Effect of Secreted *Bacteroides* Proteases on Human Intestinal Brush Border Hydrolases

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ABSTRACT Selected bacteroides species secreted various amounts of protease and glycosidase into their growth medium. *Bacteroides vulgatus*, *distasonis*, and *ovatus* secreted the most (31–60% of total). The secreted protease was similar in action to the protease within the organism, in that it had a broad pH optimum of 6–9, a $K_{m\ app.}$ for casein of 0.1 μM, and was inhibited by benzamidine, phenylmethylsulfonyl fluoride, diisopropylfluorophosphate (DIFP), and by an elastase inhibitor, $Ac(Ala)_3AlaCH_2Cl$.

Exposure of human brush border preparations to the secreted protease reduced maltase and sucrase activities; the reduction could be prevented by DIFP. In contrast, brush border alkaline phosphatase activity either did not change or increased after exposure to bacterial secretions. >90% inhibition of secreted glycosidase using EDTA and *p*-chloromercuribenzoic acid did not prevent the reduction of brush border maltase and sucrase activity, suggesting that glucosidases were not likely to be involved in the destruction of brush border enzymes. Moreover, the bacterial proteases caused only a small net release of active maltase or sucrase from the brush border. Most of the loss of activity was due to destruction of the enzyme. Proximal bowel fluid of three patients with overgrowth contained DIFP-inhibitable protease that destroyed sucrase in isolated brush borders. A *Bacteroides* species was isolated from each sample that secreted protease and destroyed brush border sucrase. We conclude that in bacterial overgrowth syndromes, brush border damage may occur from protease(s) secreted by *Bacteroides*.

INTRODUCTION

When bacterial overgrowth occurs in the small intestine, alterations in absorptive function occur. Most commonly these include steatorrhea and vitamin B_{12} malabsorption. In animals, alterations of the mucosal surface membrane have been well documented (1–5). These include morphologic as well as enzymatic changes. Variations in the levels of brush border hydrolases in rats—e.g., sucrase, maltase, lactase, and alkaline phosphatase—have been evaluated most extensively (1, 2, 4, 5). Similar changes have been demonstrated in human bacterial overgrowth syndromes (6, 7). Several hypotheses explain the mechanism of the bowel mucosal damage, such as direct damage by bacteria (1–4) or their secreted products, or changes in the luminal environment—e.g., effects of secondary bile acids (deoxycholic) with inhibition or solubilization of surface enzymes (8–10).

Bacteria are known to produce multiple exoenzymes (11–18) or secretory products, among which are proteases, glycosidases, and phospholipases. Certain proteases have the ability to solubilize or destroy the brush border disaccharidases. Papain, elastase, trypsin, and chymotrypsin all have been shown to be capable of altering certain disaccharidase and alkaline phosphatase activities in brush border preparations (19–21). Some of these proteases may be involved in maintaining normal levels of disaccharidases in the human (22).

Recently Jonas et al. (1, 4) studied the pathogenesis of mucosal injury in the experimental blind loop syndrome in rats. They found that sonicated extracts of some bacteria cultured from the blind loops possess protease(s) that released, but did not destroy, brush border maltase activity. Moreover, this releasing activity was partially inhibited by protease inhibitors. These authors concluded that proteases produced by bacteria may play a role in the etiology of disaccharidase deficiency in bacterial overgrowth. Prizont (23) has recently described findings that suggest that sugars of small intestinal mucins are degraded by enzymes present in blind loop contents of rats. Therefore, some evidence exists in rats that products of bacterial growth modify macromolecules at the intestinal cell surface.

Our results demonstrate that secreted protease

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derived from various *Bacteroides* species is capable of markedly decreasing human brush border sucrase and maltase. We propose that this protease is important in altering the surface membrane of the mucosal cell in the blind loop syndrome in humans.

METHODS

Bacteria. The bacteria used were obtained from human clinical isolates. Initial studies were performed with organisms obtained from abdominal or pulmonary abscesses. Later studies were performed on samples obtained from three patients with bacterial overgrowth syndrome complicating systemic sclerosis.

Small intestinal fluid was collected from the duodenum of patients aged 48, 52, and 57, all of whom had megaduodenum with diarrhea and steatorrhea. In all cases, broad spectrum antibiotics markedly reduced the symptoms. Total organisms numbered $>10^9$/ml in each case. Anaerobic microorganisms, predominantly *Bacteroides* species, were cultured from the duodenal fluid in each case, and the one *Bacteroides* species that predominated in each sample was subcultured. Fluid was collected over a 2-h period through a Crosby capsule that had been sterilized and from which the cutting blade and spring had been removed. Isolation and identification were performed for anaerobes by the method outlined in the Virginia Polytechnical Institute Anaerobic Bacteria Manual. The pure bacterial cultures were initially transferred to and grown in thioglycollate broth with vitamin K and bovine hemin without indicator (obtained from Remel Laboratories, Kansas City, Mo.). The cultures were grown in a 5% CO_2, 10% H_2, 85% N_2 atmosphere in an anaerobe glove tent.

To obviate the need for separation of the secreted enzymes from the growth medium, and to decrease the amount of substances that would interfere in the protease assay, a defined medium was used for subculturing. This medium was modified from the minimal medium of Blackburn (12), and satisfied the known basic growth requirements for bacteroides species (24), including vitamin B_{12} and vitamin K. The composition of the medium in grams per liter was K_2HPO_4, 0.45; KH_2PO_4, 0.45; $(NH_4)_2SO_4$, 0.90; NaCl, 0.90; $MgSO_4$, 0.09; $CaCl_2$, 0.09; L-cysteine HCl, 0.50; $NaHCO_3$, 5.00; maltose, 3.00; vitamin B_{12}, 0.0001; hemin, 0.001; and vitamin K_1, 0.00025.

10 ml of the defined medium was inoculated with $\sim 10^4$ bacteria from 24–48-h-old cultures grown in thioglycollate. Bacterial growth was allowed in the defined medium for 24–144 h. Growth was quantitated by colony counts of serial dilutions on blood agar plates, and by comparison with McFarland's barium standards on the Beckman spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.) at 530 μm. Purity of the culture was periodically reconfirmed by gram stain and reculture on blood agar plates.

Medium was prepared for enzymatic studies by centrifugation at 15,000 rpm for 30 min (20,000 *g*) in the International (model B-20) refrigerated centrifuge (International Equipment Co., Damon Corp., Needham Heights, Mass.). The decanted supernatant fraction was cell free, as determined by the fact that it was sterile when recultured. The cell-free supernatant fraction was used with or without lyophilization and reconstitution to one-tenth the original volume. The pellet containing the cells was resuspended in one-tenth the original volume of distilled water and sonicated for 1 min with a Braunsonic 1510 probe sonicator (Braun Instruments, San Francisco, Calif.). This treatment released all alkaline phosphatase activity.

To ensure that bacterial cellular autolysis or disruption was not accounting for the release of protease into the supernatant fraction, two methods were utilized. First, alkaline phosphatase, known to have significant intracellular activity, was measured by the method of Bessey et al. (25) in the sonicated pellet and in the cell-free supernatant fraction in 96-h-old cultures. Second, DNA content was measured by the method of Burton (26) in the sonicated bacterial pellet and the cell-free supernatant fraction, also from 96-h cultures.

Protease activity. Lyophilized cell-free supernatant fractions and sonicated cells were routinely assayed for protease activity using a modified caseinolytic assay. Casein (Pentex, Miles Laboratories Inc., Elkhart, Ind.) was labeled with ^{125}I (New England Nuclear, Boston, Mass.) by the method of Hunter and Greenwood (27). 1% labeled casein (300,000 cpm) was incubated with bacterial supernatant fraction containing protease activity (equivalent to ~ 10 μg of trypsin) for up to 60 min in 200 mM $NaPO_4$, pH 7.6, at 37°C. This reaction was terminated by addition of 10% TCA (final concentration). Protease-dependent hydrolysis of about 6% of the added casein occurred in 60 min. Soluble counts were measured in a Beckman gamma counter (model 4000) (Beckman Instruments Inc.). Proteolytic activity was linear with additions of protease up to 200 μg equivalent of trypsin, and was linear for up to 60 min of incubation. Protease was stable when frozen, but repeated freezing and thawing greatly diminished proteolysis. The pH optimum was evaluated with tris-maleate buffer adjusted over a range of pH from 5.5 to 9.0.

Proteolysis, as defined by the iodinated casein assay, corresponded to hydrolysis of the protein and not to deiodination alone. After incubation of the labeled casein with bacterial extract for periods up to 60 min, the mixture was precipitated with TCA and the supernate either treated with H_2O_2 and extracted with chloroform or counted as was. Few counts were removed from the TCA supernatant fraction by treatment designed to extract I_2. Moreover, thin-layer chromatography of the neutralized TCA precipitate on Silica Gel G (acetone, 65:*n*-butanol, 20:NH_3, 10:water, 5) revealed no counts with an R_f of free iodine. Finally, the use of unlabeled casein as substrate in selected assays paralleled the results obtained with the labeled substrate, but the former assay was considerably less sensitive and required larger aliquots of supernatant fluid. For this reason, the ^{125}I method was used.

Standard curves using trypsin as the enzyme were performed with each bacterial assay, and the amount of bacterial protease activity was compared with that of trypsin. Routinely, the equivalent of 10 μg of trypsin in the caseinolytic assay was used for in vitro incubations with brush borders.

To demonstrate the type of protease activity, various protease inhibitors were used to block the hydrolysis of casein. These inhibitors were diisopropylfluorophosphate (DIFP)[1], phenylmethylsulfonyl fluoride, benzamidine, iodoacetate, *p*-chloromercuribenzoic acid (PCMB), Na-tosyl-L-lysine-chloromethane HCl (TLCK), and L-1-tosyl-amino-2-phenyl ethylchloromethyl-ketone (TPCK) (all from Sigma Chemical Co., St. Louis, Mo.). $Ac(Ala)_3AlaCH_2Cl$ was obtained from Dr. J. Powers, School of Chemistry, Georgia Institute of Technology, Atlanta, Ga. Inhibitor concentration ranges varied from 100 mM to 1 μM. Preincubation for 10 min of bacterial filtrate with the inhibitors was necessary to demonstrate optimum inhibition. Trypsin, chymotryp-

[1] *Abbreviations used in this paper*: DIFP, diisopropylfluorophosphate; PCMB, *p*-chloromercuribenzoic acid; TLCK, Na-tosyl-L-lysine-chloromethane HCl; TPCK, L-1-tosyl-amino-2-phenyl ethylchloromethyl-ketone.

sin, and elastase were purchased from Worthington Biochemical Corp., Freehold, N. J.

Brush border studies. Small strips of fresh human intestinal mucosa were obtained at the time of jejunoileal bypass operations for obesity, according to a protocol approved by the Committee on Human Experimentation at Washington University. An enriched microvillus membrane (brush border) was prepared from human fresh-frozen small intestinal scrapings according to the method of Welsh et al. (28). Brush border preparations were then adjusted to a protein concentration of 1 mg/ml and stored frozen until needed. To test the effect of the bacterial extracts containing secreted enzymes, brush borders were incubated with the extracts at 37°C. Ratios of brush border to extract protein were 1:3. However, the protease equivalent of only 10 μg of trypsin was used in each assay for 0.5 mg of brush border protein. Thus, the ratio of brush border protein to protease, estimated as trypsin equivalents, was 50:1.

For some experiments, the disaccharidases were first released from the brush border by treatment with insolubilized papain (Sigma Chemical Co.). 1 mg of brush border protein was incubated with 0.6 mg of papain and 5 mM cysteine for 1 h at 37°C; the reaction was stopped by the addition of 5 mM *p*-chloromercuribenzoate, and the papain removed by centrifugation. The solubilized disaccharidases equivalent to 0.5 mg of brush border proteins were then used for each subsequent incubation. Incubation with bacterial extracts was performed in 200 mM Na_2HPO_4 buffer (pH 7.6) in the presence and absence of the inhibitor. As a control experiment, the bacterial supernatant fraction was boiled for 10 min before its addition to the incubation mixture. When inhibitors were tested, they were added to the bacterial extracts 10 min before the brush borders. After incubation for 60 min, each tube was centrifuged at 105,000 *g* for 20 min. The pellet was resuspended and washed twice with 0.2 M Na_2HPO_4 buffer, and the disaccharidases and alkaline phosphatase activities were assayed by the method of Dahlqvist (29) and Bessey (25), respectively. Bacterial glycosidases were assayed using nitrophenyl substrates, as described previously (30).

RESULTS

Secretion of proteases. Growth of the various bacteroides species was less complete in minimal medium than in Thioglycollate; the species reached a maximal concentration of 10^6 colony-forming units/ml in the minimal medium, vs. 10^8–10^9 in the enriched medium (Fig. 1). Moreover, release of protease was dependent on the medium used and on time. No secreted protease could be detected in Thioglycollate-grown *Bacteroides* cultures, even when the medium was dialyzed first to remove small molecular weight inhibitors of the caseinolytic assay. Only after the organisms reached a stationary phase of growth in the minimal defined media did protease activity appear in the cell-free medium (Fig. 1). During culture in the defined medium, intracellular proteolytic activity per cell remained constant.

By 96 h, there were many active carbohydrases in the cell-free medium (Table I). α-Glycosidase was the most active enzyme, but neither β-fucosidase nor α-mannosidase was detected. In the presence of EDTA (5 mM) and PCMB (1 mM), most of the glycosidase

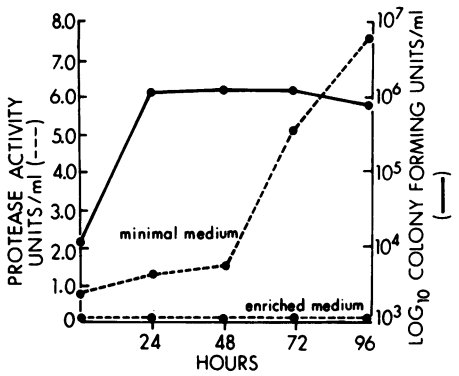

FIG. 1 Effect of growth conditions on protease activity. Individual cultures of *B. thetaiotamicron* were inoculated into minimal medium or thioglycollate broth and incubated anaerobically as described in Methods. Individual tubes of cultured bacteria were removed at designated times and assayed for colony-forming units and for protease activity, the latter assay performed on the cell-free supernate obtained after centrifugation. In thioglycollate the protease assay was performed before and after dialysis for up to 8 h without detection of proteolysis. About 30,000 cpm/ml of ^{125}I-casein were used per assay and hydrolysis proceeded in 30 min to the extent of 2–5%. The data represent the mean of triplicate assays.

activity could be inhibited although proteolysis was unaffected. For subsequent incubation with brush borders, which contain α-glucosidase and β-galactosidase not inhibited by EDTA or PCMB, these inhibitors were added.

Because many enzymes were found in the cell-free supernate after culture, we next determined that this was the result not of cell lysis, but of enzyme secretion, as reported previously for the protease from *B. amylophilus* (12, 13). First, no alkaline phosphatase was found in the medium at any time up to 96 h after culture. Moreover, only 1.9% of the total bacterial DNA was

TABLE I
Secreted Glucosidase Activity of B. distasonis

Enzyme	Enzyme activity		Inhibition
	Control	+Inhibitors	
	U/ml		%
α-Glucosidase	0.08	0.004	95
β-Glucosidase	0.028	0.0026	91
α-Galactosidase	0.034	0.0028	92
β-Galactosidase	0.0168	0.0026	85
α-Fucosidase	0.0196	0.0030	85

B. distasonis obtained from an abdominal abscess was cultured in minimal medium for 96 h, and the cell-free supernatant fraction obtained. Incubation (0.5 ml) was performed at pH 7.6 for 30 min, and the reaction stopped by adding 2 ml of 20 mM NaOH. EDTA (5 mM) and *p*-chloromercuribenzoate (1 mM) were added together as inhibitors.

found in the supernatant fraction after 24 h of culture, and this percent of noncellular DNA did not increase during prolonged culture up to 96 h. Therefore, we conclude that lysis of cells was not a factor in the release of protease or carbohydrase.

Numerous other bacterial species were examined for their ability to secrete protease. These included *Clostridum perfringens*, anaerobic lactobacilli, *Escherichia coli*, *Klebsiella*, and *Proteus vulgaris*. No protease activity was detected in the minimal culture medium after 96 h of culture.

The percent of protease activity in the medium was dependent upon the species of bacteroides cultured. Table II shows that the largest percent of total protease was secreted in cultures of *B. vulgatus* and *B. distasonis*. It is interesting that *B. melaninogenicus*, found more in oral than intestinal flora, secreted the least protease.

To characterize the proteolysis further, the cell-free supernatant fractions were concentrated 10-fold by dialysis and lyophilization. This operation increased the stability of the enzyme and allowed determinations to be made with short assay times (<30 min). Furthermore, because the growth of organisms did not exceed 10^6–10^7/ml in culture, a 10-fold increase in activity would be consistent with protease that might be secreted from 10^9–10^{10} organisms found in the blind loop syndrome (7, 31–34). Proteolysis in the concentrated form did not increase after removal by dialysis of the concentrated salt resulting from lyophilization. Therefore, in most of the experiments described below, lyophilization was not followed by dialysis. Moreover, an additional control was performed by adding 10-fold more concentrated medium to the protease assay. No effect on the activity was noted by the added salts.

TABLE II
Protease Secretion by Bacteroides Species

Species	Protease activity
	% of total activity
B. vulgatus	60±7
B. diastonis	40±5
B. ovatus	31±3
B. fragilis	21±4
B. thetaiotamicron	16±4
B. melaninogenicus	9±2

Bacteroides were cultured in minimal medium for 96 h, and the cell-free supernatant fraction assayed as described in Methods. In addition, the bacteria from which the supernatant fraction was derived were sonicated in 0.2 M Na_2HPO_4 buffer, pH 7.6, and assayed for protease activity. The total activity was the sum of the cellular and cell-free activity. The data represent the mean±1 SEM for four separate determinations. *Melaninogenicus* species was obtained from lung abscess. All other species were cultured originally from abdominal abscesses.

Characterization of proteases. The pH optimum for the secreted protease was broad, with peak activity seen between pH 6.0 and 9.0. Two minor peaks of activity were observed at pH 6.5 and 8.0. Incubations were subsequently carried out at an intermediate pH of 7.6. This pH was chosen because it permitted comparison of activity with that of trypsin and chymotrypsin. Results similar to those reported below were obtained at pH 6.5. It is not clear whether the pH curve represents more than one separate protease or one with variable activity.

The K_m for hydrolysis of casein by the secreted protease was 0.1 μM. Boiling the cell-free supernate for 10 min destroyed the protease activity. Serine protease inhibitors were variably effective (Table III). Benzamidine was inhibitory at 10 mM but not maximally effective until 100 mM. Phenylmethylsulfonyl fluoride was most effective at 1 mM, but inhibited the various proteases by only 25–60%. It is interesting that *B. thetaiotomicron* protease was the least susceptible to either inhibitor, and *B. melaninogenicus* protease was the most sensitive. DIFP at 0.1 mM prevented all enzyme activity. Iodoacetate (10 mM) was without effect.

Since the bacteroides protease appeared to be a serine protease, various chloromethylketone inhibitors were tested. Neither TPCK nor TLCK inhibited the protease activity > 15%, although the concentrations used prevented trypsin and chymotrypsin activity (Table III). $Ac(Ala)_3AlaCH_2Cl$, an elastase inhibitor, decreased activity in all the species tested, however. Once again, protease from *B. melaninogenicus* was most susceptible to inhibition.

Effect of bacterial proteases on human brush borders. The concentrated cell-free supernatant fractions containing protease were incubated with isolated human brush borders. Loss of activity began immediately after addition of the protease source, and the loss of activity was linear for 75 min, when the ratio of protease to brush border protein was 10 μg (trypsin equivalent):0.5 mg (Fig. 2). Both sucrase and maltase activity were decreased in brush borders by about 60% after 60 min of incubation. Because the 1 h point was still on the linear portion of the curve, it was chosen for further experiments. In some instances, activity was also measured after 30 min of incubation. Soluble maltase was detected during the 1st h of incubation, but reached only a level of 13% of total activity. This level persisted during the 1st h of incubation, after which no soluble activity could be detected. Table IV demonstrates that significant prevention of sucrase and maltase destruction by DIFP was found using proteases secreted by four of the *Bacteroides* species. Alkaline phosphatase activity by contrast was unchanged or in-

TABLE III

Inhibition of Bacteroides Proteases by Serine Protease Inhibitors

Species	Protease activity					
	Benzamidine	Phenylmethyl-sulfonyl fluoride	DIFP	TPCK	TLCK	$Ac(Ala)_3AlaCH_2Cl$
			% remaining			
B. fragilis	28	59	0	87	91	37
B. thetaiotamicron	47	75	0	92	84	60
B. distasonis	29	30	0	84	81	33
B. vulgatus	28	42	0	95	86	45
B. ovatus	17	45	0	94	92	55
B. melaninogenicus	0	0	0	91	94	28

The bacteroides were grown in minimal medium for 96 h and the cell-free supernatant fraction (after concentration) was assayed with benzamidine (100 mM), phenylmethylsulfonyl fluoride (1 mM), DIFP (0.1 mM), TPCK (0.3 mM), TLCK (0.3 mM), and $Ac(Ala)_3AlaCH_2Cl$ (0.1 mM). Control activity without inhibitors (100%) ranged from 380–630 cpm above background with a background of 50–60 cpm. The peptide chloromethyl ketone inhibitor was solubilized in methanol at a final concentration of 5%. The appropriate control had a similar concentration of methanol. Preincubation for 10 min was performed at pH 6.5 as previously described (31), before adjusting the pH to 7.6. Incubation was performed for 30 min at pH 7.6. The data represent the mean of three separate determinations.

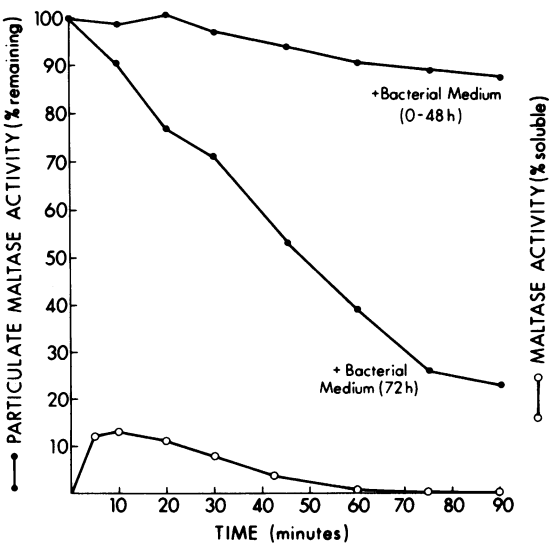

FIG. 2 The effect of bacterial medium on the maltase activity of human intestinal brush borders. Cell-free supernate medium was produced from *B. thetaiotamicron*, as described in Methods, after growth up to 48 h, and after 72 h. The equivalent of 10 μg of trypsin was added to 0.5 μg of brush border protein in a reaction mixture of 1.0 ml at pH 7.5. At times indicated, the reaction mixture was centrifuged at 40,000 *g* for 20 min, washed with 1 ml of buffer, recentrifuged, and the pellet resuspended by homogenization in 1 ml of 200 mM Na_2PO_4 buffer, pH 7.5. Aliquots of the supernate and resuspended pellets were assayed for maltase activity in the presence of 5 mM EDTA and 1 mM PCMB to inhibit bacterial maltase. The points represent the mean of duplicate experiments. Control maltase activity (100%) corresponded to 0.72 OD at 420 μm.

creased after incubation with supernatant fraction. Phosphatase activity increased after butanol or papain solubilization, presumably by making substrate available to more active sites. The same phenomenon is probably occurring in this case.

The supernatant fractions from *C. perfingens*, microaerophilic streptococci, lactobacillus, *E. coli*, *Klebsiella*, and *P. vulgaris* were added to human brush borders for up to 2 h with no loss of either sucrase or maltase activity. Furthermore, growth medium taken from *Bacteroides* strains during the first 48 h of subculture decreased maltase and sucrase activity only 8–13% after 1 h of incubation (Fig. 2). Thus, culture periods characterized by little secretion of protease (Fig. 1) corresponded to little loss of brush border disaccharidase activity.

The above experiments examined enzyme activity remaining attached to the brush border. After exposure for up to 1 h to the bacterial protease, not >2% of the enzyme activity lost from the brush border could be recovered in the supernatant fraction (Fig. 2). In this experiment, EDTA and PCMB were added (Table I) to inactivate bacterial glycosidases and to allow detection of solubilized brush border glycosidases. Thus, proteolysis could remove the enzymes from the brush border, but rapid destruction would seem to follow the solubilization. To test this hypothesis, a direct effect of the protease was measured by observing the effect of the secreted proteases on brush border enzymes previously solubilized with 1% Triton X-100. Over 50% of the solubilized maltase and sucrase ac-

TABLE IV
Effect of Bacterial Proteases on Human Brush Border Enzymes

Species	Brush border enzyme activity					
	Without inhibitor			+DIFP		
	Sucrase	Maltase	Alk phos	Sucrase	Maltase	Alk phos
	% change			% change		
B. fragilis	−62	−61	−4	−4	−7	+8
B. distasonis	−62	−68	−8	−12	−22	−8
B. ovatus	−61	−55	+28	−17	−22	−7
B. thetaiotamicron	−63	−58	+35	−7	−12	+27

Purified human brush border was added (500-μg protein) to protease from *Bacteroides* species (10-μg trypsin equivalent) in a final incubation volume of 0.5 ml at pH 7.5. DIFP was used at a concentration of 0.1 mM. After incubation for 60 min, the brush borders were recovered and washed as described in Fig. 2. Aliquots of 20 μl were removed for sucrase, maltase, and alkaline phosphatase assays. The data shown represent the mean of six separate experiments. Control brush border enzyme activity represents 0.4–0.7 OD at 420 μm.

tivity was lost within 1 h. Thus, the sensitivity of solubilized brush border enzymes was comparable to that of the membrane-bound glycosidases.

Since glycosidases were secreted in large amounts by *Bacteroides* species (Table I) it was possible that they exerted some effect of the brush border enzyme activity. Inhibition of the glycosidase activity had no effect on the loss of brush border sucrase or maltase produced by the bacteroides supernatant fraction, however.

The effect of the bacterial protease on the brush border enzymes showed specificity in that it did not inhibit alkaline phosphatase or trehelase activity (latter not shown). In addition, the action of the bacterial protease was noted at a concentration much lower than that for the intraluminal pancreatic endoproteases. Addition of trypsin, chymotrypsin, or elastase at 50 times the proteolytic activity of the bacterial supernatant fraction did not cause loss of maltase from the brush border (Fig. 3). In this experiment, the pancreatic protease:brush border protein ratio was 1:1, less than needed for alteration of brush border enzyme levels in vitro (21).

Effect of bacterial proteases produced in vivo on human brush borders. The data reported above suggested that in vivo *Bacteroides* strains might produce a protease that could destroy brush border enzymes. Three patients with stagnant loop syndrome were intubated, and small bowel fluid obtained from the area of stagnation (dŭodenum). Proteolytic activity against brush border sucrase was examined in both the original intestinal fluid and the bacterial medium obtained from the major *Bacteroides* species that grew from such fluid (Table V). In each experiment, sucrase activity was lost (48–72% in 1 h). Moreover, this loss of sucrase activity was prevented by DIFP and nearly so by the inhibitor of elastase activity, $Ac(Ala)_3CH_2Cl$.

The proteolytic activity of the small bowel fluid was comparable in effect and specificity to that studied from organisms derived from other clinical sources of infection. Disaccharidase levels were measured in a small bowel biopsy taken via a Carey capsule on a separate occasion (within 1 wk) of the intestinal fluid collection. The mean activity (±1 SEM) for lactase,

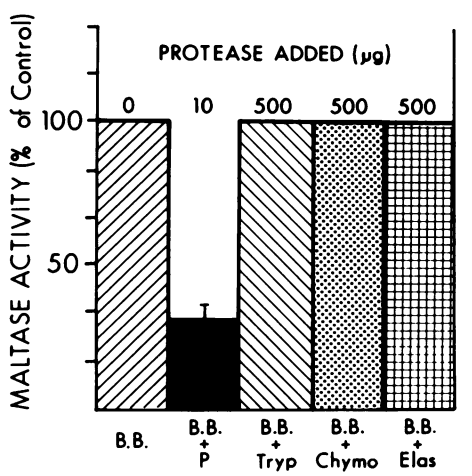

FIGURE 3 Effect of bacterial and pancreatic proteases on human brush border maltase activity. Protease was obtained from *B. fragilis*, and added to brush borders (500 μg protein) at a concentration of 10 μg trypsin equivalent per 1.0 ml of reaction mixture. Trypsin, chymotrypsin, and elastase were added (500 μg/reaction) for 30 min as well. Brush borders were recovered by centrifugation and activity of particulate maltase assayed as described in Fig. 2. Control maltase activity (100%) corresponded to 0.65 OD at 420. The data represent the mean±1 SEM of four separate experiments.

TABLE V

Effect of Intestinal Bacterial Proteases on Human Brush Border Sucrase

Source of protease		Brush border sucrase activity				
		Control	$Ac(Ala)_3AlaCH_2Cl$	TLCK	TPCK	DIFP
		% remaining				
Intestinal fluid						
1		28	82	36	38	100
2		36	80	48	52	102
3		32	88	56	66	104
B. fragilis	1	48	90	58	54	98
B. thetaiotamicron	2	53	94	63	67	99
B. fragilis	3	62	91	75	70	101

Fresh duodenal aspirates were assayed for protease activity as described in Methods. The duodenal fluid was centrifuged at 20,000 *g* for 30 min and the resulting supernates (which was cell-free) in aliquots equivalent to 10 μg of trypsin activity were added to 0.5 mg of brush border protein in the presence or absence of protease inhibitors. Incubation was carried out for 1 h at 37°C. The aspirates were also cultured immediately, and the *Bacteroides* species recovered was subcultured as described in Methods. The medium from such cultures after 72 h of growth was used for the second group of experiments. Protease activity again equivalent to 10 μg of trypsin activity was added to 0.5 mg of brush border proteins and incubated for 1 h at 37°C. Brush borders were recovered by centrifugation (Fig. 3) and sucrase activity was determined in the particulate fraction. The data represent the mean of three separate determinations. Inhibitors were added as described in Table III. The numbers 1, 2, and 3 refer to individual patients.

sucrase, and maltase was 26±4.3, 64±9, and 220 ±23 U/g protein, respectively. These values were significantly lower than the normal values of 43± 5.3, 94±12, and 307±27 U/g obtained in our laboratory. Alkaline phosphatase activity was not different from normal.

DISCUSSION

In the bacterial overgrowth syndrome, $>10^7$ microorganisms/ml can be present, consisting predominantly of bacteroides, anaerobic lactobacilli, coliforms, and enterococci (14, 33–36). The many features of this syndrome include steatorrhea and weight loss, vitamin B_{12} deficiency, anemia, and impaired mucosal function, as manifested by diminished disaccharidase activity (1–5, 37). Bacteria elaborate different enzymes; for example, *Bacteroides* are largely responsible for bile acid deconjugation (14, 38), whereas lactobacilli and *E. coli* are more dependent on vitamin B_{12} for growth (39). Jonas et al. (4) have shown in experimental blind loop syndrome that a protease is elaborated that releases maltase from the brush border. This enzyme activity was most associated with *B. fragilis*, *C. perfringens*, and *Streptococcus fecalis*. These authors found that the proteolytic activity was largely within the bacteria and released by sonication, and removed lactase, sucrase, and alkaline phosphatase as well.

Our studies show that many *Bacteroides* species secrete protease and that the amount secreted differs for the various *Bacteroides* species. Moreover, no other bacteria tested (clostridia, *E. coli*, lactobacilli) secrete a demonstrable protease with activity against brush border enzymes. This protease secreted from *Bacteroides* behaves like a serine protease, with a specificity akin to elastase, and destroys maltase and sucrase from human brush borders, but has little or no effect on alkaline phosphatase. Thus, the proteolysis has specificity, and pancreatic enzymes did not have a similar effect when given in 50-fold excess. These experiments do not directly prove that the protease detected by the caseinolytic assay is the same enzyme(s) that destroys brush border sucrase and maltase. However, two separate inhibitors, DIFP and $Ac(Ala)_3AlaCH_2Cl$, both inhibit secreted casease (Table III), and prevent the loss of sucrase (Table V). Therefore, it seems likely that both measurements of proteolysis were identifying similar proteases.

Bacteroides are often the predominant species in overgrowth areas (14, 33–35). The bacterial counts attained in the defined culture suspension approached but did not exceed actual documented counts from overgrowth areas. These counts in fact were less by a factor of 100, compared with the bacterial counts obtained in most patients with overgrowth, yet protease was elaborated even at a density of 10^7 cells/ml.

Our results indicate that *Bacteroides* species can synthesize and release protease in vitro and in vivo (Table V). Protease alone has the ability to destroy

disaccharidase activity in the absence of other intestinal luminal factors such as unconjugated bile salts or short-chain fatty acids (8–10), which have previously been implicated in small intestinal brush border hydrolase damage. An additional destructive effect by secreted carbohydrases was not demonstrated. However, in vivo these carbohydrases may render the glycocalyceal enzymes more vulnerable to digestive enzymes by removal of the polysaccharide or by digestion of the mucus covering the cell surface (23). The evidence that the damage of selected disaccharidases in vitro was solely due to the protease in the bacterial cell-free supernate or luminal fluid is supported by the fact that certain protease inhibitors could completely prevent any effect of the protease.

Brush border enzymes are either removed from the surface of the cell and rapidly destroyed (Fig. 2) or destroyed *in situ* by the action of *Bacteroides* protease(s). The enzyme activity of the brush border is decreased in vivo (in three patients) as well as in vitro. However, it is not clear how many of the symptoms produced by the stagnant loop syndrome are referable to this abnormality. Perhaps the major deleterious effects of *Bacteroides* growth are on other brush border proteins or even by a mechanism other than protease productions (e.g., toxin production).

Our conclusions can be summarized as follows. Intestinal *Bacteroides* species secrete one or more proteases. The amount of secretion is dependent upon the bacterial species and the degree of its growth, which may account for variable resultant damage in areas of overgrowth. The secreted protease alone can destroy human brush border disaccharidase. The potential for destruction by the bacterial protease is much greater than that of the pancreatic proteases in equivalent amounts. Therefore, bacterial proteases can account for the altered brush border enzyme activity seen in human disease. Species other than *Bacteroides* may also secrete proteases in vivo, but our experiments suggest that *Bacterioides* species have the capacity to cause brush border damage. Further characterization of the bacterial proteases produced in various disease states may prove helpful in determining the mechanism of damage to the small bowel.

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