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ENZYMES OF THE HUMAN INTESTINAL BRUSH BORDER MEMBRANE

IDENTIFICATION AFTER GEL ELECTROPHORETIC SEPARATION*

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SUMMARY

The position of a number of human intestine brush border membrane enzyme activities in polyacrylamide gels after electrophoresis has been determined. These activities are, in order from the origin, maltase/glucoamylase, lactase/phlorizin hydrolase, maltase/sucrase/isomaltase, enteropeptidase, trehalase and γ-glutamyltransferase. Leucylnaphthylamide hydrolyzing activity was inactivated by sodium dodecylsulfate and its position was not determined. The positions of the activities have been correlated with the positions of protein bands previously determined. One such band situated between enteropeptidase and alkaline phosphatase has not been identified.

INTRODUCTION

We have previously described methods for the preparation of purified human intestinal brush border membranes [1] and their solubilization and fractionation by gel electrophoresis [2]. The current paper describes the results of our efforts to identify enzyme activities associated with the specific bands obtained by gel electrophoresis. The activities studied include maltase (α-D-glucoside glucohydrolase, EC 3.2.1.20), glucoamylase (exo-1,4-α-glucosidase, EC 3.2.1.3), lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23), phlorizin hydrolase (phlorizin glucohydrolase, EC 3.2.1.62), sucrase (sucrose α-glucohydrolase, EC 3.2.1.48), isomaltase (oligo-1,6-glucosidase, EC 3.2.1.10), trehalase (α,α-trehalase, EC 3.2.1.28), enteropeptidase (EC 3.4.21.9), alkaline phosphatase (EC 3.1.3.1), γ-glutamyltransferase (EC 2.3.2.2) and leucylnaphthylamide-hydrolyzing activity.

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MATERIALS AND METHODS

Chemicals

Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate and *N,N,N',N'*-tetramethylenediamine were from Eastman Kodak Co. Sodium dodecylsulfate was from Mallinckrodt. Coomassie brilliant blue and *o*-dianisidine were from Schwartz-Mann. L-γ-Glutamyl-*p*-nitroanilide, β-naphthylamine, ammonium sulfamate, sodium nitrite, *N*-1-naphthylethylenediamine dihydrochloride, L-leucyl-β-naphthylamide, horseradish peroxidase, Tris, bovine serum albumin, and Triton X-100 were from Sigma Chemical Co. Amylogen was from Merck and Co. Disaccharides and phlorizin were from the same commercial sources previously noted [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 15 patients. 11 jejunal and 4 ileal specimens are included in this study.

Membrane preparation

Brush border fragments (Fraction P_2) and microvillus membranes (Fraction F_{II}) were prepared and characterized as previously described [1]. The fractions were suspended in distilled water and dry sodium dodecylsulfate or concentrated Triton X-100 was added, immediately prior to electrophoresis, to achieve the desired detergent concentration.

Acrylamide gel electrophoresis

Electrophoresis was performed as previously described [2] except that the lower reservoir buffer was kept at 4 °C. 50 μg of protein were generally applied. Immediately after completion of the electrophoresis the gels were removed and the migration front of the gels used for the enzyme localization was measured. Duplicate gels used to visualize the protein bands were treated as previously described [2]. The relative mobility was calculated knowing the position of the slices or of the protein band relative to the bromophenol blue front. Molecular weights were calculated from the migration of markers of known molecular weights [2].

Assays

The principles laid down by Gabriel for identification of electrophoretically separated bands by biochemical assay were followed [4]. 1-mm slices of gel were taken by hand and placed in a grinding tube containing 0.5 ml of cold distilled water or an appropriate buffer. After homogenization and centrifugation at 3000 rev./min during 10 min the supernatant and the pellet obtained were assayed for various enzymic activities. 90 % of the recovered enzyme remained in the supernatant. Disaccharidase activities were assayed according to a modification by Lloyd and Whelan [5] of Dahlqvist's method [6] except that the substrate concentration used was 0.1 M. Leucylnaphthylamide-hydrolyzing activity and alkaline phosphatase activity were assayed according to Goldbarg and Rutenburg [7] and Eichholz [8], respectively.

Phlorizin hydrolase activity was assayed as suggested by Malathi and Crane [3]. γ-Glutamyltransferase activity was assayed according to Naftalin et al. [9]. Glucoamylase activity was assayed at 37 °C at pH 6 in 0.05 M sodium maleate buffer using amylogen as the substrate [10]. Enteropeptidase activity was assayed as previously described [11].

Protein was assayed according to Lowry et al. using crystalline bovine serum albumin as standard [12].

Studies of the effects of sodium dodecylsulfate and Triton X-100 on the enzyme activities of the intestinal brush border membrane were carried out on brush border fragments (0.42 mg brush border protein per ml) rather than purified microvillus membranes. Detergent-treated and untreated fragments were kept at 4 °C. Activities of the detergent-treated fragments were calculated as percentages of the activities found in the untreated.

Localization of enzyme activities was studied with 30 gels. 15 of the gels were prepared from brush border fragments; 15 were prepared from purified membranes. The segments which were positive for enzyme activity were identified with protein bands by comparison with duplicate gels stained for protein.

RESULTS

Solubilization with sodium dodecylsulfate

As in our previous studies [2] solubilization of membranes was obtained with 2 % sodium dodecylsulfate. The sample was then diluted to 1 % sodium dodecylsulfate and by subsequent gel electrophoresis in a discontinuous system containing 0.1 % sodium dodecylsulfate in the upper electrophoresis buffer only, it was possible to fractionate the brush border membrane into its constituents. 23 bands, at least, were found corresponding to a heterogeneous group of polypeptides of molecular weight ranging from 25 000 to over 400 000. Jejunal and ileal brush border fragments and brush border membrane showed differences in banding patterns but these differences were not in that region of the gel in which the enzyme activities under study are to be found.

The distribution of maltase, sucrase, and alkaline phosphatase activities in gels obtained from jejunal brush border membranes is shown in Fig. 1. In this experiment 42 and 38 %, respectively, of the alkaline phosphatase and sucrase activities applied were recovered from the gels. Two peaks of maltase activity were found centered at positions corresponding to molecular weights of 440 000 and 250 000. Sucrase and isomaltase activities were exclusively associated with the faster-migrating maltase band. Alkaline phosphatase activity seemed to correspond to a single band. By similar means, the bands corresponding to maltase/glucoamylase, lactase/phlorizin hydrolase, maltase/sucrase/isomaltase, enteropeptidase, alkaline phosphatase, and trehalase have been identified (see Table I). Glucoamylase activity is associated with the heaviest maltase. Lactase activity is associated with phlorizin hydrolase and is found in between the two maltases. Enteropeptidase, alkaline phosphatase and trehalase activities migrate further down the gel. Not indicated is one major unidentified band in the high-molecular-weight region migrating between enteropeptidase and alkaline phosphatase. In Table I, relative mobility and molecular weight are assigned on the basis of the location of enzymic activity. Alkaline phosphatase activity showed an

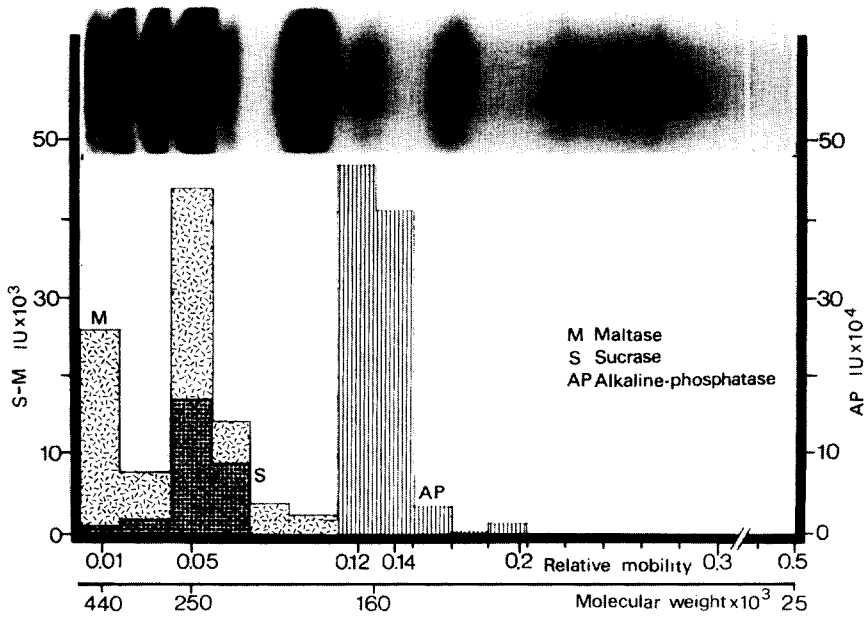

Fig. 1. Polyacrylamide gel electrophoretic pattern of purified jejunal microvillus membrane. The sample (50 μg of protein) was solubilized in 2 % sodium dodecylsulfate, diluted and immediately placed on the gel. Eight different gels were run simultaneously. Two gels were stained for protein. The other gels were immediately sliced into 1-mm fractions and used for maltase (M) sucrase (S) and alkaline phosphatase (AP) activity estimations. The position of the bands after protein staining is indicated in the upper part of the diagram. The histograms in the lower part indicate the enzyme activity. Relative mobility and molecular weight were calculated as described in Methods. Enzyme activities are expressed in international units.

average apparent molecular weight of 160 000 (allowing ± 20 000 variance). When the gel extract having only alkaline phosphatase activity was run on another gel and subsequently stained for protein a single band was obtained with an apparent molecular weight corresponding to 150 000. A similar re-electrophoresis was done with a gel extract having sucrase and isomaltase activities. The sucrase · isomaltase complex showed an apparent molecular weight of 250 000.

Attempts were made to dissociate the sucrase · isomaltase complex with β-mercaptoethanol but no observable subunits were formed although the enzymic activity in the 250 000 region decreased with β-mercaptoethanol concentration (100 %, 80 % and 50 % recovery at 0.1, 1 and 10 % β-mercaptoethanol, respectively).

After solubilization with 2 % sodium dodecylsulfate, not all samples (17 out of 22) showed lactase activity, a smaller proportion (9 out of 14) showed trehalase activity, and only one trial out of six showed γ-glutamyltransferase activity. Leucyl-naphthylamide-hydrolyzing activity was always inactivated and the band corresponding to this activity has not been identified.

Enzyme inactivation as a function of sodium dodecylsulfate concentration

The fact that some brush border membrane enzyme activities are preserved while others are inactivated during detergent solubilization suggested the possibility

TABLE I

RELATIVE MOBILITY AND CALCULATED MOLECULAR WEIGHT ASSOCIATED WITH ENZYME ACTIVITIES IN GELS AFTER ELECTROPHORESIS OF SODIUM DODECYL-SULFATE-SOLUBILIZED MEMBRANES

Membranes were solubilized in 2 % sodium dodecylsulfate. The solution was diluted with water to 1 % sodium dodecylsulfate before being submitted to electrophoresis as described in Methods. Relative mobilities and molecular weights were assigned as described in methods based on data previously published [2]. Total number of gels assayed is given in parentheses if different from the number of positive enzyme identifications.

Enzyme	Relative mobility (mean ± S.E.)	Number of determinations	Molecular weight
Maltase	0.015 ± 0.000	22	440 000 ± 20 000
Glucoamylase		6	
Lactase	0.030 ± 0.000	17 (22)	380 000 ± 40 000
Phlorizin hydrolase	0.050 ± 0.003	4	320 000 ± 30 000
Sucrase	0.068 ± 0.004	16	260 000 ± 30 000
Maltase	0.072 ± 0.003	20	250 000 ± 20 000
Isomaltase	0.089 ± 0.012	6	230 000 ± 20 000
Enteropeptidase	0.091 ± 0.006	7	220 000 ± 30 000
Alkaline phosphatase	0.144 ± 0.008	16	160 000 ± 20 000
Trehalase	0.201 ± 0.025	9 (14)	120 000 ± 20 000
γ-Glutamyltransferase	0.284	1 (6)	80 000

that conditions other than those routinely used might be found in which at least enough leucylnaphthylamide-hydrolyzing activity for band identification would survive the necessary procedures.

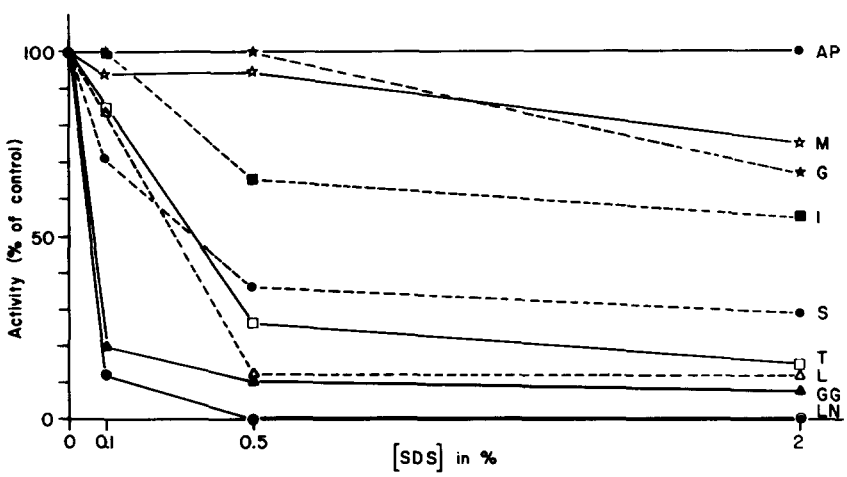

Fig. 2. The effect of sodium dodecylsulfate on brush border enzyme activities. Brush border fragments were suspended in cold distilled water. Aliquots were treated with sodium dodecylsulfate (SDS) at the concentration shown in the figure and enzymic assays performed immediately after the addition of the detergent. The enzyme activities in the detergent-treated fraction were compared with that of the same amount of the untreated preparation. Each point in the figure represents the mean of six determinations. AP, alkaline phosphatase; M, maltase; G, glucoamylase; I, isomaltase, S, sucrase, T, trehalase; L, lactase; GG, γ-glutamyltransferase; LN, leucylnaphthylamide-hydrolyzing activity.

Accordingly we studied the immediate effects of graded levels of sodium dodecylsulfate on enzyme activities. The results are shown in Fig. 2. 0.5 % and 2 % sodium dodecylsulfate gave similar results although enzyme activities were somewhat lower after treatment with the latter concentration. γ-Glutamyltransferase, trehalase and lactase activities were greatly reduced but enough apparently still remained for band identification, though not every time (see Table I). Leucylnaphthylamide-hydrolyzing activity was completely inactivated at both concentrations. Some leucylnaphthylamide-hydrolyzing activity survived treatment with 0.1 % sodium dodecylsulfate and other enzymic activities were correspondingly higher.

Membrane solubilization appears to be complete with 0.5 % as well as 2 % sodium dodecylsulfate and the same gel patterns are obtained. Membrane solubilization is not complete with 0.1 % sodium dodecylsulfate and this concentration gives an incomplete gel pattern. Consequently, we did not use this concentration for band identification, despite the partial survival of leucylnaphthylamide-hydrolyzing activity.

Enzyme inactivation as a function of time of incubation with sodium dodecylsulfate

The results on enzyme activities of incubation of membrane fragments with 0.5 % sodium dodecylsulfate for various time periods is shown in Fig. 3. Immediately following sodium dodecylsulfate treatment alkaline phosphatase and maltase activities were scarcely affected whereas sucrase, trehalase and γ-glutamyltransferase activities were greatly reduced. Leucylnaphthylamide-hydrolyzing activity was completely inactivated. Prolonged exposure of the brush border fragments to 0.5 % sodium dodecylsulfate brought about further slow decreases of enzyme activities. The fact that the 10 % γ-glutamyltransferase activity remaining immediately after solubilization in 0.5 % sodium dodecylsulfate was reduced to 4 % and 0 % after 2 and 24 h, respectively, would seem to explain why γ-glutamyltransferase was usually not detectable. In the normal course of the work, gel extracts are obtained not earlier than 2 h after sodium dodecylsulfate solubilization. Recoveries of enzyme activities from the

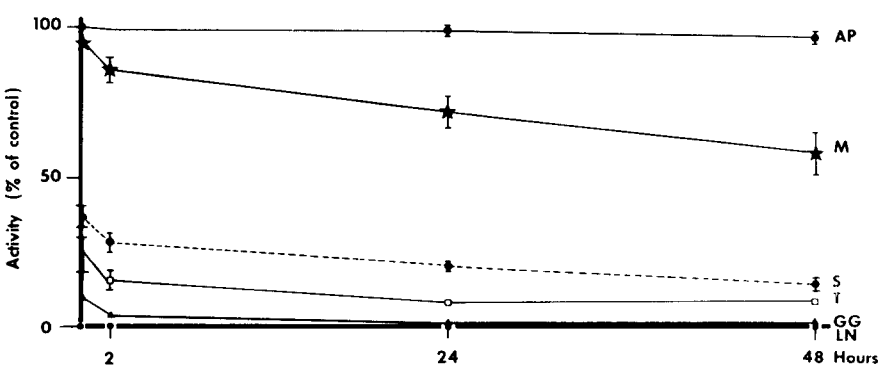

Fig. 3. The effect of 0.5 % sodium dodecylsulfate on brush border enzyme activities. Brush border fragments were incubated at 4 °C, with and without 0.5 % sodium dodecylsulfate. Aliquots of the incubation medium were removed for analysis of brush border enzyme activities immediately and at 2, 24 and 48 h as explained in the text. Enzymic determinations and abbreviations are the same as for Fig. 2.

gel, moreover, are only about 50 % in the best circumstances. Similar results to the above were obtained after solubilization of the brush border fragments with 0.1 % and 2 % sodium dodecylsulfate.

Solubilization with 1 % Triton X-100

Enzyme activities after incubation of brush border fragments with 1 % Triton X-100 for various time periods were measured. Immediately after solubilization with 1 % Triton X-100, almost 90 % of all enzyme activities was recovered and at least 70 % of all activities remained 24 h later. Phlorizin hydrolase and glucoamylase were slightly activated. Trehalase, alkaline phosphatase and isomaltase were not affected. Lactase, sucrase, leucylnaphthylamide-hydrolyzing activity, maltase, and γ-glutamyltransferase were slightly reduced. Solubilization of brush border fragments and microvillus membranes with 1 % Triton X-100, and subsequent polyacrylamide gel electrophoresis in the presence of 0.1 % Triton X-100 allowed the visualization of five to six major protein bands in the gel patterns. Five of these bands were located near the point of application of the original sample; the other migrated further down the gels. Studies of the enzyme activities on duplicate gels showed that γ-glutamyltransferase, leucylnapthylamide-hydrolyzing activity, sucrase, alkaline phosphatase, maltase and lactase activities were localized in a single band at the top of the gel, while trehalase migrated further. Triton X-100 solubilization is being studied further but as currently used is not useful for protein banding.

DISCUSSION

Solubilization and electrophoresis of membrane-bound enzymes with detergents often brings about inactivation [13, 14] although some membrane-bound enzymes are not affected and others are stimulated by detergent treatment [15]. Rat liver plasma membrane has been extensively studied in this regard [13–16]. Similar data on the intestinal brush border membrane are few [17].

Results reported in this paper reveal marked differences in the response of the intestinal brush border enzymes to sodium dodecylsulfate and Triton X-100 and it is clear that the conditions required for optimal solubilization are not necessarily the same as those needed for the preservation of the enzyme activities. Sodium dodecylsulfate gives excellent solubilization of brush border membrane but inactivates some enzymes. Triton X-100 does not cause important inactivation but its solubilization of the membrane is poor. In spite of these difficulties, we were able to identify most of the high-molecular-weight bands of the electrophoretic patterns of human intestinal brush border membrane in line with similar results reported for hamster and rat brush borders [17–19]. The reason for our success is that enzymes bound to intestinal brush border membranes seem to be more resistant to sodium dodecylsulfate treatment than are enzymes bound to other membranes. However, the intestinal membrane-bound enzymes are not uniform in their response to sodium dodecylsulfate or Triton treatment and their individuality, in this regard, as well as the overall difference between brush border and other membranes, may be related to the degree of dependence of an individual membrane enzyme upon lipids for its stability and/or function [15, 20].

Previous papers have reported the solubilization and isolation of the brush

border membrane sucrase/isomaltase complex from rat and rabbit small intestine [21-23]. The rabbit complex (molecular weight of 220 000) has been dissociated into two subunits similar in size but having only one or the other enzyme activity [24]. There is also a preliminary report that the human complex separates into the two enzymic moieties when treated with urea/mercaptoethanol [25]. In our study, dissociation of the complex was not observed at any concentration of mercaptoethanol used. Also alkaline electrophoretic systems, particularly those containing Tris, are reported to dissociate the rat sucrase/isomaltase complex [10]. This also seems not be the case for the human complex. In our study, electrophoresis was performed using a multiphasic borate/sulfate system with a running pH of 9.50 in gels containing 0.41 M Tris. Under these conditions the human sucrase/isomaltase complex was not split. On the contrary, the only band on the gel having sucrase and isomaltase activities was clearly defined and centered at the position corresponding to a molecular weight of 230 000. We did not test whether increasing the sodium dodecylsulfate concentration or the incubation time may have led to dissociation of the complex. Since Tris is a fully competitive inhibitor of the disaccharidases [26] the substrate concentration of the disaccharides used was increased to 0.1 M to reverse this inhibitory action.

In rat, hamster and man, lactase and phlorizin hydrolase are bound together into an oligo-enzyme complex [27, 28] but they have different stabilities towards heat. The present results confirm these earlier findings. Lactase and phlorizin hydrolase activities are found in the same band and they have different stabilities to sodium dodecylsulfate and Triton treatment.

Human glucoamylase has maltase and isomaltase but no sucrase activity [29, 30]. Our findings agree well since glucoamylase activity is localized in the same band as the heat-stable maltase, i.e. the band corresponding to a molecular weight of 440 000. On the other hand, heat inactivation studies have shown that amylase and maltase activities decrease at the same rate at a given temperature [21, 29] but in our experience these activities seem to have different sensitivities to sodium dodecylsulfate or Triton treatment.

The molecular weights assigned to the enzyme bands in the present work were obtained on the basis of the curve previously described [2] and their reliability depends on the adequacy of the reference proteins. It must be kept in mind that most of the brush border enzymes are glycoproteins whose molecular weights cannot be accurately ascertained from the gels because of the large proportion of carbohydrate they carry [31]. However, the molecular weight for the heaviest maltase agrees well with the value found in the rat [32], the molecular weight found for enteropeptidase agrees well with the finding of Baratti et al. [33] and the molecular weight found for alkaline phosphatase agrees with the values previously found [34].

The separation procedure which was the basis of this study may well serve as a starting point for characterizing the function and properties of the proteins of the intestinal brush border membrane. The procedures employed are reasonably mild and preserve some biological activities. The systematic evaluation of the bands of the electrophoretic patterns may yield information of value in the purification and the study of membrane enzymes in normal individuals and may allow the identification of the molecular basis of some brush border membrane diseases. For example, using modifications of the electrophoretic conditions described in this paper, it has been possible to study the membrane proteins of peroral biopsies of sucrase

isomaltase-deficient individuals. It was found that the band corresponding to this complex was missing [35].

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