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Significance of Microflora in Proteolysis in the Colon

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Protease activities in human ileal effluent and feces were compared by using a variety of native and diazotized protein substrates. In many cases the diazotized proteins had altered susceptibilities to hydrolysis compared with the native proteins. Proteolytic activity was significantly greater ($P < 0.001$) in small intestinal effluent than in feces (319 ± 45 and 11 ± 6 mg of azocasein hydrolyzed per h per g, respectively). Moreover, fecal proteolysis was qualitatively different in that ileal effluent did not hydrolyze the highly globular protein bovine serum albumin, whereas all fecal samples tested degraded this substrate. Inhibition experiments provided further evidence that fecal protease activity differed from that in the small intestine. Physical disruption of fecal bacteria released large quantities of proteases, indicating that the lysis of bacteria in the colon may contribute to the extracellular proteolytic activity in feces. Protease inhibition studies with washed fecal bacteria showed that they produced serine, cysteine, and metalloproteases, and experiments with synthetic *p*-nitroanilide substrates indicated that low levels of trypsin- and chymotrypsin-like activities were associated with whole cells. An elastase-like enzyme was bound to the outer membranes of some fecal bacteria.

There is increasing interest in the activities of bacteria that inhabit the human large intestine. To a large extent this has resulted from a growing awareness of the intimate association that exists between the gut microflora and the host and the role in human physiology played by the products of bacterial metabolism (5, 23).

There have been many studies on the fermentation of polysaccharides or dietary fiber and the production of short-chain fatty acids by the colonic microflora (6, 8, 20), but in addition to carbohydrate, protein is also available in large quantities for fermentation in the gut (4, 19). Unlike the metabolism of carbohydrate, however, the degradation of proteins results in the formation of a number of potentially toxic metabolites, such as ammonia, phenols, indoles, and amines (7, 12, 17).

The initial stages in the breakdown of proteins in the large gut are carried out by a range of host-produced and bacterial peptide hydrolases. Recent work has shown that high levels of proteolytic activity occur in gut contents (19), but efforts to determine the contribution made by the microflora in these experiments were complicated by the fact that variable levels of pancreatic proteases (trypsin, chymotrypsin, and elastase) were present in samples. Nevertheless, it was clear from these studies and later investigations (18) that a substantial proportion of large intestinal protease activity was of bacterial origin.

In this paper we have investigated the abilities of small gut proteases and proteases in different fractions of feces to hydrolyze a range of protein substrates. The distribution of proteases within bacterial cells and their potential contribution to proteolytic activity in feces are also examined.

MATERIALS AND METHODS

Collection of ileal effluent and feces. Small intestinal contents were collected directly from the ileal stoma of five healthy individuals with ileostomies. The material was diluted 10-fold with 0.1 M sodium phosphate buffer (pH 7.4) and immediately tested for proteolytic activity. Fecal slurries (10%, wt/vol) were prepared by suspending fresh feces from five normal individuals in phosphate buffer that had been sparged with oxygen-free nitrogen gas. A sample of each slurry (50 ml) was retained for a determination of total proteolytic activity. The remainder was centrifuged to yield a washed particulate fraction, a washed bacterial cell fraction, and a cell-free supernatant fraction as previously described (19).

Hydrolysis of native proteins by ileal effluent. The soluble protein substrates casein, bovine serum albumin (BSA), and ovalbumin were dissolved in the phosphate buffer to a concentration of 10 mg/ml. Portions (50 μl) of the diluted ileal effluent were added to 4.95 ml of protein solution, in triplicate, to start the reaction. A suspension of collagen (Sigma type I) containing 49.5 mg of protein in 4.95 ml of buffer was used to study the breakdown of this substrate. Samples were taken at zero time and at regular intervals up to 3 h. The samples (0.5 ml) were inactivated by the addition of 50 μl of 50% (wt/vol) trichloroacetic acid and left at room temperature for 30 min. After centrifugation at 11,600 × *g* (5 min) to remove precipitated material, supernatant fluid was tested for trichloroacetic acid-soluble peptides by the method of Lowry et al. (16), using the appropriate protein standard.

Hydrolysis of native proteins by fecal slurries. The hydrolysis of casein, BSA, ovalbumin, and collagen by different fractions of feces was determined as described above except that the protein solutions used were at a concentration of 20 mg/ml. The reactions were initiated by the addition of 2.5 ml of the protein solution to 2.5 ml of fecal preparation.

Hydrolysis of diazotized proteins. Measurements of the hydrolysis of azocasein, azoalbumin, azocoll, and the proteins in azosoybean flour by ileal effluent and feces were carried out as described previously (18).

Effects of inhibitors on ileal and fecal protease activity. Experiments were carried out with small intestinal contents and fecal slurries, using procedures described by Macfarlane et al. (18).

Inhibition of proteolytic activity in whole bacteria and bacterial extracts. Suspensions of washed fecal bacteria (0.8

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mg [dry weight] per ml) were prepared as described above. Portions were retained for assays with whole cells; the remaining cells were disrupted by two passages through a French pressure cell (1.1×10^5 kPa). The resultant crude homogenate was centrifuged at $40,000 \times g$ for 30 min to separate cell wall material. The pellets were washed twice and suspended to their original volumes with the phosphate buffer. The supernatants which contained cytoplasmic and membrane-associated proteases were also retained. Protease assays, together with determinations of the effect of inhibitors, were carried out on each fraction as described previously (18).

Measurement of trypsin-, chymotrypsin-, and elastase-like activities associated with fecal bacteria. Whole bacteria and fractions from disrupted cells were tested for arylamidase activity against the trpysin substrate benzoylarginine *p*-nitroanilide, the chymotrypsin substrate glutarylphenylalanine *p*-nitroanilide, and the elastase substrate succinylalanylalanylalanine *p*-nitroanilide as described by Gibson and Macfarlane (10).

Chemicals. All chemicals and reagents were obtained from Sigma.

RESULTS

Hydrolysis of native and diazotized proteins by small intestinal effluent and feces. Casein, collagen, azocasein, azoalbumin, and the proteins in azosoybean flour were extensively hydrolyzed by small gut proteases (1,214, 281, 319, 208, and 333 mg of protein hydrolyzed per h per g of ileal effluent, respectively) (Table 1). The rate of breakdown of these substrates by feces was 20- to 60-fold lower, however. Pancreatic proteases in ileal effluent did not hydrolyze the highly globular protein BSA and only poorly degraded ovalbumin. In contrast, fecal proteases broke down BSA with relative ease (2 mg of protein hydrolyzed per h per g of feces), although the hydrolysis of this protein was less efficient than that of casein (20 mg of protein hydrolyzed per h per g of feces). The diazotized proteins had characteristics different from those of their native protein counterparts with respect to proteolysis. Azocasein and azocoll were hydrolyzed more slowly than the native proteins by ileal effluent, whereas azoalbumin hydrolysis was greater than that of either ovalbumin or BSA. Essentially similar effects were observed with feces; however, the rates of collagen breakdown were slightly higher than those of azocoll.

Hydrolysis of native and diazotized proteins by different fractions of feces. All proteins tested were broken down by washed fecal bacteria, washed particulate material, and cell-free fecal supernatants (Table 1). The proteins were hydrolyzed to various degrees by the different fractions, however. Casein, azocasein, azoalbumin, and the proteins in azosoybean flour were broken down maximally by the washed particulate fraction, while collagen, azocoll, and ovalbumin were most efficiently degraded by the cell-free supernatants.

Inhibition of small intestinal and fecal proteases. Results (Table 1) showed not only that protease activity was higher in small gut contents compared with feces, but also that fecal proteolysis was qualitatively different with respect to the types of protein substrate hydrolyzed. Studies with protease inhibitors (Table 2) confirmed that there were fundamental differences in small and large gut proteolysis. Small intestinal proteases were strongly inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), together with the trypsin and chymotrypsin inhibitors soybean trypsin inhibitor (STI) and chymostatin. The aspartic protease inhibitor pepstatin A, the cysteine protease inhibitors thimerosal and iodoacetate, and the metalloprotease inhibitors EDTA and cysteine did not significantly reduce protease activity. The pattern of inhibition was considerably different in fecal slurries, however, where the major inhibitors were PMSF and thimerosal. In comparison with small gut contents, the degree of inhibition by STI and chymostatin was almost halved, but inhibition was between 2- and 25-fold greater with iodoacetate, EDTA, and cysteine. These data confirmed that whereas ileal effluent contained only serine proteases, the fecal slurries contained a mixture of serine, cysteine, and metalloproteases.

Inhibition of proteolytic activity of fecal bacteria. Physical disruption of washed fecal bacteria released large amounts of proteases. Compared with that of whole cells, protease activity in the crude cell homogenates was greater by a factor of about 6 (Table 3). The supernatant remaining after removal of the cell walls retained approximately 74% of the original activity. Experiments with protease inhibitors demonstrated the predominance of serine proteases in all cell fractions (46 to 58% inhibition with PMSF). Inhibition by thimerosal, iodoacetate, cysteine, and EDTA (ca. 20 to 40%) showed the presence of cysteine and metalloproteases, while the increased inhibition of proteolysis in the crude homogenate by STI and chymostatin indicated the presence of intracellular trypsin- and chymotrypsinlike enzymes.

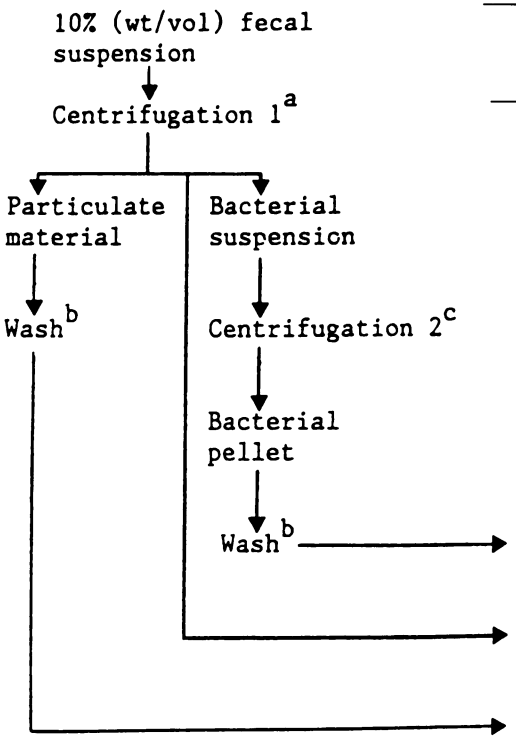
Hydrolysis of synthetic protease substrates by fecal bacteria. Studies with protease inhibitors suggested that trypsin- and chymotrypsin-like enzymes were associated with washed fecal bacteria (Table 3). This was confirmed using synthetic *p*-nitroanilide substrates (Table 4). Whole cells hydrolyzed glutarylphenylalanine *p*-nitroanilide and benzoylarginine *p*-nitroanilide, which are respective substrates for chymotrypsin and trypsin (10, 18). Hydrolysis of the elastase substrate succinylalanylalanylalanine *p*-nitroanilide was also detected in whole-cell preparations. When the bacteria were disrupted, glutarylphenylalanine *p*-nitroanilide hydrolysis increased fourfold in the crude homogenate and soluble fractions, supporting the inhibition data that suggested the presence of intracellular chymotrypsinlike enzymes. Trypsin and elastase activities did not significantly increase, however. In general, only a minor proportion of these hydrolytic activities were associated with the washed cell walls, indicating that the enzymes were cytoplasmic or, more likely, membrane bound.

DISCUSSION

A wide variety of proteins such as collagen, serum albumins, plant proteins, and microbial proteins are potentially available as substrates for bacteria in the human large intestine. Quantitatively, pancreatic secretions, containing a variety of hydrolytic enzymes (proteases, peptidases, amylase, and lipase), are probably among the most significant sources of protein. It has been estimated that about 1 to 3 g of both trypsin and chymotrypsin and 0.5 g of elastase can be produced by the pancreas each day (15). Pancreatic proteases probably have a dual role in the colon, acting as hydrolytic enzymes that take part in protein breakdown and also serving as substrates for bacterial proteases, thereby contributing to the pool of organic nitrogen available to the microflora.

Results obtained in this study demonstrated that proteolysis in the small intestine and large bowel differed both quantitatively and qualitatively (Table 1). Quantitatively,

TABLE 1. Hydrolysis of native and diazotized proteins by small intestinal contents and different fractions of human feces



Sample from:	Hydrolysis of substrate[d]							
	Casein	BSA	Collagen	Ovalbumin	Azocasein	Azoalbumin	Azocoll	Azosoybean flour
Ileal effluent	1,214 ± 546	0 ± 0	281 ± 35	131 ± 48	319 ± 45	208 ± 34	150 ± 21	333 ± 55
Feces	20 ± 2	2 ± 1	6 ± 2	2 ± 0.5	11 ± 6	9 ± 7	5 ± 4	6 ± 3
Washed cell fraction	5.20 ± 2.70	0.09 ± 0.13	0.50 ± 0.26	0.23 ± 0.16	0.41 ± 0.15	0.03 ± 0.02	0.19 ± 0.14	0.29 ± 0.04
Cell-free supernatant	3.00 ± 2.00	0.09 ± 0.13	0.67 ± 0.26	0.47 ± 0.27	0.17 ± 0.24	0.01 ± 0.01	0.31 ± 0.15	0.34 ± 0.23
Washed particulate fraction	7.10 ± 3.30	0.08 ± 0.09	0.38 ± 0.36	0.18 ± 0.17	0.89 ± 0.47	0.07 ± 0.03	0.29 ± 0.14	0.54 ± 0.23

[a] At 1,500 × *g* (15 min).
[b] Material was washed twice with anaerobic phosphate buffer and finally suspended to the original volume.
[c] At 30,000 × *g* (15 min).
[d] Milligrams of protein or azoprotein hydrolyzed per hour per gram (wet weight). Values are means of measurements on feces from five healthy individuals and ileal contents from five ileostomy subjects ± standard deviation.

TABLE 2. Inhibition of proteolysis ileal effluent and fecal slurries

Sample from:	% Inhibition of proteolysis[a]							
	PMSF	STI	Chymostatin	Pepstatin A	Thimerosal	Iodoacetate	EDTA	Cysteine
Ileal effluent[b]	97 ± 5.8	43 ± 4.9	53 ± 29.5	11 ± 4.2	15 ± 7.6	12 ± 3.7	2 ± 0.2	1 ± 0.4
Fecal slurry (10%, wt/vol)[b]	42 ± 6.5	28 ± 9.6	23 ± 8.8	6 ± 3.2	42 ± 10.1	23 ± 5.1	17 ± 2.6	25 ± 3.1

[a] Results are presented as mean values ± standard deviation.
[b] Samples were obtained from five persons.

proteolysis in feces was an order of magnitude lower than that in ileal effluent, suggesting that considerable breakdown or inactivation of pancreatic proteases occurs during large gut transit. Apart from their breakdown by colonic bacteria, pancreatic proteases are known to be adsorbed onto intestinal epithelial cells (1, 11) while chymotrypsin binds firmly to particulate material in gut contents (14). Inactivation by host-produced antiproteases may also influence the recoveries of the enzymes in feces (2, 3).

Fecal protease activity was qualitatively different from that in the small gut, as evidenced by the range of proteins hydrolyzed and the susceptibility of the enzymes to protease inhibitors. Results (Table 1) showed that globular proteins were poorly degraded by proteases in ileal effluent and, in the case of BSA, completely recalcitrant to hydrolysis, whereas this substrate was hydrolyzed by proteases in feces. Highly globular proteins such as ovalbumin and serum albumin have also been shown to be resistant to hydrolysis by rumen microorganisms (21, 22). From these and later studies it was concluded that protein structure was an important determinant of the degradability of a protein (25). In the large gut, however, other factors that may affect the breakdown of proteins include gut pH and possibly their occurrence in complexes with polysaccharides that restricts the accessibility of the protein to hydrolytic enzymes.

Experiments with protease inhibitors (Table 2) demonstrated that fecal and small gut proteases had different sensitivities to these substances. The serine proteases in ileal effluent were in large part replaced by cysteine and metalloproteases in feces, as shown by the increased inhibition by thimerosal and iodoacetate and by EDTA and cysteine, respectively. The inhibitory effects of the trypsin and chymotrypsin inhibitors (STI and chymostatin) were correspondingly lower in feces, confirming that there was a reduction in the contribution of pancreatic enzymes to fecal proteolysis.

With the exception of azoalbumin, proteases in ileal effluent and feces hydrolyzed native proteins to a greater extent than did their diazotized derivatives (Table 1). It is unlikely that this was due to an enzymatic reduction of the released azodyes by fecal bacteria, since this process has been found to be insignificant over the short incubation periods used in the protease assays (18). The decreased susceptibility to proteolysis of the diazotized proteins could be explained by steric effects caused by the sulfanilamide adduct on the protein molecule at or near the sites of peptide cleavage. Alternatively, changes in conformation of the proteins as a result of diazotization may restrict access for the proteases. Alterations in protein tertiary structure due to diazotization probably explain the reverse effect observed with azoalbumin, that is, facilitation of hydrolysis by making sites of peptide cleavage more accessible for proteases.

A significant proportion of the protease activity in feces was associated with bacterial cells (Table 1). The activities of proteolytic bacteria may be important in the microbial ecology of the colon, since the breakdown of proteins resistant to the action of pancreatic proteases would also increase the nutrients available to nonproteolytic species. Experiments with protease inhibitors showed that trypsin- and chymotrypsinlike enzymes did not occur to a significant extent on the surface of fecal bacteria (Table 3). However, protease activities increased by a factor of about 6 when the cells were disrupted, and the increased inhibitory effects of chymostatin and STI in the extracts indicated the presence of intracellular trypsin- and chymotrypsin-like enzymes. Cysteine proteases also appeared to be important intracellularly, as indicated by the increased inhibition by thimerosal. The occurrence of intracellular trypsin- and chymotrypsin-like enzymes was supported by measurements with synthetic *p*-nitroanilide substrates (Table 4); moreover, the hydrolysis of succinylalanylalanylalanine *p*-nitroanilide demonstrated that there was also elastase-like activity associated with the bacteria.

Intracellular bacterial proteases are probably important in the colon in that they not only will function in protein turnover inside the bacteria, but also, after cell lysis, will contribute to general proteolysis in gut contents. While there is evidence that large amounts of protease are released by aging cultures of colonic bacteroides (10) and that similar enzymes occur in feces (18), it is still not clear to what extent the bacterial component of protease activity in the cell-free supernatant fraction of feces is due to true extracellular proteolytic enzymes such as those produced by clostridia or propionibacteria (13, 18) or to cell lysis.

TABLE 3. Effect of inhibitors on proteolysis by whole bacteria and bacterial cell extracts obtained from the washed cell fraction of feces

Bacterial fraction	Mean protease activity	% Inhibition of proteolysis[a]							
		PMSF	STI	Chymostatin	Pepstatin A	Thimerosal	Iodoacetate	EDTA	Cysteine
Whole cells	74 ± 15	56 ± 8	14 ± 1	10 ± 5	8 ± 3	27 ± 9	25 ± 7	24 ± 6	29 ± 16
Crude homogenate	460 ± 58	48 ± 8	20 ± 22	20 ± 23	12 ± 6	26 ± 22	12 ± 4	28 ± 14	20 ± 23
Suspended cell wall material	58 ± 35	46 ± 7	19 ± 9	9 ± 12	5 ± 4	22 ± 20	4 ± 3	7 ± 6	16 ± 14
Soluble fraction	342 ± 72	52 ± 6	21 ± 14	15 ± 12	7 ± 4	44 ± 7	11 ± 2	18 ± 11	15 ± 13

[a] Samples were prepared from feces obtained from five different individuals, using procedures described by Macfarlane et al. (18). Values are given as mean ± standard deviation.

TABLE 4. Chymotrypsin-, trypsin-, and elastaselike activities associated with fecal bacteria

Bacterial fraction[b]	Hydrolysis of substrate[a]		
	Chymotrypsin substrate (GPPNA)	Trypsin substrate (BAPNA)	Elastase substrate (SAAAPNA)
Whole cells	2 ± 1	2 ± 1	221 ± 166
Crude homogenate	8 ± 3	2 ± 1	259 ± 173
Suspended cell wall material	2 ± 1	2 ± 2	72 ± 68
Soluble fraction	8 ± 2	3 ± 2	225 ± 292

[a] Nanomoles of *p*-nitroaniline released per hour per milliliter. Values are given as means ± standard deviation. Abbreviations: GPPNA, glutarylphenylalanine *p*-nitroanilide; BAPNA, benzoylarginine *p*-nitroanilide; SAAAPNA, succinylalanylalanylalanine *p*-nitroanilide.

[b] Samples were prepared from feces from five different individuals as described in Materials and Methods.

In the disrupted cells the majority of the protease activity occurred in the soluble fraction, which contained cytoplasmic and membrane-bound enzymes. Only a minor proportion of the activity was recovered with the washed cell wall material (Table 4). This was not surprising since there is little documentation of cell wall-associated proteases in the literature, although they have been reported to occur in *Lactococcus lactis* (9, 24).

The data presented in this paper show that human gut contents contain a diverse range of proteolytic enzymes. In a previous survey of the proteolytic bacteria in feces (19), bacteroides, clostridia, and propionibacteria were found to be the numerically predominant organisms, yet none of these bacteria produce cell-associated proteases that have characteristics of either the trypsin-, chymotrypsin-, or elastaselike enzymes found in mixed populations of fecal bacteria in this study.

We have previously ascribed to the bacteroides a major role in protein turnover in the large gut; however, the serine, cysteine, and metalloproteases of these bacteria do not hydrolyze proteins such as collagen and the globular proteins BSA and ovalbumin (10). It seems likely, therefore, that hitherto unrecognized subpopulations of proteolytic bacteria may have a key role in the breakdown of similar proteins in the gut.

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