Studies on the Proteolytic Activity of *Bacteroides fragilis*

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The proteolytic activity of the intestinal bacterium *Bacteroides fragilis* NCDO 2217 was cell-bound during exponential growth, but was progressively released from the cells in stationary phase. Proteins hydrolysed included casein, trypsin, chymotrypsin, azocasein and the proteins in azosoya bean flour. Collagen, azocoll, elastin, gelatin, ovalbumin and bovine serum albumin were either weakly degraded or completely refractory to proteolysis. Arylamidase activity was exhibited against leucine *p*-nitroanilide (LPNA), leucine β -naphthylamide, glycyl-proline *p*-nitroanilide and valyl-alanine *p*-nitroanilide. The bacterium grew with ammonia, peptone or casein as sole nitrogen source. Azocasein- and LPNA-hydrolysing activities were consistently higher when grown on casein. Cell-bound protease activity increased concomitantly with growth rate in both carbon- and nitrogen-limited continuous culture. Leucine arylamidase activity was also growth-rate-dependent, being 3-fold greater at $D = 0.18 \text{ h}^{-1}$ compared to $D = 0.03 \text{ h}^{-1}$. Extracellular proteolytic activity was only detected at low growth rates, accounting for about 25% of total protease activity.

INTRODUCTION

Growth of saccharolytic bacteria in the human large intestine appears to be effectively limited by the availability of fermentable carbohydrate, while comparatively high levels of protein and ammonia occur throughout the length of the gut (Macfarlane *et al.*, 1986). Intestinal contents are strongly proteolytic and although much of this activity is undoubtedly pancreatic in origin (Bohe *et al.*, 1983), recent evidence has indicated that colonic bacteria, particularly *Bacteroides* spp., may contribute substantially to protein breakdown (Macfarlane *et al.*, 1985, 1986; Macfarlane & Allison, 1986). At present, however, little is known concerning the factors that control bacterial proteolysis in the large gut, or of the significance of this process in the microbial ecology of the colon. *Bacteroides fragilis* group organisms are the numerically predominant anaerobes in the large gut, and as such account for about 30% of bacteria isolated from faeces (Macy & Probst, 1979; Salyers, 1984). The major activity of these bacteria in the colon appears to be that of polysaccharide degradation, a process that has been extensively studied (Salyers *et al.*, 1977; Salyers & O'Brien, 1980; Gherardini *et al.*, 1985). The proteolytic capabilities of colonic bacteroides have so far received little attention, however.

Preliminary investigations in our laboratory indicated that different strains from the *Bacteroides fragilis* group possessed similar proteolytic activities, although the levels of protease activity varied considerably between individual species (Cummings *et al.*, 1986). In this paper, we report the results of further studies on the proteolytic activity of *Bacteroides fragilis* NCDO 2217.

METHODS

Organism. *Bacteroides fragilis* NCDO 2217 (Loesche *et al.*, 1964) was obtained from the National Institute for Research in Dairying, Shinfield, Reading, UK. Confirmation of the identity of the bacterium was obtained using procedures described by Holdeman *et al.* (1977).

Abbreviations: LPNA, leucine *p*-nitroanilide; N-CBZ-, N-carbobenzoxy-; VAPNA, valyl-alanine *p*-nitroanilide; VFA, volatile fatty acids.

Location of proteases. Bacteria were grown in batch culture on Wilkins-Chalgren broth in 1 l fermenters. Anaerobic conditions were maintained by sparging cultures with a gas mixture containing 10% H₂, 10% CO₂ and 80% N₂. Temperature (37 °C) and pH (6.8) were controlled as described by Macfarlane & Englyst (1986). Bacterial growth was determined by measuring the optical density at 650 nm. For measurement of proteolytic activity, 20 ml vols of culture were centrifuged at 20000 *g* for 30 min. The cell pellets were washed and resuspended in 20 ml anaerobic 0.1 M-sodium phosphate buffer, pH 7.4. Protease activities were determined in the resuspended cells and cell-free culture supernatants. Protease activities were measured as follows. Samples (1 ml) were dispensed in triplicate into 1.5 ml capacity Eppendorf tubes and mixed with 0.2 ml phosphate buffer. The reactions were begun by the addition of 0.3 ml azocasein stock solution, which gave an initial substrate concentration of 10 mg ml⁻¹. The tubes were capped and incubated for 2 h at 37 °C. The reactions were stopped by transferring the tube contents into centrifuge tubes that contained 1 ml 10% (w/v) TCA. Control tubes were incubated as above, but without azocasein, which was added after inactivation of the sample with TCA. After samples had been allowed to stand for 30 min, they were centrifuged at 27000 *g* for 8 min. Thereafter, 1 ml of supernatant was mixed with an equal volume of 1.0 M-NaOH. The absorbance of this solution was then read at 450 nm.

Hydrolysis of different protein substrates. *B. fragilis* was grown and harvested as described above. Cells from 200 ml of culture were resuspended and washed twice in 100 ml phosphate buffer. The ability of the cell-bound proteases to hydrolyse a range of native and diazotized proteins was then examined. Samples of bacteria (5 ml) were added to 5 ml of a solution containing one of the following protein substrates (all 2%, w/v): casein, BSA grade V, ovalbumin grade V, collagen grade V, gelatin, trypsin, chymotrypsin, azocoll, azoalbumin or azosoya bean flour. Bacteria were incubated with the proteins at 37 °C. Samples (1 ml) were taken at zero time, and at 2, 4 and 6 h, and added to 1 ml 10% (w/v) TCA. Peptides released from native proteins were measured using the Lowry method. Dye released from the diazotized proteins was measured spectrophotometrically at 450 nm as described previously. The release of orcein dye from elastin-orcein was determined using the method of Appel (1984a). Breakdown of trypsin and chymotrypsin was followed by the decrease in the activities of these enzymes towards their *p*-nitroanilide substrates (benzoyl-arginine *p*-nitroanilide and glutaryl-phenylalanine *p*-nitroanilide respectively) during incubation with bacteria. Boiled cells were used as controls.

Hydrolysis of *p*-nitroanilide substrates. The ability of *B. fragilis* proteases to hydrolyse a range of synthetic *p*-nitroanilide substrates was investigated using a modification of the method of Appel (1984b). The following L-amino acid *p*-nitroanilides were tested: benzoyl-arginine *p*-nitroanilide, leucine *p*-nitroanilide (LPNA), valyl-alanine *p*-nitroanilide (VAPNA), glutaryl-phenylalanine *p*-nitroanilide, glutamyl *p*-nitroanilide, succinyl-alanyl-alanyl-alanine *p*-nitroanilide, benzyl-cysteine *p*-nitroanilide, glycyl-proline *p*-nitroanilide, benzyl-tyrosine *p*-nitroanilide and lysine *p*-nitroanilide. Washed cells of *B. fragilis* were obtained as described above, except that the bacteria were resuspended in 0.05 M-Tris/HCl, pH 7.4. Samples (0.5 ml) were dispensed in triplicate into 1.5 ml capacity Eppendorf tubes. Samples (1.0 ml) of substrate (1.2 mM in 0.05 M-Tris/HCl, pH 7.4) were then added to the tubes to start the reaction. The capped tubes were incubated at 37 °C for 15 min in the case of VAPNA, and 1 h for the other *p*-nitroanilides. Reactions were stopped by the addition of 0.1 ml perchloric acid (70%, w/v). After standing for 30 min the samples were centrifuged at 11600 *g* for 10 min. The supernatant was retained. *p*-Nitroaniline released by hydrolysis of the synthetic substrates was measured by diazotization (Appel, 1984b).

Hydrolysis of β -naphthylamide substrates. The ability of *B. fragilis* to hydrolyse the following substrates was tested: alanine β -naphthylamide, leucine β -naphthylamide, serine β -naphthylamide, pyrrolidonyl β -naphthylamide, proline β -naphthylamide, cystine β -naphthylamide and valine- β -naphthylamide. Release of β -naphthylamine, which indicated a positive reaction, was determined using procedures for measuring leucine-aminopeptidase activity, described in Sigma Technical Bulletin 251.

Carboxypeptidase measurements. These assays were done using the colorimetric method described by Appel (1984c) in which the amino acid or peptide released by C-terminal hydrolysis is detected by the ninhydrin reaction. The following substrates were used: N-CBZ-glycyl-alanine, N-CBZ-glutamyl-phenylalanine, N-CBZ-valyl-alanine methyl ester, N-CBZ-proline and N-CBZ-aspartate.

Utilization of casein by *B. fragilis*. The bacteria were grown in batch culture as before. The mineral salts medium used contained (l⁻¹): glucose, 10.0 g; KH₂PO₄, 4.0 g; Na₂HPO₄, 2.0 g; NaCl, 9.0 g; cysteine, 0.8 g; NaHCO₃, 1.5 g; CaCl₂, 0.1 g; MgCl₂, 0.15 g; MnCl₂, 0.10 g; CoCl₂, 0.10 g; FeSO₄, 1.0 mg; haemin, 5.0 mg; vitamin B₁₂, 0.5 mg. Casein (0.5%) was the sole nitrogen source. Bacterial growth was followed by direct counting methods using a Neubauer counting chamber (Cruickshank, 1965). Protein and peptides were measured as follows. Samples were centrifuged at 20000 *g* for 30 min to remove bacteria. The supernatants were retained and divided into two equal portions, one of which was treated with an equal volume of 10% (w/v) TCA. The other was added to an equal volume of 0.1 M-sodium phosphate buffer, pH 7.4 and was used to measure the soluble protein remaining in the culture. The TCA-treated samples were allowed to stand for 30 min at room temperature, then centrifuged at 27000 *g* for 10 min. The resulting supernatants were used for measurement of TCA-soluble peptides and the pellets were resuspended in phosphate buffer for determination of TCA-insoluble protein. Protein and peptides were measured by the Lowry method.

Effect of nitrogen source on protease and arylamidase activity. Bacteria were grown in batch culture on the mineral salts medium containing NH_4Cl , peptone water or casein as nitrogen source (all 0.5%). In experiments where the effects of protein and peptides on proteolysis were studied, casein, BSA or peptone (5%, w/v) were dissolved in 0.05 M-Tris/HCl, pH 7.4. Samples (75, 150, 225, 300 or 450 μl) of each solution were dispensed in triplicate into 1.5 ml capacity Eppendorf tubes containing 500, 425, 350, 275, 200 or 50 μl of the Tris buffer. The washed bacteria (0.5 ml) were then added to the tubes and the mixtures were incubated for 15 min at 37 °C. The reactions were initiated by addition of the Tris buffer (0.5 ml) containing VAPNA or LPNA (both 2.4 mM). Arylamidase activities were determined as described above.

Effect of ammonia, volatile fatty acids and haemin on protease and arylamidase activity. Batch cultures of *B. fragilis* were grown on mineral salts medium in experiments where the effect of ammonia on protease production was studied and in Wilkins-Chalgren broth to determine the effect of volatile fatty acids (VFA). Filter sterilized fatty acid mixtures (acetate, propionate and butyrate, molar ratio 2:1:1) were added to the medium after autoclaving. To determine its influence on protease production, haemin was omitted from the mineral salts medium. Fermentation products were measured using the methods of Holdeman *et al.* (1977).

Continuous culture studies. Bacteria were grown in continuous culture using 0.5 l (working volume) glass chemostats. Temperature (37 °C), pH (6.8) and the maintenance of anaerobic conditions were controlled as described by Macfarlane & Englyst (1986). The basal growth medium was the mineral salts medium described above. Glucose and NH_4Cl concentrations in the carbon-limiting medium were 5 g l⁻¹ and 1 g l⁻¹ respectively, and in the nitrogen-limiting medium were 15 g l⁻¹ and 1 g l⁻¹. Bacteria were harvested by centrifugation as before. The cell-free supernatants were retained for measurement of extracellular protease and arylamidase activities. After washing, the cell pellets were used for determination of cell-bound protease and arylamidase activities.

Dry weight measurements. Culture dry weights were determined using methods described by Keith & Herbert (1983).

Chemicals. Casein was obtained from BDH. All other chemicals were supplied by Sigma. Bacteriological media were purchased from Oxoid.

RESULTS

Location of proteases. *B. fragilis* proteases were totally cell-associated during the initial stages of growth in batch culture (Fig. 1). Cell-bound proteolytic activity increased concomitantly with growth until stationary phase was reached. As growth slowed, however, protease activity accumulated in the culture medium. This was accompanied by only a slight decrease in culture optical density and cell-bound protease activity.

Proteins hydrolysed by B. fragilis proteases. *B. fragilis* proteases exhibited a degree of specificity with respect to the hydrolysis of protein substrates (Table 1). Of the proteins tested, only casein, trypsin, chymotrypsin, azocasein and the proteins in azosoya bean flour were hydrolysed to any significant extent. The highly globular proteins ovalbumin, BSA and azoalbumin were poor substrates. It was considered possible that low levels of BSA hydrolysis had occurred, but had been concealed by assimilation of the peptide products by the bacteria. However, an advantage of using diazotized substrates to detect proteolysis by *B. fragilis* was that the dye released during protein breakdown was stable and was not reduced by the bacteria. The absence of dye release from azoalbumin therefore provided corroboratory evidence as to the resistance of these globular proteins to *B. fragilis* proteases. The insoluble proteins elastin, gelatin, collagen and azocoll (diazotized collagen) similarly did not serve as substrates for *B. fragilis* proteases.

Arylamidase and carboxypeptidase activities. As well as peptide bonds, some proteolytic enzymes can catalyse the hydrolysis of the amino-acyl bond between amino acids and *p*-nitroaniline or β -naphthylamine. *B. fragilis* proteases hydrolysed LPNA, VAPNA, glycyl-proline *p*-nitroanilide and leucine β -naphthylamide (Table 1). No activity was detected against the trypsin substrate benzoyl-arginine *p*-nitroanilide, the chymotrypsin substrate glutaryl-phenylalanine *p*-nitroanilide or the elastase substrate succinyl-alanyl-alanyl-alanine *p*-nitroanilide. Carboxypeptidase activity was not found against any of the N-CBZ amino acid substrates (Table 1).

Utilization of casein during growth. Casein was hydrolysed by *B. fragilis* in exponential and stationary phase cultures (Fig. 2). The proteolysis occurring during active growth did not result in the accumulation of TCA-soluble peptides in the culture medium, indicating that *B. fragilis*

Fig. 1. Location of *B. fragilis* proteases. ●, OD_{650} ; ▲, cell-bound protease activity; ■, extracellular protease activity. Data are means of three experiments.

Table 1. *Proteins and synthetic substrates hydrolysed by B. fragilis proteases*

Exponentially growing bacteria were harvested and washed before use in enzyme assays. Breakdown of trypsin and chymotrypsin was monitored by following the decrease in arylamidase activity against their respective substrates, benzoyl-arginine *p*-nitroanilide and glutaryl-phenylalanine *p*-nitroanilide. Results are presented as the means of between two and five experiments $\pm$ SD, where appropriate.

Protein substrate	Hydrolysis*	Carboxypeptidase substrate	Hydrolysis	Arylamidase substrate	Hydrolysis†
Casein	188 ± 56	N-CBZ-Glycyl-alanine	—	Leucine <i>p</i> -nitroanilide	22.3 ± 7.6
BSA	—	N-CBZ-Glutamyl-phenylalanine	—	Benzoyl-arginine <i>p</i> -nitroanilide	—
Ovalbumin	1.0 ± 0.4	N-CBZ-Valyl-alanine methyl ester	—	Glutaryl-phenylalanine <i>p</i> -nitroanilide	—
Collagen	2.1 ± 0.5	N-CBZ-Proline	—	Glutamyl <i>p</i> -nitroanilide	0.2 ± 0.1
Trypsin	+	N-CBZ-Aspartate	—	Valyl-alanine <i>p</i> -nitroanilide	368 ± 102
Chymotrypsin	+			Succinyl-alanyl-alanyl-alanine <i>p</i> -nitroanilide	—
Gelatin	—			Benzyl-cysteine <i>p</i> -nitroanilide	—
Elastin-orcein	—			Glycyl-proline <i>p</i> -nitroanilide	243 ± 41
Azsoya bean flour	152 ± 15			Benzyl-tyrosine <i>p</i> -nitroanilide	—
Azocasein	215 ± 12			Lysine <i>p</i> -nitroanilide	—
Azoalbumin	—			Pyrrolidonyl β -naphthylamide	—
Azocoll	—			Alanine β -naphthylamide	—
				Proline β -naphthylamide	—
				Leucine β -naphthylamide	18.7 ± 6.8
				Cystine β -naphthylamide	—
				Serine β -naphthylamide	0.3 ± 0.2
				Valine β -naphthylamide	—

* μ g protein hydrolysed h^{-1} (mg dry wt cells) $^{-1}$.

† nmol *p*-nitroaniline or β -naphthylamine produced h^{-1} (mg dry wt cells) $^{-1}$.

+, Protein breakdown; —, no hydrolysis.

assimilated the products of casein hydrolysis. Protein breakdown continued after growth ceased but the rate of peptide uptake was markedly reduced, resulting in a progressive accumulation of these hydrolysis products.

Effect of nitrogen source on protease and arylamidase activities. *B. fragilis* grew with ammonia, peptone or casein as nitrogen source, with growth being fastest on ammonia (Table 2). Analysis of fermentation products showed that spent medium did not contain branched-chain VFA and that only marginally higher levels of acetate and propionate were present after growth on

Fig. 2

Fig. 2. Breakdown of casein by *B. fragilis* during growth in batch culture. ●, Total protein and peptides; ■, casein; ▲, TCA-soluble peptides; ○, cell numbers. Results are the means of duplicate experiments.

Fig. 3

Fig. 3. Effect of protein and peptides on arylamidase activities of *B. fragilis* proteases. Data are shown as fractions of control activities. Closed symbols, LPNA activities; open symbols, VAPNA activities. Cell-bound arylamidase activities are shown in the presence of BSA (■, □), casein (▲, △) and peptone (●, ○).

Table 2. Effects of nitrogen source on growth and protease activities

Bacteria were grown in batch culture on mineral salts medium with glucose (1%, w/v) as carbon source and either ammonia, peptone or casein (all 0.5%) as nitrogen source. Samples were taken for measurement of protease and arylamidase activities during the exponential phase of growth. Results are the means of three experiments $\pm$ SD.

Nitrogen source in growth medium	Doubling time (h)	Protease activity*	Arylamidase activity†	
			LPNA	VAPNA
Ammonia	1.61 $\pm$ 0.38	43 $\pm$ 14	13 $\pm$ 2	224 $\pm$ 24
Peptone	2.94 $\pm$ 0.86	38 $\pm$ 7	23 $\pm$ 1	299 $\pm$ 40
Casein	2.44 $\pm$ 0.83	141 $\pm$ 39	78 $\pm$ 5	364 $\pm$ 10

* μg azocasein hydrolysed h^{-1} (mg dry wt cells) $^{-1}$.

† nmol *p*-nitroaniline produced h^{-1} (mg dry wt cells) $^{-1}$.

peptone or casein. Protease activity was greatest when the bacteria were grown on casein and this was accompanied by a 6-fold increase in leucine arylamidase activity and a 63% increase in VAPNA hydrolysis compared to ammonia-grown cultures. Glycyl-proline *p*-nitroanilide was not used as an arylamidase substrate in these and subsequent studies since it was found that the enzyme which catalysed the hydrolysis of this substrate was identical to that which hydrolysed VAPNA (unpublished results).

Stimulation of arylamidase activity by protein and peptone. BSA stimulated LPNA hydrolysis by *B. fragilis* at all concentrations tested (Fig. 3), however no effect on valyl-alanine arylamidase activity was observed. Casein concentrations up to 5 mg ml^{-1} stimulated leucine arylamidase but higher concentrations had progressively less effect. In contrast, casein (15 mg ml^{-1}) inhibited the hydrolysis of VAPNA by up to 30%. The inhibition of both LPNA and VAPNA hydrolysis by peptone was concentration dependent (90% at 15 mg ml^{-1}).

Table 3. *Effect of haemin on the expression of protease activity*

Bacteria were grown on mineral salts medium in batch culture at 37 °C for 24 h. Glucose (1%, w/v) was the carbon source and ammonia (0.5%, w/v) was the nitrogen source. Results are the means of two experiments.

Growth medium	Protease activity*	Arylamidase activity†		Fermentation products (mM)				Cell yield (mg dry wt cells ml ⁻¹)
		LPNA	VAPNA	Acetate	Propionate	Fumarate	Succinate	
With haemin	181	56	840	9.0	4.0	ND	2.3	0.96
No haemin	174	54	789	4.1	ND	3.4	0.2	0.19

* μg azocasein hydrolysed h^{-1} (mg dry wt cells)⁻¹.

† nmol *p*-nitroaniline produced h^{-1} (mg dry wt cells)⁻¹.

ND, Not detected.

Table 4. *Effect of growth rate and nutrient limitation on protease and arylamidase activities*

Bacteria were grown under carbon limitation (5 g glucose l⁻¹, 1 g NH₄Cl l⁻¹) and nitrogen limitation (15 g glucose l⁻¹, 1 g NH₄Cl l⁻¹) at various dilution rates. Anaerobic conditions were maintained by sparging cultures with a gas mixture containing 10% CO₂, 10% H₂ and 80% N₂. Results are the means of four experiments.

Dilution rate, <i>D</i> (h ⁻¹)	Limiting nutrient	Protease activity*		Specific cell-bound protease activity†	Arylamidase activity‡				Specific cell-bound arylamidase activity§	
					Extracellular		Cell-bound			
		Extracellular	Cell-bound		LPNA	VAPNA	LPNA	VAPNA	LPNA	VAPNA
0.03	Ammonia	25	73	88	ND	29	26	248	32	303
	Glucose	32	98	108	ND	29	31	207	34	227
0.06	Ammonia	24	74	91	ND	28	23	249	28	308
	Glucose	24	96	110	ND	34	27	139	31	160
0.12	Ammonia	14	204	215	ND	23	75	306	79	322
	Glucose	25	105	148	ND	29	40	185	56	260
0.18	Ammonia	ND	248	223	ND	ND	116	336	105	305
	Glucose	ND	110	170	ND	ND	31	176	78	270

* μg azocasein hydrolysed h^{-1} ml⁻¹.

† μg azocasein hydrolysed h^{-1} (mg dry wt cells)⁻¹.

‡ nmol *p*-nitroaniline produced h^{-1} ml⁻¹.

§ nmol *p*-nitroaniline produced h^{-1} (mg dry wt cells)⁻¹.

ND, Not detected.

Effect of ammonia, VFA and haemin on the expression of protease and arylamidase activities. The human large gut is characterised by the presence of high levels of VFA and ammonia. Experiments were therefore undertaken to ascertain whether these metabolites had an effect on protease activity. Increasing ammonia concentrations from 1 mM to 100 mM in growth media repressed valyl-alanine arylamidase and azocasein hydrolysing activity by 47% and 19% respectively. Leucine arylamidase was unaffected, however. High concentrations of VFA (up to 80 mM) neither stimulated nor repressed protease activity. When haemin was omitted from the growth medium, cell yields were greatly reduced and fumarate accumulated in the medium (Table 3). Cell-bound protease specific activities were completely unaffected, however.

Continuous culture experiments. Extracellular protease activity was observed in continuous cultures of *B. fragilis* grown at low dilution rates ($D = 0.03 \text{ h}^{-1}$ – $D = 0.12 \text{ h}^{-1}$) under both carbon and nitrogen limitation. This accounted for about 25% of total protease activity in the cultures (Table 4). Extracellular LPNA hydrolysis was never found, although low levels of extracellular

VAPNA-hydrolysing activity occurred (23–24 nmol *p*-nitroaniline produced h⁻¹ ml⁻¹). Cell-bound protease activity was strongly growth-rate-dependent, particularly in nitrogen-limited cultures. Leucine arylamidase activity was stimulated 3-fold by increasing the growth rate from a generation time of 23.1 h to that of 3.8 h, but valyl-alanine arylamidase activity appeared to be little affected.

DISCUSSION

Although *B. fragilis* proteases were completely cell-bound during active growth in batch culture, substantial quantities of proteolytic activity were released from stationary phase bacteria. In this respect, *B. fragilis* proteases behaved similarly to those of the rumen species *B. ruminicola* (Hazlewood & Edwards, 1981).

Proteolytic activity in human faeces is cell-bound and extracellular (Macfarlane *et al.*, 1986). The release of proteolytic activity from older cells of *B. fragilis* suggests that colonic bacteroides may contribute towards extracellular proteolysis in the gut, as well as that associated with the bacteria.

A comparatively restricted range of proteins and synthetic substrates was hydrolysed by *B. fragilis*, indicating that the proteases of this bacterium are different to those of other *Bacteroides* spp. For example, BSA is hydrolysed by the proteases of *B. ruminicola* (Hazlewood & Edwards, 1981) and collagen is degraded by the extracellular trypsin-like proteases of *B. melaninogenicus* (Gibbons & Macdonald, 1961) and by other black-pigmented *Bacteroides* species (van Steenberg & de Graaff, 1986). The apparent weak hydrolysis of collagen by *B. fragilis* was probably not a true collagenolytic activity, since many general proteases can hydrolyse parts of the collagen molecule outwith the helical domain of the protein (Siefter & Harper, 1971).

The inability of *B. fragilis* to degrade collagen and elastin is perhaps not surprising since these proteins are efficiently hydrolysed by pancreatic proteases in the small intestine and in normal circumstances they are unlikely to be readily available as substrates for colonic bacteria. The pancreatic proteases trypsin and chymotrypsin do enter the colon in substantial amounts, however, and are extensively degraded by gut bacteria (Bohe *et al.*, 1983). These proteins may well serve as substrates for *B. fragilis* type organisms *in vivo*. Indeed, we have found that *B. fragilis* can grow with trypsin or chymotrypsin as sole nitrogen source (unpublished results).

Studies on casein utilization showed that the protein was hydrolysed during exponential and stationary phases of growth. TCA-soluble peptides only accumulated in the culture medium in the stationary phase, however, suggesting that protein utilization by actively growing bacteria was limited by the rate of casein hydrolysis rather than by the rate of peptide assimilation.

Azocasein- and LPNA-hydrolysing activities were higher in bacteria grown on casein compared to those grown on peptone or ammonia (Table 2). BSA, a protein which is refractile to hydrolysis by *B. fragilis* proteases, also stimulated leucine arylamidase activity (Fig. 3) but when BSA was washed from the cells, this stimulation was abolished (data not shown), indicating that the effect was reversible, as well as non-specific with respect to the stimulating protein. The reason for stimulation of protease and leucine arylamidase activity is unclear. A possibility is that exogenous protein associates with and stabilizes the outer membrane thereby facilitating the activity of outer membrane proteases. Casein appeared to be less stimulatory than BSA, which together with the peptone inhibition data suggests that there was inhibition of LPNA arylamidase activity by casein hydrolysis products.

B. fragilis proteases were constitutive and were formed under a variety of growth conditions. Experiments to determine factors that affected protease activity showed that more protease was produced at high growth rates in continuous cultures, and that ammonia, VFA or haemin did not significantly affect proteolysis. In contrast to *B. fragilis*, ammonia has been shown to inhibit proteolysis in other Gram-negative bacteria such as *Vibrio alginolyticus* (Long *et al.*, 1981), *Pseudomonas aeruginosa* (Whooley *et al.*, 1983), *Aeromonas hydrophila* (Pansare *et al.*, 1985) and *Butyrivibrio fibrisolvens* (Cotta & Hespell, 1986).

It is not clear whether the proteases of *B. fragilis* type bacteria have an important nutritional function, since colonic contents contain large amounts of both ammonia and protein. However,

proteolytic activity has been related to pathogenicity in some bacteria, such as the clostridia (Hentges & Smith, 1985), *Pseudomonas aeruginosa* (Moriwaka & Homma, 1985) and *Legionella* (Gul'nik *et al.*, 1986). Amongst the bacteroides, the proteases of *B. melaninogenicus* and *B. gingivalis* are thought to be associated with periodontal disease (Hausmann & Kaufman, 1969; Yoshimura *et al.*, 1984).

Although part of the normal gut flora (Finegold *et al.*, 1983), *B. fragilis* group organisms such as *B. vulgatus*, *B. thetaiotaomicron*, *B. distasonis* and particularly *B. fragilis* itself can be pathogenic, and are frequently isolated from clinical specimens (Dowell & Lombard, 1981). In view of the role in the aetiology of disease of proteolytic enzymes produced by other bacteria, it is possible that the proteases formed by colonic bacteroides may similarly be a contributing factor towards their pathogenicity in man.

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