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STUDIES ON THE ORGANIZATION OF THE BRUSH BORDER IN INTESTINAL EPITHELIAL CELLS

V. SUBFRACTIONATION OF ENZYMATIC ACTIVITIES OF THE MICROVILLUS MEMBRANE

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SUMMARY

Isolated brush border membranes of the intestinal epithelial cell have been partially digested by treatment with low concentrations of papain. A multienzyme particle containing maltase, sucrase and isomaltase activities is released. Further treatment with papain releases leucyl naphthylamidase activity in particulate form. Trehalase activity remains with the residue of the membrane. The disaccharidase-containing particles can be separated from those containing leucyl naphthylamidase on Sephadex G-200 columns. Alkaline phosphate activity is rapidly destroyed by papain treatment. Leucyl glycine-splitting activity can be separated from leucyl naphthylamide-splitting activity by papain treatment. Thus there is now evidence for two brush-border enzymes involved in the digestion of peptides.

The association of membrane enzymes with particles containing multiple activity is in accord with the view of a mosaic substructure of the membrane.

INTRODUCTION

Starting with brush borders isolated from intestinal epithelial cells, we have separated, in substantially pure form, the membranes of the microvilli[1-3]. These membranes appear to carry out the transport activities of the cell[4]. They also contain a number of hydrolytic and digestive enzymes such as alkaline phosphatase (EC 3.1.3.1), ATPase (EC 3.6.1.3) and leucyl naphthylamidase (EC 3.4.1.1), as well as the activities of several disaccharidases[3]. Recently cholesteryl and retinyl ester hydrolases were also found to be present in the membrane fraction of the brush border[5,6]. In view of the presence of such a diversity of activities within this membrane, a unique possibility is offered for deciphering its structural and molecular arrangement. It has been previously proposed[4] that the various enzymes within the membrane are arranged, as in a mosaic, into discrete morphochemical subunits and are not diffusely distributed throughout the protein portion of the membrane matrix. It seemed possible that isolation of the enzymatic activities in a particulate state as multienzyme complexes would help to reveal the functional organization of the total structure.

We have now obtained such a multienzyme subfractionation of the isolated membrane by use of controlled papain (EC 3.4.4.10) digestion. The results appear to support the concept of a specific localization of membrane enzymes into mosaic-like arrangement. Preliminary results have been previously reported[7].

METHODS AND MATERIALS

Isolation of brush border membranes

As previously reported[1], microvillus membranes were obtained from hamster intestinal brush borders isolated by a modification[3] of the procedure of MILLER AND CRANE. Briefly, isolated brush borders were disrupted with 1 M Tris at 0° and various fractions were separated on a 20–60% discontinuous glycerol gradient containing 0.05 M $MgCl_2$. Fraction C, the membrane fraction, was collected and diluted 3–4-fold with cold distilled water and subjected to centrifugation at 39000 × *g* for 30 min. After a second wash in cold water, the membrane fraction was resuspended in a small volume and its protein content determined.

Papain treatment of isolated membranes

Treatment with soluble papain

The papain used for membrane fractionation was obtained from the Sigma Chemical Co. It was their twice recrystallized product, suspended in sodium acetate. Before use, the papain was activated with 0.1 M cysteine in the presence of 0.05 M EDTA at pH 6.2. It was subsequently diluted with an EDTA solution to make a papain solution of 10 μg/ml in 0.005 M EDTA and 0.001 M cysteine. The incubation volume for papain digestion was set at 3 times the volume of the membrane fraction used in the experiment.

To the washed membrane fraction, enough cysteine and K_2HPO_4 (brought to pH 7.0) were added to make their respective concentration 2.5 and 12 mM after addition of papain. The mixture was then brought to room temperature and enough papain solution was added to keep the papain to membrane protein ratio 1:100. The final pH of the mixture was between 6.9 and 7.0. After incubation the mixture was immediately cooled in a $CaCl_2$–ice mixture and subjected to centrifugation at 12300 × *g* for 30 min at 4°. This force has been established as the minimal necessary to sediment all of the enzymatic activity in an untreated sample.

Treatment with papain complexed with cellulose

In order to stop the reaction rapidly by removal of papain from the reaction mixture we have complexed the papain with cellulose. The isolated membrane fraction containing 0.8 mg protein was treated with the complexed papain containing 0.808 unit activity in the presence of 12 mM K_2HPO_4, 2.5 mM cysteine in 5 mM EDTA. Total volume of the reaction mixture was 9 ml and the pH was 6.5. The incubation was carried out in a capped tube which was shaken in a horizontal position at 37°.

To remove the complexed papain, the reaction mixture was subjected to 10-min centrifugation in the International centrifuge at 800 × *g*. The supernatant was re-centrifuged in the Servall angle rotor at 19200 × *g* for 45 min to separate the unreacted membrane from the released particles.

Preparation of the cellulose–papain complex

In order to render the papain insoluble it was chemically linked to a cellulose

matrix. A *p*-aminobenzyl derivative of cellulose (Cellex PAB, Bio Rad Laboratories) has been diazotized by addition of 1 g of cellulose to 25 ml of cold 2 M HCl *plus* 5 ml of 15 % $NaNO_2$. The mixture was kept at 0° for 1 h and then aspirated on a Büchner funnel, washed with 50 ml of 5 % sodium acetate, 5 % urea solution and finally with deionized water. This constituted the reactive supportive matrix. The diazotized cellulose was then placed into 60 ml of 0.075 M K_2HPO_4 buffer (pH 7.7) in the presence of 5 mM cysteine and 2 mM EDTA to which 99.2 mg of crystallized papain (6.2 ml crystallized papain suspension from the Sigma Chem. Co.) were added. The mixture was stirred overnight at 5° and then subjected to centrifugation at 1500 rev./min for 10 min in the International centrifuge. The supernatant was decanted and the precipitate was washed repeatedly in 50 ml of phosphate buffer with cysteine and EDTA as prepared above. Additional washing was carried out on a Büchner funnel. The papain–cellulose complex was then dried and stored in the refrigerator.

Enzyme assays

Disaccharidase activities were measured as described by DAHLQVIST[8]. Glucose was estimated with the glucose oxidase reagent. Alkaline phosphatase was measured by the rate of splitting of *p*-nitrophenyl phosphate (Sigma Chemical Co.) in a glycine buffer as previously described[1]. Leucyl naphthylamidase was determined by a slightly modified procedure of GOLDBERG AND RUTENBERG[9]. All enzymatic activities are expressed in μM substrate reacted per min at 37°.

Protein determination

Protein was estimated by the use of the method of LOWRY *et al.*[10] using Versatol (General Diagnostics) or albumin as a standard.

Column chromatography

Sephadex G-200 (Pharmacia) was equilibrated with 0.15 M NaCl in the presence of 0.001 M imidazole at pH 7.0. Usually 0.5 ml of sample was applied on a 1.6 cm $\times$ 40 cm column. Samples were collected at a rate of about 10 ml/h at 4°.

Centrifugation

All high-speed centrifugation and density gradient work was carried out with a Beckman Model L preparative centrifuge.

RESULTS

Papain has been previously used for solubilization of disaccharidases by AURRICHIO, DAHLQVIST AND SEMENZA[11]. These authors observed solubilization of several disaccharidases from human mucosal homogenates after treatment with relatively high concentrations of papain in the presence of potassium phosphate. We have confirmed the requirement of K^+ for the release of enzymes from the isolated membrane fraction of the hamster intestine. Fig. 1 illustrates this fact, and the importance of maintaining the K^+ concentration within a range from 10 to 80 mequiv/l. KCl effectively substitutes for the phosphate salt under these conditions. Fig. 2 illustrates the release of maltase and alkaline phosphatase activities from the brush border membrane as the function of time, using soluble papain. A lag in release of phos-

phatase activity is apparent from the graph. It is noteworthy that a considerable destruction of phosphatase activity occurred during its release, since in these experiments the recoveries of this enzyme were lower than 30 %. In the same experiment complete recoveries of maltase activity were obtained.

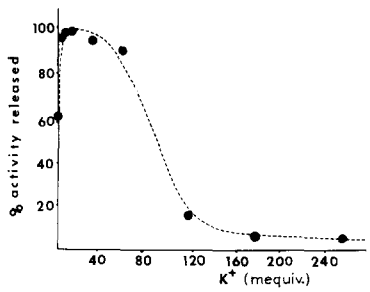

Fig. 1. Release of sucrase activity from the membrane at various concentrations of K^+.

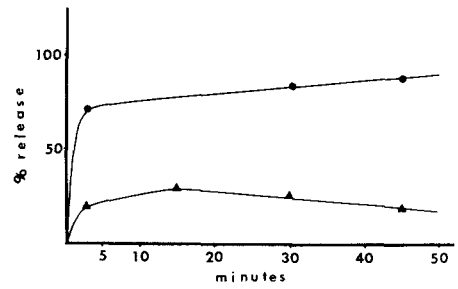

Fig. 2. Release of enzyme activities from the membrane after treatment with papain in solution. ●—●, maltase activity; ▲—▲, alkaline phosphatase activity. Incubation as described in METHODS AND MATERIALS.

Results with short-interval papain digestion

From the results in Fig. 2 it is clear that maltase and alkaline phosphatase can be separated by stopping papain digestion at any time before significant release of the latter has occurred. In early experiments we attempted to stop papain action by oxidation of the incubation mixture with ascorbic acid but found that this procedure also inactivated some of the disaccharidases, especially lactase. Subsequently, we have employed rapid cooling and immediate removal of the intact membrane residue by differential centrifugation. Under these conditions an incubation for 5 min and centrifugation at 4°, as described in METHODS AND MATERIALS, resulted in nearly complete separation of enzymatic activities. This is illustrated in Table I for the several enzymatic activities measured.

The supernatant fraction after papain digestion was found to be sedimented by centrifugation at 151000 × *g* for 2 h. Table II shows the distribution of enzymatic activity in successive fourths of the contents taken from the top of the centrifuge tube after such centrifugation. It is apparent that most of the enzymatic activities assayed were found in the bottom fraction of the centrifuge tube. When the bottom

TABLE I

PAPAIN TREATMENT OF BRUSH BORDER MEMBRANE FRACTION 3–5 min

Enzyme	% *Released*	% *Recovery*
Sucrase	95–98	80–111
Alkaline phosphatase	7–12	100–108
Maltase	77–80	95–104
Isomaltase	80–84	96– 98
Lactase	86–96	91–103
Trehalase	10–14	86–120
Leucyl naphthylamidase	10–14	87– 93

fraction is subsequently applied to a Sephadex G-200 column, most of the material is recovered in a single peak containing all of the disaccharidases (Fig. 3) except lactase which is retarded in the column and presumably is attached to a smaller fragment of the membrane.

TABLE II

DISTRIBUTION OF ENZYMATIC ACTIVITIES IN THE TUBE AFTER HIGH-SPEED CENTRIFUGATION

Fraction No. from the top	*% Enzymatic activity*				
	Protein	*Sucrase*	*Lactase*	*Maltase*	*Isomaltase*
1	14.5	9.0	7.1	14.5	14.7
2	14.7	10.5	8.3	12.0	14.7
3	14.7	10.1	7.9	14.7	13.3
4	56.0	70.4	76.5	56.0	60.0

TABLE III

SEPARATION OF DISACCHARIDASES FROM LEUCYL NAPHTHYLAMIDASE AND FROM ALKALINE PHOSPHATASE + TREHALASE BY SEQUENTIAL TREATMENT WITH PAPAIN

Enzyme	*Distribution of activity in %*			
	5-min supernatant	*45-min supernatant*	*Ppt.*	*Total recovery*
Sucrase	84	16	0	80–111
Leucyl naphthylamidase	12	74	13	75–110
Trehalase	8	15	76	87
Alkaline phosphatase	24	18	58	26
Leucyl glycine hydrolase	29	13	57	13

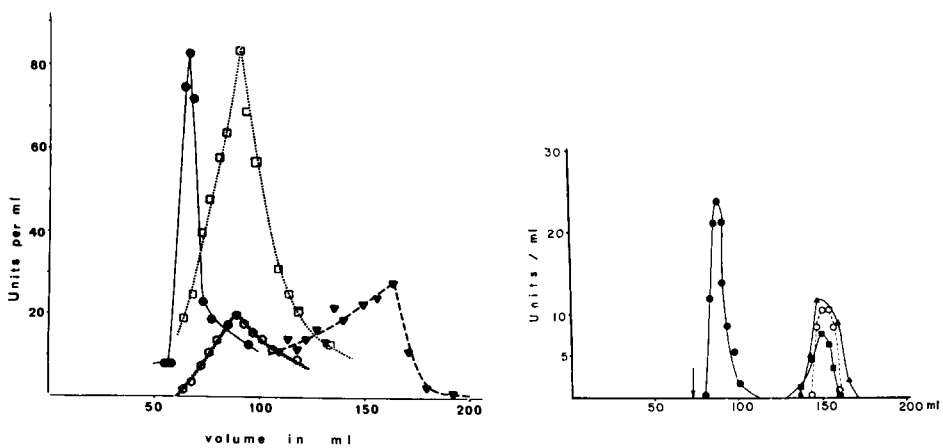

Fig. 3. Separation of enzyme activities on Sephadex G-200 column after treatment with complex papain. ●—●, dextran blue; □···□, maltase; ○···○, sucrase and isomaltase; ▼---▼, lactase.

Fig. 4. Separation of leucyl naphthylamidase activity from disaccharidase activities on Sephadex G-200. ●—●, leucyl naphthylamidase; ▲—▲, maltase; ■—■, lactase; ○---○, isomaltase.

Results with long-interval papain digestion

If the digestion with papain is prolonged to 40 min under identical conditions (Table III) most of the leucyl naphthylamidase is released to the supernatant leaving trehalase activity with the precipitate. A small amount of alkaline phosphatase activity is also released leaving the remaining undigested activity also with the precipitate.

The separation between leucyl naphthylamidase and the disaccharidase particles can also be obtained by fractionation on a Sephadex column, as can be seen from Fig. 4. Because of the relative position of the particles it appears likely that the disaccharidase particle is smaller even though it contains several enzymatic activities.

It is of interest that the release of leucyl glycine-splitting activity during papain digestion does not follow the release of leucyl naphthylamidase, suggesting that two different peptidases are present in the brush border. This conclusion is also supported by the vast difference in the resistance to papain digestion of the two enzymes.

DISCUSSION

Although papain digestion had been used for several years for solubilization of disaccharidases[11], the first indication that it may actually represent removal of particles was reported by ODA AND SATO[12]. They have treated the whole intestine with papain and observed by use of an electron microscope the disappearance of surface particles from the microvilli after release of disaccharidase activity. Recently similar results for disaccharidases were obtained by JOHNSON[13] from hamster intestine.

Using low papain concentrations and short incubation times we have applied papain digestion to the isolated membrane fraction as previously reported in a preliminary communication[7]. This paper gives the full account of these findings. Such a digestion with dilute papain and short incubation times leads to the release of some of the disaccharidases. We are showing now that additional prolonged treatment with papain leads to the separation of leucyl naphthylamidase from the membrane and from other enzymatic activities known to be present. This differs from the observation reported by ODA AND SEKI[14] of the separation from rabbit intestine of a particle containing both the disaccharidase and leucyl naphthylamidase activities. As can be seen from Tables II and III and Fig. 4, at low papain concentrations and with varied intervals of digestion the two activities are not only sequentially released from the hamster intestine but the released particles separate on a Sephadex column. The difference in our results and those obtained by ODA AND SEKI[14] may be due to a difference in species. However, it seems more likely to be due to the much higher concentration of papain used by ODA and his co-workers. Whether the particles removed under our conditions are identical to the membrane particles previously described[2,12,13] still remains to be shown by further investigation.

Of special interest is the separation of trehalase activity from the activity of other disaccharidases. It should be borne in mind that this is an enzyme for which no substrate is usually present in the intestine. Its "true" substrate and role to digestive-absorptive process may still be unknown.

The data presented in this paper are in accord with the concept of a structural organization of the enzymatic activity within the brush border membrane, as in a

mosaic. We have been able to dissect the membrane by enzymatic means and to remove quantitatively particulate multienzyme complexes. Presumably these complexes were held to the membrane by covalent peptide links, but this is by no means proved.

The specific activities of the enzymes in papain-digested fractions of the brush border membrane could not be accurately measured. The amounts of protein were exceedingly small and had to be measured in the presence of a relatively high correction factor due to cysteine present in the incubation mixture. Rough measurement of specific activities indicates a 20-fold increase in specific activities of the enzyme in released particles over the brush border membrane. Since the isolation of brush borders by our procedure yields a 20-fold increase and subsequent membrane isolation a 4-fold increase in specific activities, the over-all purification from the original mucosal scrapings is about 1600-fold.

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