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SUGAR HYDROLASES OF THE INFANT RAT INTESTINE AND THEIR ARRANGEMENT ON THE BRUSH BORDER MEMBRANE

KENNETH K. TSUBOI *, STEVEN M. SCHWARTZ **, PETER H. BURRILL ***, LINDA K. KWONG and PHILIP SUNSHINE

Department of Pediatrics, Section of Developmental Medicine, Stanford University Medical Center, Stanford, CA 94305 (U.S.A.)

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Summary

Lactase and maltase, the predominant sugar hydrolases associated with the intestinal brush border membrane of the suckling rat, were purified essentially free of the other to near homogeneity (lactase at specific activity 23, maltase at specific activity 58), and their specific physicochemical properties determined. Antisera prepared to each showed by immunodiffusion a single common precipitin line with pure enzyme and solubilized proteins of the brush border membrane. Brush border membranes were purified 26—35-fold from infant rat intestine. Membranes prepared from 10-day-old rats contained 32% protein, 43% lipid and 25% carbohydrate with lactase and maltase estimated to comprise in excess of 10% and 2%, respectively, of the membrane protein.

Immunotitration curves of lactase and maltase showed equivalent antibody binding by the membrane-bound and free enzyme forms. Furthermore, antibody binding to one enzyme did not affect the immunotitration curve or the extractability (by papain or Triton X-100) of the other membrane-bound enzyme. It was concluded that the lactase and maltase molecules are attached singly on the external membrane surface in a spatially independent manner with their antigenic sites as freely available to antibody binding as exhibited by their papain-solubilized counterparts.

Introduction

The digestive and absorptive functions of the intestinal epithelial cell are localized with the brush border membrane which contains transport systems

* To whom reprint requests should be addressed.

and digestive hydrolases as integral components of its structure. The development of suitable isolation techniques (e.g. see Refs. 1—4) has allowed the study of this membrane in an apparently highly purified form. The membrane has been examined particularly in relation to the associated digestive hydrolases with changes in their developmental patterns accompanying postnatal growth described in several reports [5,6].

The membrane consisting of a typical trilaminar unit is superimposed by a carbohydrate-rich surface coat [7], which has been variously viewed by electron microscopy as a layer of fine filaments radiating from the outer leaflet [8] or composed of 60-Å knob-like structures [9,10]. Parallel removal of these surface projections and solubilization of the digestive hydrolases by papain action has been reported [10—12]. The hydrolases which contain large amounts of carbohydrate [13—15] may be assumed on this basis to be located on the external membrane surface and to comprise a significant fraction of the surface coat. The membrane-bound hydrolases have also been solubilized by Triton X-100 [12,14,16—18], yielding catalytically similar products but of greater hydrophobicity than their papain-released counterparts. The available evidence would indicate that these enzymes like other membrane glycoproteins [19,20] are anchored to the lipid matrix by relatively small apolar segments from which they can be separated by proteolytic cleavage.

Lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23) and maltase (α-D-glucoside glucohydrolase, EC 3.2.1.20) constitute the predominant sugar hydrolases of infant rat intestine. The present report summarizes the results of a study concerning the surface arrangement of these enzymes on the intestinal brush border membrane of the suckling rat. The experimental approach involved obtaining lactase and maltase in high purity, the preparation of specific antiserum to each and determining the immunoreactive properties of these enzymes in their membrane-bound state.

Materials and Methods

Materials. Infant Wistar rats provided the source of enzymes and brush border membrane preparations. Antisera were produced in New Zealand rabbits. Agarose beads (Bio-Gel A-0.5 m0 and hydroxyapatite were purchased from Bio-Rad Labs., Glucostat from Worthington, papain Type I and α-lactose from Sigma Chemical Co., maltose and cellobiose from Calbiochem, soluble potato starch from J.T. Baker Chemical Co., Ampholine from LKB Labs., methanolic HCl and 3.8% SE-30 on 80/100 Gas-chrom Q from Applied Science Labs and Tri-Sil Z from Pierce Chem. Co.

Enzyme assays and units. Routine enzyme assays were performed at 37°C in 250 μl volume in 0.05 M sodium phosphate buffer at pH 6.0, 0.002% Triton X-100 and 0.15 M lactose or 0.0125 M maltose. Glucose released was determined by incubation for 30 min at 37°C with 250 μl glucostat reagent prepared in 0.5 M Tris at pH 7.0. Termination of lactase and maltase activity was effected by the added Tris. Units of enzyme refer to μmol substrate hydrolyzed/min and specific activity to units of enzyme/mg protein. Protein was estimated by the method of Lowry et al. [22] unless specified otherwise.

Enzyme preparations. Lactase and maltase (glucoamylase) were prepared

from 50 g of freshly excised small intestine from 17-day-old infant rats by modification of published procedures [23]. The whole intestines were homogenized in a Waring blender with 5 vols. of 0.025 M potassium citrate (buffer medium) at pH 6.0 and centrifuged for 1 h at 20 000 × *g*. The sediment was washed and resuspended to 100 ml volume in the buffer medium. Lactase and maltase were solubilized by incubating the resuspended sediment for 30 min at 37°C with 100 mg papain plus 2 mg cysteine-HCl and separated by centrifugation for 1 h at 20 000 × *g*. To the papain-solubilized enzyme solution was added room temperature acetone to 68% (v/v) and the precipitated enzymes sedimented by centrifugation for 20 min at 20 000 × *g*. The enzymes were dissolved in buffer medium and centrifuged to remove considerable acetone-denatured protein.

The enzyme solution was concentrated by ultrafiltration (UM-10 Amicon filter) to 5 ml volume and fractionated on a 2.5 × 100 cm column of agarose (Bio-Gel A-0.5 m) at a flow rate of 30 ml/h using 10 mM potassium phosphate buffer (pH 6.0) as the elution medium. Separation of a major peak of maltase activity at 240 ml elution volume preceded a peak of lactase activity at 295 ml volume. Coincident protein distribution within these enzyme peaks suggested a high degree of homogeneity. Whereas most of the maltase activity was well separated from lactase, considerable contamination of the lactase fractions by a secondary overlapping peak of maltase activity was present. The lactase and maltase peak fractions were combined, concentrated and fractionated further on a 1.7 × 12 cm column of hydroxyapatite. Enzyme solutions and hydroxyapatite were equilibrated with 10 mM potassium phosphate buffer. Maltase is not retained by hydroxyapatite under these conditions and was collected in the effluent and wash. Lactase is retained and was eluted as a sharp coincident peak of enzyme activity and protein by linear potassium phosphate gradient to 0.2 M. The lactase and maltase fractions were concentrated, dialyzed with 10 mM potassium phosphate buffer (pH 6.0) and represented the final isolated products.

Determination of enzyme properties. Amino acid composition of lactase and maltase was determined with a model D-500 Durrum Analyzer (AAA Labs.). Samples were hydrolyzed for 24, 48 and 72 h following performic acid oxidation. Molar ratios of amino acids were computed relative to cystine/2 (least amino acid present in each case), yielding estimates of minimal molecular weight units (see Table II). Sedimentation velocity experiments were performed in a Model E analytical ultracentrifuge by Dr. C. Chervenka (Beckman Instruments, Spinco Division) with standard sedimentation coefficients computed in the usual fashion. Estimates of molecular weights were obtained from distribution coefficients (K_D) on a column of Bio-Gel A-0.5 m, standardized by proteins of known molecular weight.

Electrophoresis was performed in acrylamide gel (7.5%) by the method of Davis [24]. The gels were stained with Coomasie Blue G for protein and with the periodate/Schiff reagent for carbohydrate [25]. Isoelectric focusing was performed in an LKB 8101 electrofocusing column using a mannitol density gradient (0—15%) containing 1% carrier Ampholyte (pH 4—6) and 0.01% Triton X-100.

Bound carbohydrates were determined by gas-liquid chromatography by

procedures described by Rheinhold [26], using methanolysis to liberate carbohydrate residues. Dried membrane and enzyme samples containing added mannitol as an internal standard and reference sugar standards were methanolyzed for 4 h at 65° C in 0.5 N HCl in anhydrous methanol and following re-*N*-acetylation and trimethylsilation (Tri Sil Z) were chromatographed on a 3.8% SE-30 on 80/100 Gas-Chrom Z column. Sum of carbohydrate units was computed in each case as a single polymer (i.e. minus equivalent mol of water).

Kinetic constants for lactase and maltase were derived from Lineweaver-Burk plots of initial rate measurements in the usual way. Inhibition constants (K_i) for Tris, imidazole and colchicine (which acts as competitive inhibitors where inhibition was found) were determined from slope changes induced at various concentrations of the inhibitor. All reactions were at 37° C in 50 mM sodium phosphate buffer at pH 6.0 and 0.002% Triton X-100.

Brush border membrane isolations. Membranes were isolated from infant (and adult) rat intestine by a modification of published procedures [3,27]. whole infant small intestine (or adult mucosal scrapings) was homogenized with 50 vols. 5 mM EDTA in 1 mM Tris *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.4 in a Waring blender at the lowest speed setting yielding an absence of visible large particles within a 20 s blending period. The homogenate was centrifuged at 2500 × *g* for 10 min and the sediment washed three times by gentle resuspension in homogenizing medium. Excessive membrane loss, measured as either lactase or sucrase loss (stable marker enzymes for infant and adult membrane), into the initial supernatant solution and washes occurred with over-homogenization. The washed, crude brush border fraction was suspended in 2.5 vols. of 100 mM mannitol in 1 mM Tris/Hepes at pH 7.4 and centrifuged at 20 000 × *g* for 20 min. The sediment was suspended in 2.5 vols. of the buffered mannitol solution and homogenized in a glass-teflon homogenizer at 1000 rev./min for 30 s to effect membrane release from the brush border. Solid $CaCl_2$ was added to 10 mM and the mixture centrifuged at 3000 × *g* for 10 min to sediment residual mitochondria, basolateral plasma membranes and endoplasmic reticulum [3]. Bursh border membranes were collected from the resultant supernatant solution by centrifuging at 25 000 × *g* for 30 min. The isolated membranes were dialyzed against 100 mM D-mannitol or water and either used directly or stored frozen at —20° C.

Preparation of papain and Triton X-100 membrane extracts. Membranes were digested with papain at 10/1 ratio of membrane protein to papain for 30 min at 37° C in 10 mM sodium phosphate at pH 6.0 plus 1 mM EDTA. Papain was activated with cysteine-HCl (1/50 ratio of activator by weight) and added in two equal portions at 0 and 15 min incubation. Membranes at 1.0 mg protein or less were extracted in 0.1% Triton X-100 containing 1 mM EDTA for 30 min at room temperature. Solubilized membrane components were separated in each case by centrifugation at 12 000 × *g* for 10 min.

Immunological procedures. Rabbit antisera were prepared by injecting into footpads 25 μg lactase (specific activity 23) and 100 μg maltase (specific activity 58) mixed with 0.5 ml complete Freund's adjuvant. The injections were repeated after 2 weeks and 3 weeks later the rabbits were bled by cardiac puncture. Antisera were stored at —70° C and dialyzed against saline prior to

use. Antisera were tested by gel diffusion [28] on slides prepared with 1% agarose in phosphate-buffered saline containing 0.1% Triton X-100.

Immunotitrations were performed at 250 μl volume in 1.5 ml plastic-capped centrifuge tubes for 18—24 h at near 0°C in 0.1% bovine serum albumin, 50 mM NaCl, 10 mM sodium phosphate at pH 6.0 and 0.1% Triton X-100 where indicated. Titration curves were obtained by reacting a constant 0.01 unit of enzyme with increasing amounts of antisera. In the case of the soluble enzyme form, titration curves were obtained by determining enzyme remaining in solution, after removal of the catalytically active immunprecipitate formed by centrifugation at 12 000 × *g* for 5 min. Titration curves for membrane bound enzyme forms were determined as follows. Amounts of membrane containing a constant 0.01 unit of bound enzyme were first reacted for 24 h at near 0°C with increasing amounts of its antiserum, following which the membranes were sedimented by centrifugation at 12 000 × *g* for 10 min. The supernatant solutions were transferred quantitatively to fresh tubes and excess antibody back-titered by reacting for 18—24 h at near 0°C with a constant amount (0.01 unit) of free enzyme, yielding a combined immunotitration curve for both free and bound enzyme. Membranes were preloaded with antibodies to lactase or maltase in certain experiments by incubating for 18 h at near 0°C with respective antiserum at three-fold excess. The titration curve for bound enzyme was obtained by difference between the combined and free enzyme curves.

Results

Enzyme isolation and properties

Purification summaries of infant rat intestinal lactase and maltase (glucoamylase) are compiled in Table I. Each of the enzymes appeared near homogeneous based on constant specific activity across their elution peaks accompanying column fractionation on agarose and on hydroxyapatite in the case of lactase. Additional evidence of purity was obtained by acrylamide gel electrophoresis (see Fig. 1) with lactase showing a single diffuse band and maltase a major and minor catalytically active bands. All bands showed coincident protein and carbohydrate staining. The isolated enzyme products were prepared at greater or equal specific activity to those obtained previously from infant rat intestine [23], with the added assurance of minimal contamination of one by the other.

Specific properties of infant lactase and maltase were determined and the information compiled in Table II for comparative and reference purposes. Their acidic nature is confirmed by their isoelectric points (p*I*) and their apparent size indicated from distribution coefficients on gel filtration and sedimentation coefficients by ultracentrifugation. Due to the recognized difficulty of obtaining reliable molecular weight estimates of these carbohydrate-rich proteins, minimal molecular weight units were computed from amino acid and carbohydrate analyses. Lactase contains 10% and maltase 19% carbohydrate by our analyses. Similar carbohydrate levels were reported for maltase of adult rat intestine by others [13,29]. From the molecular weight estimates by gel filtration, lactase would consist of 13 and maltase 24 minimal molecular weight units.

TABLE I

PURIFICATION OF LACTASE AND MALTASE (GLUCOAMYLASE) FROM 17-DAY-OLD INFANT RAT INTESTINE

Details relating to the purification procedures have been described in Materials and Methods. Homogenate was prepared from 50 g freshly excised small intestine.

Fraction	Volume (ml)	Protein (mg)	Lactase		Maltase	
			Units	Spec. act.	Units	Spec. act.
Homogenate	300	5900	400	0.07	320	0.05
Supernatant fraction	265	2900	15	—	130	—
Sedimented fraction	100	2600	360	0.14	150	0.06
Papainized extract	91	290	320	1.1	130	0.45
Acetone fraction	20	11	220	20	90	8.2
Agarose fractions:						
Lactase, conc.	2.0	7.0	154	22	5.8	—
Maltase, conc.	2.0	1.5	<0.1	—	75	50
Hydroxyapatite fractions:						
Lactase, conc. *	1.0	5.7	130	23	<0.1	—
Maltase, conc. **	1.0	1.2	<0.05	—	70	58

* Represents final isolated lactase, showing 330-fold purification, 33% recovery and contamination by less than 0.1 unit maltase.

** Represents final isolated maltase, showing 1160-fold purification, 22% recovery and contamination by less than 0.05 unit of lactase.

The isolated lactase degraded lactose, cellobiose specifically, *o*-nitrophenyl-D-galactopyranoside and phlorizin and was inhibited competitively by Tris, imidazole and colchicine. The isolated maltase hydrolyzed starch and maltose but not palatinose, dextran or isomaltose and was also inhibited competitively by Tris.

Brush border membranes

Information on the purification, gross composition and disaccharidase content of brush border membranes of infant rat intestine is compiled in Table III. Membranes were obtained at 26—35-fold purification (using lactase or sucrase as a stable membrane marker) from rats ranging in age from 5 days to adult by the procedures described. The purification factor achieved indicated a high degree of membrane purity based on established criteria [3,27]. Gross analyses indicated that as much as 25% of the infant membrane dry weight may consist of carbohydrate. The carbohydrate components present and average distribution by weight were: *N*-acetylglucosamine (32%), galactose (26%), *N*-acetylgalactosamine (21%), sialic acid (12%), mannose (4.0%), glucose (4.0%) and fucose (less than 1.0%). Lactase activity of the membrane showed about 1/10 of the specific activity (i.e. units enzyme/mg protein) of the pure enzyme. Assuming that lactase activity does not differ between free and membrane-bound enzyme forms, it is evident that lactase could comprise in excess of 10% and maltase 2.6% of the 10-day-old infant membrane protein. Since little difference in lactase and maltase activity is shown before and after their solubilization from the membrane by Triton X-100 (Table IIIC), catalytic

Fig. 1. Polyacrylamide gel electrophoresis of infant rat intestinal lactase (left) and maltase (right). Electrophoresis patterns were obtained with 50 μg lactase and 15 μg maltase. Duplicate gels showed identical staining areas for protein (shown) and carbohydrate (not shown).

activity of both free and bound enzyme forms has been considered to be similar. Apparent incomplete solubilization of lactase by papain may be attributed to its instability accompanying release.

Antisera specificity

Rabbit antisera prepared to lactase and maltase were tested against these enzymes in pure solution and in the presence of other membrane extractives solubilized by papain or Triton X-100. Immunodiffusion patterns obtained with the antilactase serum are shown in Fig. $2A_1$ and A_2 and with antimaltase serum in Fig. 2B. Antilactase serum showed a single common precipitin line with pure lactase and papain and Triton X-100 membrane extracts. A second precipitin line shown with the Triton X-100 extract (Fig. $2A_1$) was abolished following papain digestion of this extract (Fig. $2A_2$). Antimaltase serum showed only a single common precipitin line with pure maltase and papain and Triton X-100 membrane extracts. No reactivity was evident between antilactase serum and maltase and antimaltase serum and lactase. Although the antisera were obtained to enzymes prepared by papain digestion, precipitin

TABLE II

SUMMARY OF LACTASE AND MALTASE (GLUCOAMYLASE) PROPERTIES

Details relating to the determination have been summarized in Materials and Methods.

Properties	Lactase	Maltase
Physicochemical		
Sedimentation coefficient ($s_{20,w}$)	11.5 S	14.1 S
pI (apparent)	4.6	4.5
Mol. wt. by gel filtration	170 000	192 000
Minimal mol. wt. unit, — carbohydrate *	11 500	6 400
Carbohydrate **	1 300	1 474
Minimal mol. wt. unit, + carbohydrate	12 800	7 883
Kinetic		
pH optimum	5—6	6.5
K_m	15 mM (lastose)	1.1 mM (maltose) 3.2 % (starch)
V ***	28	58 (maltose) 25 (starch)
K_i (Tris)	4 mM	6.5 mM
K_i (imidazole)	1.8 mM	no inhibition
K_i (colchicine)	0.3 mM	no inhibition

* Based on amino acid residues relative to cystine/2.

** Based on sum of carbohydrate units (determined by gas-liquid chromatography) present/mg of enzyme protein. Sum of carbohydrate units were computed minus equivalent mol H_2O. Carbohydrate groups include: fucose (7.0, 22), xylose (5.6, 13), Man (44, 26), Gal (17, 63), Glc (5.2, 15), *N*-acetylglucosamine (44, 90) and *N*-acetylgalactosamine (1.8, 27). Numbers in parenthesis refer to μg/mg lactase and maltase, respectively.

*** Computed as μmol substrate converted (or glucose released in the case of starch)/min per mg enzyme protein.

lines of either papain or Triton X-100-extracted enzyme forms were indistinguishable. The secondary precipitin line in the Triton X-100 extract reacting with antilactase serum was also presumed to be lactase, convertible to a common lactase form by papain.

Immunotitration of soluble lactase and maltase

Quantitative relationships involving the immunoprecipitation of lactase and maltase from pure solution and from papain and Triton X-100 extracts of brush border membranes are shown by the immunotitration curves plotted in Figs. 3A and 4A. Equivalent precipitation of each of these enzymes was found per unit of antiserum added, whether alone in pure solution or in the presence of accompanying membrane extractives. Reactivity of the lactase and maltase antibodies with other surface membrane components could be excluded on this basis. Furthermore, an absence of other non-immunoreactive forms of these enzymes on the membrane was confirmed by the ability of each antiserum to effect precipitation of all lactase or maltase activity present in the membrane extracts.

Immunotitration of membrane-bound lactase and maltase

Antibody binding by lactase and maltase in their free state and while attached to isolated brush border membrane vesicles was determined from the

TABLE III

BRUSH BORDER MEMBRANE OF RAT INTESTINE

A. Purification. B. Gross chemical composition. C. Disaccharidase content and solubilization. Details relating to membrane purification, analyses and enzyme solubilization have been described in Materials and Methods. Units of enzyme were computed per g of original intestine protein. Specific activity refers to units of marker enzyme/mg protein.

A. Purification

Age (days)		Fraction	Membrane marker enzyme			
			Units	Spec. act.	Purification (-fold)	% recovery
15	(a)	original homogenate	66 (lactase)	0.07	(1.0)	(100)
	(b)	2nd homogenate	35 (lactase)	0.32	4.6	51
	(c)	B.B. membrane	14 (lactase)	2.1	30	21
5		B.B. membrane	25 (lactase)	2.5	27	27
10		B.B. membrane	15 (lactase)	2.4	34	21
17		B.B. membrane	14 (lactase)	2.7	35	24
19		B.B. membrane	2.1 (sucrase)	0.49	26	17
Adult		B.B. Membrane	24 (sucrase)	3.0	29	17

B. Gross composition *

n	As % of dry weight		
	Protein	Lipid	Carbohydrate
2	32 ± 1	43 ± 3	25 ± 2

C. Dissaccharidase content and solubilization

Enzyme	Spec. act. (units/mg protein)		% of membrane protein	% solubilized **	
	Membrane	Pure enzyme		Papain	Triton X-100
Lactase	2.4	23	10.4	82	96
Maltase	1.5	58	2.6	98	100

* Protein was determined by ammonia nitrogen, lipid by gravimetric analysis and carbohydrate by gas-liquid chromatography. Membrane preparations were from 10-day-old rats. *n*, number of determinations.

** Refers to percent of membrane-associated enzyme activity recovered in the extracts. Membrane preparation was from 17-day-old rats.

immunotitration curves shown in Figs. 3B and 4B. Titration curves are presented showing the relationship between antisera addition and precipitation of 0.01 unit of free lactase and maltase determined (a) alone and (b) in combination with an equivalent 0.01 unit of membrane-bound enzyme. The combined titration curves show the total amounts of antisera required to both saturate antigenic sites exposed by 0.01 unit of membrane-bound enzyme and to precipitate 0.01 unit of the free enzyme from solution (for experimental details see Materials and Methods). The difference between the titration curves obtained with the free enzyme alone and in combination with membrane-bound enzyme is shown by the broken line curves, which represent the titration curves for the

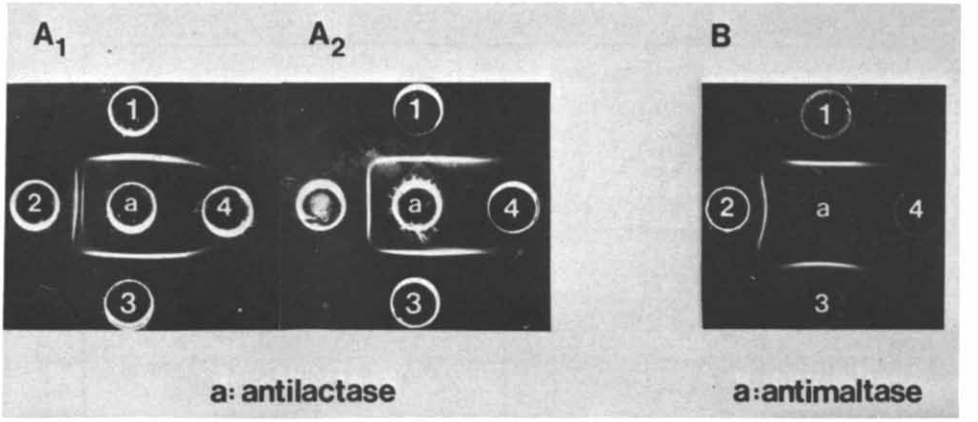

Fig. 2. Immunodiffusion patterns showing specificity of rabbit antilactase (A_1, A_2) and antimaltase (B) antisera determined in the presence of other solubilized membrane components. Center wells contained 5 μl antiserum and outer wells 10 μl of the following: 1, papainized membrane extract; 2, Triton X-100 membrane extract before (A_1 and B) and after (A_2) its papainization; 3, pure lactase (A_1 and A_2) or pure maltase (B); 4, pure maltase (A_1 and A_2) or pure lactase (B). Membranes were prepared from 25-day-old infant rats. Extracts and enzyme solutions contained between 0.05 and 0.1 unit of maltase and lactase activity/10 μl volume.

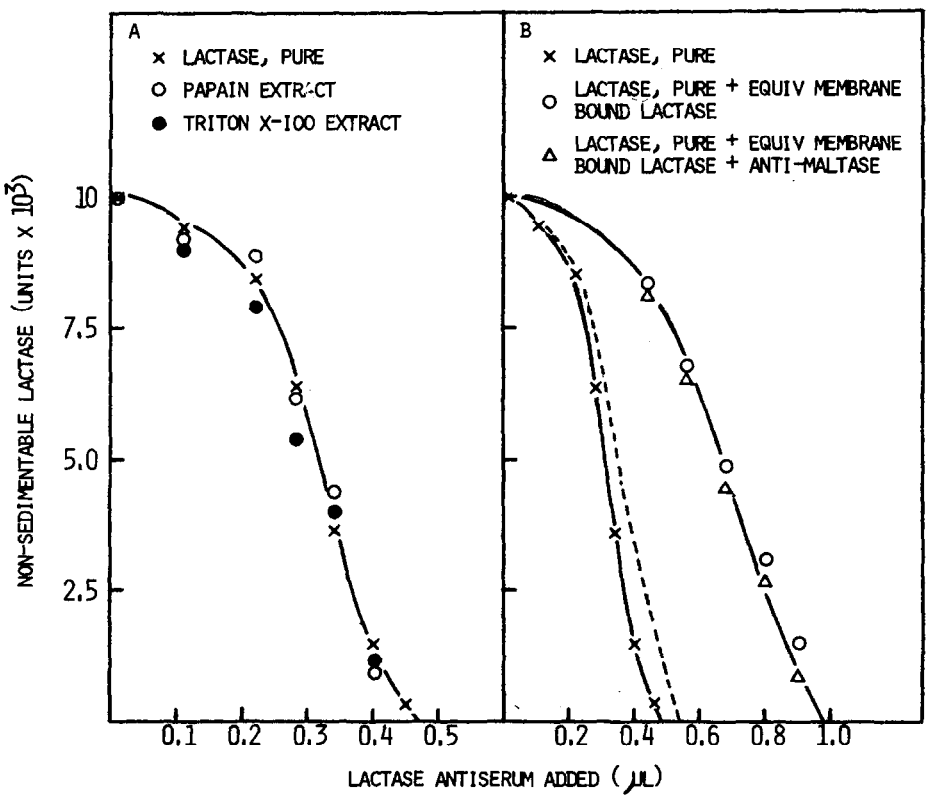

Fig. 3. Immunotitration curves of (A) free and (B) membrane-bound lactase. (A) Immunotitration curves are shown for free lactase present at equal activity (a) in pure solution, (b) in papain, and (c) Triton X-100 extracts of brush border membranes of 15-day-old infant rat (for details see Materials and Methodes). (B) Immunotitration curves are shown for (a) free lactase (0.01 unit), (b) membrane-bound plus free lactase (0.01 unit each), and (c) membrane-bound plus free lactase (0.01 unit each), determined with membranes preloaded with maltase antibody. ------, titration curve for membrane-bound lactase (obtained by difference). Intestinal brush border membrane vesicles were preapred from 15-day-old infant rats (for details see Materials and Methods).

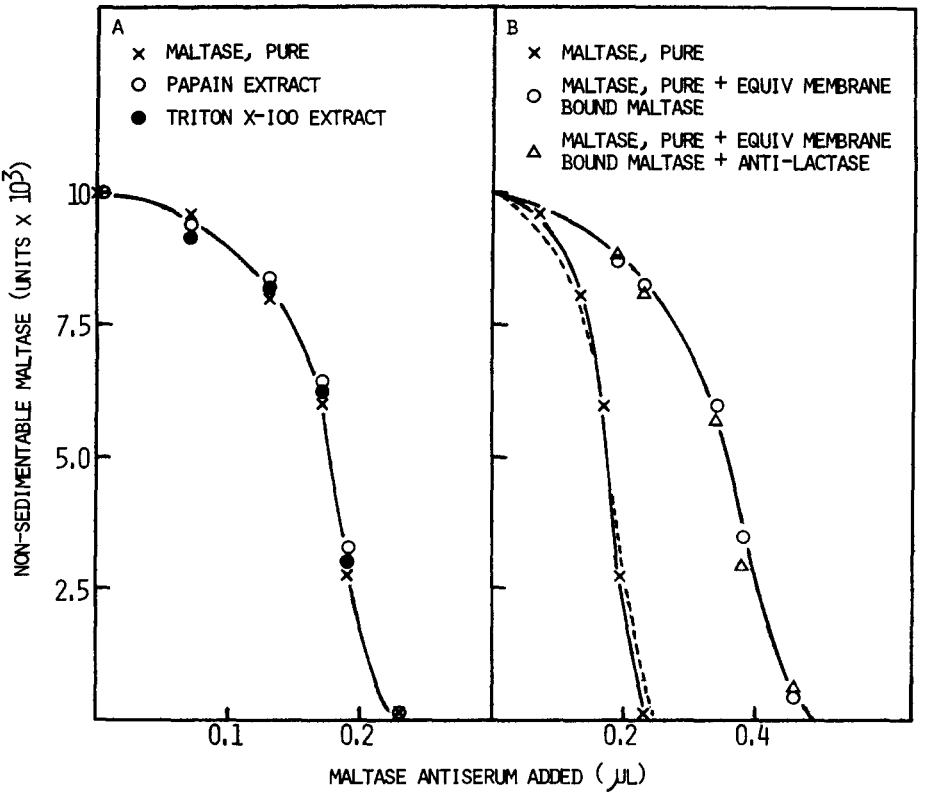

Fig. 4. Immunotitration curves of (A) free and (B) membrane-bound maltase. (A) Immunotitration curves are shown for free maltase present (a) in pure solution, (b) in papain, and (c) in Triton X-100 extracts of brush border membranes of the 15-day-old infant rat (for details see Materials and Methods). (B) Immunotitration curves are shown for (a) free maltase (0.01 unit), (b) membrane-bound plus free maltase (each at 0.01 unit), determined with membranes preloaded with lactase antibody. - - - - -, titration curve for membrane-bound maltase (obtained by difference). Intestinal brush border membrane vesicles were prepared from 15-day-old infant rats (for details see Materials and Methods.

membrane-bound enzymes. the titration curves of membrane-bound enzymes, determined in this manner did not differ significantly from the titration curves obtained with the free enzymes alone. Equivalent binding of antibody by membrane-bound and free forms of both lactase and maltase was indicated on this basis. These findings suggest that both lactase and maltase are placed independently on the external brush border membrane surface, involving a minimal attachment in each case, such that their antigenic sites are as accessible to antibody binding as those of their solubilized counterparts.

Further evidence of the structural independence of lactase and maltase in their arrangement on the external membrane surface is shown by the immunotitration curves included in Figs. 3B and 4B. In this study, membranes were preloaded with antibody to one of the enzymes and the effect on antibody binding by the second enzyme determined. Essentially identical immunotitration curves were obtained for membrane-bound lactase or maltase when determined in the absence (open circles) or presentce (triangles) of bound antibody

TABLE IV

LACK OF INTERFERENCE BY BOUND ANTIMALTASE ON LACTASE AND BOUND ANTILACTASE ON MALTASE EXTRACTION BY PAPAIN AND TRITON X-100 FROM THE BRUSH BORDER MEMBRANE OF THE INFANT RAT INTESTINE

Equivalent aliquots of membrane (20 μg membrane protein) were placed in two sets of tubes and incubated (a) alone, (b) with excess antimaltase (1.5 μl antiserum) and (c) with excess antilactase (1.5 μl antiserum) for 18 h at near 0°C in 250 μl medium containing 0.1% bovine serum albumin, 0.05 M NaCl and 10 mM sodium phosphate buffer, pH 6.0. Following incubation, one set of tubes was extracted by papain digestion and the other with 0.1% Triton X-100. The resulting extracts were assayed for lactase and maltase activity. Results are expressed as munits/20 μg membrane protein.

Membrane modification	Papain extract		Triton X-100 extract	
	Lactase	Maltase	Lactase	Maltase
(a) Membrane alone	35	28	50	27
(b) Membrane + bound antimaltase	36	1	53	2
(c) Membrane + bound antilactase	0.1	28	2	28

to the second enzyme as shown. These findings would indicate that the lactase and maltase molecules are spatially separated on the membrane to the extent that the immunoreactivity of one is not impaired by attaching antibodies to the other.

Extraction properties of lactase and maltase from membranes containing bound antibodies

Brush border membranes were separately reacted with excess antiserum to lactase and to maltase and the effect on the extraction properties of these enzymes determined. As shown in Table IV, preloading of membranes with antibody to one enzyme clearly prevented its extraction in soluble form, but had little effect on the extractability of the other enzyme by either papain or Triton X-100. It is evident that the presence of bound antibody on one of the enzymes did not prevent Triton X-100 extraction or interfere sterically with papain cleavage of the other. These findings are consistent with those presented previously indicating that lactase and maltase are arranged independent of each other on the external membrane surface. Whether the antibody-complexed enzyme remained immobilized on the membrane or was detached in insoluble form by the extractants was not determined further. These studies also confirm the specificity of the antisera preparations relative to the enzyme to which the antiserum was prepared.

Discussion

Among the well-studied postnatal developmental changes of mammalian intestine are those involving the sugar hydrolases. Lactase is maintained at elevated activity during the suckling period of high dietary lactose, falling to low levels after weaning in the rat [30,31]. In contrast, other sugar hydrolases are either absent or in the case of maltase present at low activity during suckling with increases in activity occurring with weaning [31]. These enzymic activity changes in the developing intestine have been shown [5,6] to reflect

corresponding changes in amounts of the enzyme protein bound to the brush border membrane. Since lactase and maltase undergo reciprocal changes, it was of interest to examine their structural relationship to each other and the developing membrane.

In these studies we have prepared lactase and maltase in high purity from the infant intestine, determined their specific kinetic and molecular properties, obtained highly specific antibodies to each and applied the antisera in immunological studies designed to determine the immunoreactivity of the membrane-bound forms of these enzymes. The enzymes were isolated by simplified procedures providing good recoveries and minimal contamination of one by the other. Contamination of a lactase preparation by as little as 2—3% maltase produced an unsatisfactory antiserum showing an excessive antimaltase titer. Amino acid profiles of lactase and maltase were remarkably similar. Maximal differences of 1.5—2-fold greater amounts of cystine, methionine and threonine were found in maltase. Both enzymes contained high levels of the acidic amino acids, consistent with their low isoelectric points. Lactase contained 10% and maltase 19% bound carbohydrate, consisting of the same sugars attached in dissimilar proportions to each. From the amino acid and carbohydrate analyses, minimal mol. wt. units of 12 800 for lactase and 7900 for maltase were obtained and are presented for comparative reference purposes. We have also isolated maltase from adult rat intestine and found it to be essentially identical to the enzyme from infant intestine in amino acid and carbohydrate composition as well as all other properties studied. Our maltase preparations showed comparable purity, carbohydrate content, p*I* and $S_{20,w}$ values with those presented in a recent report [29] on this enzyme.

Brush border membranes were prepared in consistent good yield and purity by the procedures described. The membrane of the infant intestine contained about twice the lipid and half the protein (per unit dry weight) of the mature adult intestine, consistent with their lesser density observed by others [5,6]. The catalytic activity of lactase and maltase did not differ whether these enzymes were attached to the membrane or released in soluble form. On this basis, it may be assumed that these enzymes are positioned on the membrane with catalytic sites exposed fully and functionally unimpaired.

Lactase and maltase were present on the infant membrane at 1/10 and 1/40 of their respective specific activities in pure form, suggesting that they may constitute corresponding fractions of the total membrane protein. An arrangement suitable for accommodating such quantities of enzyme proteins (i.e. 12.5% of the membrane protein) over a limited surface area and providing for maximal catalytic exposure of each, would have the molecules positioned independently with catalytic sites extending free beyond the plane of the lipid bilayer into the luminal cavity. That this may be the case is supported from various lines of evidence. That they and other digestive hydrolases are localized to the external membrane surface and may project beyond the plane of the lipid bilayer is indicated by electron micrographs which show structural projections on this surface which disappear with papain digestion in parallel with the solubilization of these enzymes [10—12]. Since the catalytic activities of lactase and maltase do not differ between their membrane-bound forms and solubilized intact (by Triton X-100) and partially degraded (by papain) forms,

that portion of their molecules binding to the membrane (i.e. hydrophobic anchors) would not appear to be involved in their hydrolytic function.

Further insight regarding the arrangement of lactase and maltase in relation to each other and the external surface membrane is provided by our immunological studies. In these studies, antisera of high specificity for lactase and maltase were applied to determine the immunoreactivity of these enzymes in their bound state on isolated brush border membrane vesicles. Membrane vesicles were found to bind antilactase and antimaltase antibodies in amounts equivalent to that required to precipitate comparable amounts of the free enzymes from solution. Since precipitation of these enzymes from either pure solution or from crude membrane extracts (papain or Triton X-100) required equivalent amounts of antisera, antibody binding by the membrane vesicles could be assumed to be highly selective involving only the enzymes to which they were directed. That the membrane-bound and soluble forms of lactase and maltase react with equivalent amounts of their antibodies is presented as evidence for the arrangement of each on the membrane surface in a maximal immunoreactive form. This and other evidence also indicate that the enzyme molecules are positioned on the membrane surface in a spatially independent manner to the extent that the presence of bound antibody on one molecule does not interfere sterically with further antibody binding by adjacently placed lactase or maltase molecules. That lactase and maltase are attached on the membrane in structural independence of each other is verified further by the inability to demonstrate in situ an effect of bound antibody to one on the catalytic activity, the antibody-binding capacity or the extractability (by papain or Triton X-100) of the other.

Evidence has been reported concerning the arrangement of other digestive hydrolases on the microvillus membrane. Using immuno-electron microscopic observations with labeled [32] and unlabeled [33] antibodies to sucrase-isomaltase, it has been concluded that this enzyme complex protrudes 12—15 nm from the outer membrane surface. Amino peptidase has been estimated by similar technique to extend about 10 nm beyond the outer membrane [34]. That most of the aminopeptidase surface emerges from the bilayer was confirmed by the availability of all but a few antigenic determinants for antibody binding [35]. Furthermore, it has been shown that aminopeptidase is a transmembrane protein with exposure at the inner surface membrane [36]. Whether lactase and maltase similarly span the lipid bilayer remains to be determined.

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