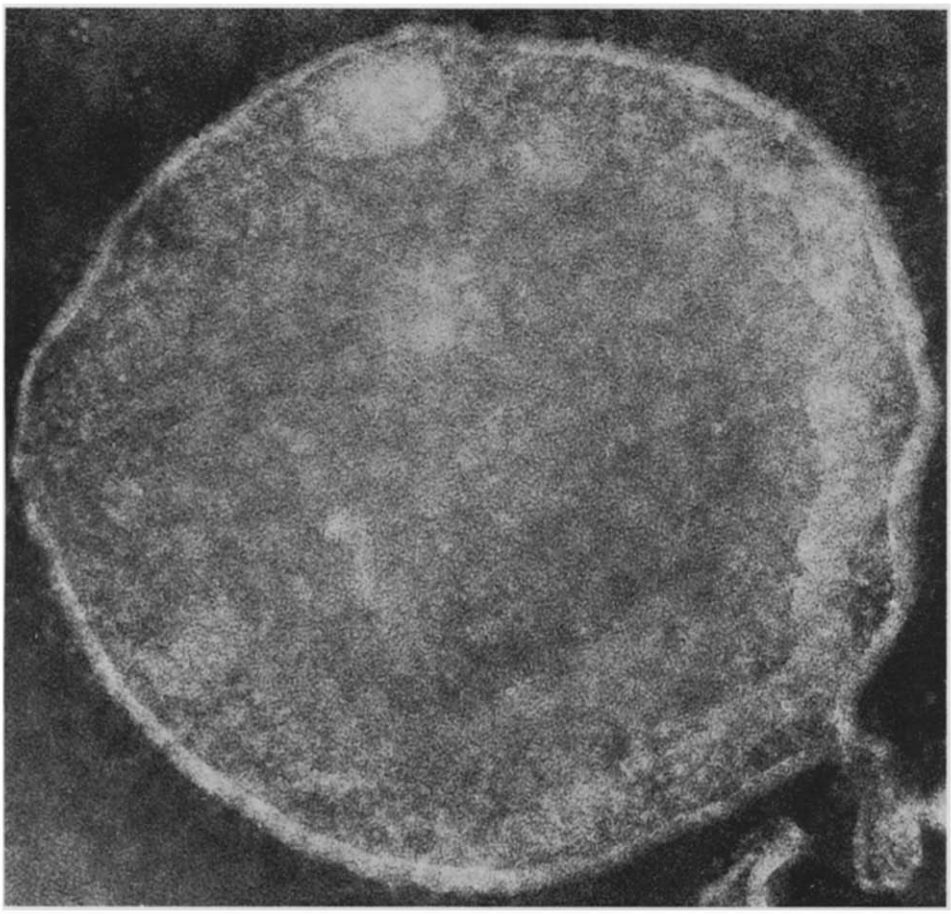

Fig. 6. Electron micrographs of negatively stained brush border membrane preparations. Stained with 2 % phosphotungstic acid pH 7.1. High magnification micrograph of the membrane shows that vesicles have lost their granular appearance after 30 min of papain digestion and present a particle-free smooth surface (×260 000).

physiological concentrations and elastase or papain at low concentrations. Under these conditions, trypsin and chymotrypsin caused no release of most of enzymic activity (only leucylnaphthylamide-hydrolyzing activity is released by trypsin); no proteolytic treatment releases trehalase and alkaline phosphatase whereas papain and elastase solubilize maltase, sucrase, leucylnaphthylamide-hydrolyzing activity, and lactase. Papain and elastase treatments differ only in that γ-glutamyltransferase activity is released by papain. Our results agree with earlier data on homogenates [15], and when compared with those obtained after papain digestion in pig, rat and rabbit jejunum brush borders [16] show that maltase, sucrase and aminopeptidase are fully released while the releases of alkaline phosphatase and trehalase would appear to vary with the species. As the brush border membranes used in our study appeared (under electron microscopy) to be right-side-out vesicles, it is concluded that the molecules released are external components of the membrane. Release of sucrase,

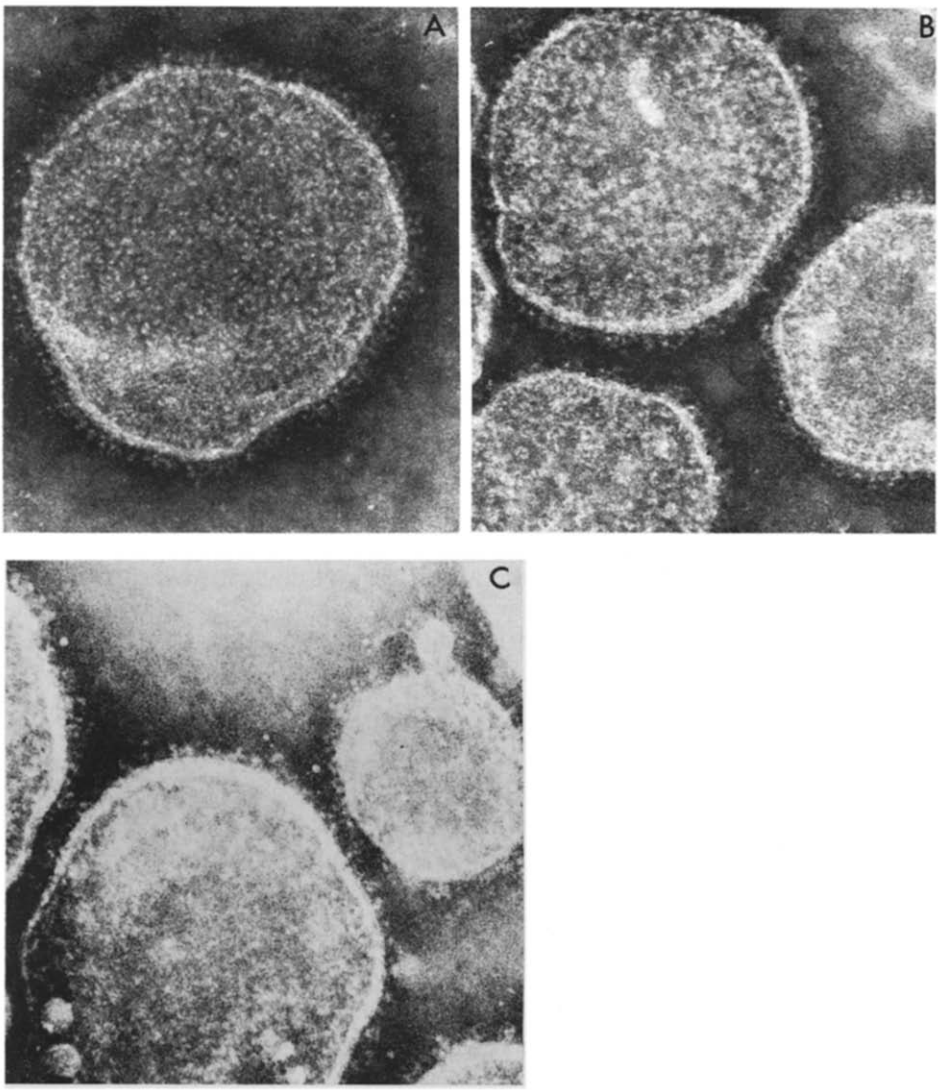

Fig. 7. Electron micrographs of negatively stained brush border membrane preparations. Stained with 2 % phosphotungstic acid pH 7.1. Untreated (A) and elastase-treated (B and C) brush border membrane vesicles. Control vesicles (A) were incubated for 30 min without elastase. A high proportion of particles remained attached to the membrane after 30 min of digestion (B), while the number of particles present is diminished after 60 min of digestion (C) (× 135 000).

maltase, lactase and leucylnaphthylamide hydrolyzing activity may have both a structural and chemical explanation. The linkages of these enzymes to the surface membrane may differ chemically from those of alkaline phosphatase and trehalase in that they are more sensitive to papain and elastase. Digestion of brush border membranes using papain, elastase, trypsin or chymotrypsin does not release alkaline phosphatase or trehalase activity even after an hour of treatment. This and the fact

that all surface particles are removed without disruption of the membrane after papain digestion would seem to indicate that alkaline phosphatase and trehalase are buried in the lipid bilayer and thus less accessible than other enzymes. The amount of soluble protein, after correction for the proteolytic agent added, increased during trypsin, chymotrypsin and elastase digestion. Trypsin and chymotrypsin digestion released 28 and 34 % respectively of the total proteins with no dislocation of the membrane. Moreover, electron microscopy shows no disappearance of the particles on the external side of the membrane, and trypsin and chymotrypsin digested vesicles have nearly the same aspect as untreated vesicles. After papain and elastase treatments up to 50 % of the total protein is released with no appreciable dislocation of the microvillus membrane. Electron microscopy shows that during papain treatment enzyme removal is concomitant with the disappearance of the particles; a finding in agreement with the results of Johnson [13]. In contrast, correlation of release of enzymes with removal of particles after elastase treatment is impossible because some particles remain attached to the external side of the membrane even after 60 min of digestion. Although the only difference in the release patterns obtained after the papain and elastase treatments is the fact that γ-glutamyltransferase is not released by elastase, more experiments are needed before assuming that the particles remaining after elastase treatment correspond to γ-glutamyltransferase. However, since it is difficult to quantitate with great precision the particles remaining on the membrane and since not all brush border membrane enzymes have been studied, the possibility that a specific fraction of the particles is associated with this enzymic activity has not been excluded. Some variation in stability of the enzymes studied was noted during the solubilization. Significant inhibition of alkaline phosphatase and lactase activity occurred during their release by papain, while trehalase was inhibited by trypsin. However, most of the enzymes are fully active after the proteolytic treatment and their molecular weights remained identical.

ACKNOWLEDGEMENTS

Thanks are due to Mrs. M. E. Dagorn and Mr. P. Magny for their excellent technical assistance. Pieces of human intestine have been supplied by Drs. G. Devroede, G. Konok, P. Lejeune, B. Perey, J. Poisson, A. Rioux, W. Saleh and J. Susset, from the department of Surgery, Sherbrooke Medical Center, Sherbrooke, P.Q. This work was supported by Grants from the Medical Research Council of Canada and the Banting Research Foundation. Dr. Maestracci held a I. W. Killam Memorial scholarship of the Canada Council.

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