Proteolytic activity of *Clostridium difficile*

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Summary. Ten isolates of *Clostridium difficile* expressing different degrees of toxigenicity and virulence in an animal model were assayed for the production of proteolytic enzymes by various methods. All strains demonstrated some activity in one or more of the assay systems. There was no direct correlation between toxigenic status and enzyme production. However, those strains known to be highly virulent in a hamster model were the most proteolytic. The most commonly detected enzyme was cell associated, and its substrate specificity suggested it was a trypsin-like enzyme. Initial purification of the enzyme from strain VPI 10463 gave a 10% yield with a 14-fold increase in purity. Inhibition studies on this preparation indicated that the enzyme was a thiol protease. The enzyme has pH and temperature optima of 7.5 and 37°C, respectively. These characteristics suggest that the enzyme is more related to clostripain, the thiol clostridio-peptidase of *C. histolyticum*, than to trypsin. Whilst the role of this enzyme remains unclear, it is possible that it may be a contributory factor in the virulence of the organism as described for other clostridial infections.

Introduction

Clostridium difficile is now recognised as the major cause of pseudomembranous colitis and is implicated in many cases of antibiotic-associated colitis and diarrhoea.^{1–3} The pathogenicity of the organism has been explained in part by the identification of several known and putative virulence factors—toxin production,^{4,5} adhesion to the gut wall,⁶ expression of fimbriae⁷ and capsule production.⁸ The possible role in pathogenicity of hydrolytic enzymes produced by the organism has been highlighted recently.⁹ However, although protease formation by other anaerobic pathogens has been well documented,^{10,11} there is a paucity of information about the proteolytic properties of *C. difficile*. Furthermore, recent studies on the metabolism and nutritional requirements of *C. difficile* have indicated that the species is asaccharolytic, and, whilst it can ferment free amino acids, the preferred substrates are low-mol.-wt peptides.¹² Therefore, it is reasonable to hypothesise that *C. difficile* would produce proteases capable of ensuring a supply of peptides suitable for metabolism. The inability to compete for necessary substrates for nutrition may explain in part the phenomenon of colonisation resistance, the breakdown of which is so characteristic of *C. difficile* infections. In this paper we report the results of investigations to detect and identify proteases produced by *C. difficile*.

Materials and methods

Bacteria and media

Ten isolates of *C. difficile* (table I), described in detail previously,⁹ were maintained by weekly subculture on defibrinated sheep blood 5% agar (Difco). All isolates were grown at 37°C in an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10%. For the fluorogenic and chromogenic assays, cells were harvested from 48-h cultures in Brain Heart Infusion (BHI) Broth (Oxoid) by centrifugation at 4000g for 20 min. The cell pellet was then resuspended in phosphate-buffered saline (PBS) and sonicated on ice in an MSE Soniprep (13 microns; 4 × 30 s) until at least 80% of cells appeared to be disrupted when examined by light microscopy. Both sonicated cell-pellet suspensions and the culture supernates were standardised to a protein concentration of 100 µg/ml by the dye-binding method¹³ before assaying for proteolytic activity. *Porphyromonas* (*Bacteroides*) *gingivalis* strain W83 (a clinical isolate), a known highly proteolytic organism,¹⁴ was used as a positive control. All assays were performed in triplicate or more frequently.

Plate assay

Filter sterilised casein (Difco), skimmed milk powder (Oxoid), or gelatin (Sigma) were added to BHI agar to a final concentration of 2%. After inoculation and incubation for 4 days, plates were observed for any clearing before being flooded with a Coomassie

Blue (Sigma) 0.001% solution for 5 min. Translucent areas, >6 mm diameter, surrounding bacterial colonies were recorded as positive reactions; areas of clearing <6 mm or a reduction only in the opacity of the surrounding media were considered weak reactions.

Azo-dye assay

Cells harvested from sheep blood 5% agar after anaerobic incubation for 48 h at 37°C were resuspended in PBS to a protein concentration of 100 µg/ml. Samples (5 ml) of these bacterial suspensions were added to 5 ml of an aqueous solution containing azocollagen, azoalbumin or azocasein (all 2% w/v; Sigma), and incubated aerobically at 37°C for 6 h. Dye released from the diazotised proteins was measured spectrophotometrically at 450 nm as described elsewhere.¹⁵ A positive reaction was recorded for values >1 µg of diazoprotein hydrolysed, a weak result for <1 µg diazoprotein hydrolysed.

Hydrolysis of fluorogenic substrates

Synthetic fluorescent labelled substrates were used to detect specific protease activities of the test isolates. The substrates employed were: N-Benzoyl-L-arginine-7-amido-4-methylcoumarin (BAAMC), a trypsin and papain substrate; L-Lysine-alanine-7-amido-4-methylcoumarin (LAAMC), a dipeptidyl aminopeptidase substrate; proline-phenylalanine-arginine-7-amido-4-methylcoumarin (PPAMC), a kallikrein substrate; and N-succinyl-glycine-proline-leucine-glycine-proline-7-amido-4-methylcoumarin (SAMC), a collagenase-like peptidase substrate. Reactions were performed in microtitration trays with 20 µl of aqueous substrate (4 µg/ml; Sigma) and 30 µl of sonicated cell-pellet suspension or culture supernate. Plates were prepared in duplicate. One set was incubated anaerobically and the other aerobically, for 2 h at 37°C, before being examined under UV irradiation (300 nm); a positive result was indicated by fluorescence. Results were expressed qualitatively as positive, weak or negative.

Hydrolysis of chromogenic substrates

Enzyme activities were measured quantitatively with chromogenic substrates. The substrates used were: N-CBZ-glycyl-glycyl-L-leucine-*p*-nitroanilide (GGLN), a subtilisin substrate; N-CBZ-leucine-leucine-glutamine- β -naphthylamide (LLGN), a cation-sensitive endopeptidase; N α -benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA), a trypsin substrate; and N α -*p*-tosyl-L-arginine methyl ester (TAME), a chymotrypsin substrate. To 1.3 ml of the reaction mixture, containing 50 mM Tris-HCl (pH 8.5), 20 mM cysteine hydrochloride and 1.2 mM substrate, was added 0.6 ml of culture

supernate or sonicated cell-pellet suspension. The reaction was followed by monitoring the absorbance at 412 nm for the *p*-nitroanilide substrates, 380 nm for the β -naphthylamide substrates and 340 nm for the methylester. The amount of chromophore released was determined from its extinction at the appropriate wavelength. Test results were corrected for spontaneous hydrolysis of substrate by replacing the *C. difficile* test sample with buffer.

Preliminary protease purification

Strain VPI 10463 was cultivated anaerobically in dialysis bags for 48 h as described previously.¹⁶ Cells were harvested by centrifugation at 4°C (8000*g* for 15 min), washed in PBS and suspended in 10 ml of PBS. This cell suspension was sonicated as described above, passed through a 0.22-µm filter, and loaded on a Q sepharose FF anion-exchange gel (Pharmacia) fitted in a fast protein liquid chromatography apparatus (Pharmacia). Elution by a 0–1.0M NaCl gradient in 20 mM Tris-HCl (pH 7.5) was performed and 2-ml fractions were collected. All fractions were assayed for proteolytic activity in a microtitration-plate assay, with L-BAPNA as the substrate, as described above. Positive fractions were pooled and loaded on to Mono Q (Pharmacia), a second anion exchange gel column; elution was as described above except that 1-ml fractions were collected and screened for proteolytic activity against L-BAPNA.

Protease characterisation

The substrate specificity of the partially purified enzyme was investigated with the range of chromogenic and fluorogenic substrates described above plus a thiol protease substrate, benzoyl-arginine ethyl ester (Sigma), whose breakdown was followed at 340 nm.

The effect of the addition of various protease inhibitors on the enzyme's ability to hydrolyse the L-BAPNA substrate was investigated by adding to the reaction mixture 20 µl of a 10 mM aqueous suspension of inhibitor 10 min before addition of substrate; the reaction was followed as described above. The inhibitors studied included three serine protease inhibitors—soyabean trypsin inhibitor, N- α -tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)—and three thiol protease inhibitors—ethylguanidine, ethylalanine and butylamine (all supplied by Sigma).

To investigate the effect of pH on the enzyme activity, 1 ml of partially purified enzyme was freeze-dried and 10 µg was resuspended in a range of biological buffers (BDH). The effect of pH on the stability of L-BAPNA was previously determined. The temperature optimum of the enzyme was determined in tests incubated in water baths set at various temperatures, again with L-BAPNA as substrate.

Results

Protease activity

The spectrum of protease produced by *C. difficile* varied between strains. Strains 2B, VPI 10463 and P-1, in particular, possessed a wide range of activities (tables I, II and III), whereas strain DD demonstrated no activity against any of the substrates, except very weak degradation of three of the four chromogenic substrates. There was no correlation between toxigenic status and protease activity. However, strains known to be highly virulent in the hamster model¹⁷—strains 2B, P-1¹⁷ and VPI 10463—displayed the greatest proteolytic activity.

The substrate-incorporated agar method proved less sensitive than assays with diazotised, fluorogenic and chromogenic substrates. None of the test isolates caused clearing on gelatin plates (table I) but this could in part be explained by the poor sensitivity of this method. However, the skimmed milk plates

demonstrated the presence of non-specific proteolytic activities very clearly with translucent zones surrounding bacterial colonies. With diazotised substrates, the two strains that gave positive results in the plate assay for casein degradation were confirmed to give positive reactions and five others were shown to have weak activity (table I). The more globular azoalbumin proved relatively resistant to breakdown (table I). Six of the 10 isolates degraded azocollagen (table I) and the same six isolates also degraded the collagen-like peptidase fluorogenic substrate, SAMC (table II).

Only two isolates, 2B and VPI 10463, readily degraded the dipeptidyl aminopeptidase substrate (LAAMC) (table II); five strains degraded both kallikrein (PPAMC) and trypsin (BAAMC) substrates, and two strains (S-1 and MA) degraded BAAMC but not PPAMC.

Whilst a positive result in tests with the chromogenic substrates indicated that some enzyme activity was present, only values greater than one third of that recorded for *P. gingivalis* W83 were considered strongly

Table I. Virulence characteristics and proteolytic activity of *C. difficile* isolates and *P. gingivalis* against various substrates

Bacterial strain	Toxigenic status	Virulence*	Casein plates	Skimmed milk plates	Gelatin plates	Azocasein	Azoalbumin	Azocollagen
<i>C. difficile</i>								
2B	+	H	+	+	—	+	—	+
VPI 10463	+	H	—	W	—	W	W	+
P-1	+	H	—	W	—	W	—	+
Tra 5/5	+	L	—	—	—	—	W	+
SC	+	L	—	—	—	W	—	—
DD	+	L	—	—	—	—	—	—
MA	+	L	—	W	—	W	W	+
KN	—	AV	W	—	—	+	W	—
M-1	—	AV	—	—	—	—	W	—
S-1	—	AV	—	+	—	W	—	+
<i>P. gingivalis</i>								
W83	—	...	+	+	W	+	—	+

+, Positive; W, weak; —, negative; * in hamster model;^{9,17} H, highly virulent; L, less virulent; AV, avirulent.

Table II. Hydrolysis of fluorometric substrates by *C. difficile* isolates and *P. gingivalis*

Bacterial strain	Hydrolysis of							
	BAAMC		LAAMC		PPAMC		SAMC	
	S	P	S	P	S	P	S	P
<i>C. difficile</i>								
2B	W	+	—	+	—	+	W	+
VPI 10463	W	+	—	+	—	+	+	+
P-1	W	+	—	W	—	+	—	W
Tra 5/5	—	—	—	—	—	—	—	+
SC	—	+	—	—	—	+	—	—
DD	—	—	—	—	—	—	—	—
MA	—	W	—	—	—	—	W	+
KN	—	—	—	W	—	—	—	—
M-1	W	+	—	—	—	+	—	—
S-1	—	+	—	W	—	—	W	+
<i>P. gingivalis</i>								
W83	+	+	—	W	—	—	W	+

S, supernatant; P, cell pellet; +, positive; W, weak; —, negative.

Table III. Protease activity of *C. difficile* isolates and *P. gingivalis* with various chromogenic substrates

Bacterial strain	Mean (SD) activity (μ mol substrate released/min/mg of protein) with							
	GGLN		LLGN		BAPNA		TAME	
	S	P	S	P	S	P	S	P
<i>C. difficile</i>								
2B	18.8 (0.8)	52.8 (1.8)	10.0 (0.3)	17.7 (0.5)	29.6 (1.3)	178 (6.4)	0.0	0.0
VPI 10463	12.6 (0.6)	78.0 (2.3)	8.8 (0.2)	15.6 (0.4)	28.7 (0.9)	154 (5.8)	0.0	0.0
P-1	7.0 (0.1)	33.0 (1.0)	11.9 (0.2)	28.8 (0.8)	33.8 (1.1)	200 (6.0)	0.0	0.0
Tra 5/5	2.0 (0.1)	6.8 (0.1)	0.0	6.0 (0.1)	12.1 (1.0)	89 (1.3)	0.0	0.0
SC	3.2 (0.1)	4.9 (0.1)	3.1 (0.1)	4.7 (0.1)	18.0 (0.4)	167 (11.0)	0.0	0.0
DD	0.0	8.1 (0.1)	0.0	5.0 (0.1)	0.0	32 (1.1)	0.0	0.0
MA	6.7 (0.2)	23.7 (0.8)	5.9 (0.2)	8.4 (0.2)	17.5 (0.9)	87 (1.8)	0.0	0.0
KN	5.3 (0.3)	6.9 (0.1)	1.2 (0.1)	5.5 (0.1)	5.0 (0.2)	18 (0.9)	0.0	0.0
M-1	4.7 (0.2)	14.4 (0.3)	6.4 (0.3)	7.5 (0.2)	28.0	137 (7.5)	0.0	0.0
S-1	1.4 (0.1)	7.7 (0.1)	6.0 (0.2)	7.0 (0.2)	27.0 (1.2)	119 (8.6)	0.0	0.0
<i>P. gingivalis</i>								
W83	67.5 (1.6)	154.5 (5.1)	9.8 (0.4)	14.9 (0.5)	57.7 (2.1)	489 (15.6)	27.0 (0.98)	53.6 (1.32)

S, supernatant; P, cell pellet.

positive. Performing the assays anaerobically did not appear to increase the levels of detectable activity with any of the substrates. Only two isolates, 2B and VPI 10463, gave high levels of subtilisin activity (GGLN; table III). *P. gingivalis* W83 possessed only low levels of a cation-sensitive endopeptidase and *C. difficile* strains 2B, VPI 10463 and P-1 demonstrated greater activity against this substrate (table III). Trypsin-like activity was quite widely detected, though only three strains (2B, SC and P-1) gave strongly positive reactions, and two strains, DD and KN, showed very low activity (table III). However, levels of trypsin-like activity were high for five other strains even in culture supernates (2B, VPI 10463, P-1, M-1, S-1). None of the strains tested proved capable of degrading the chymotrypsin substrate TAME.

In general, greater activity was detected in the resuspended pellet samples than in the culture supernates when assayed with either fluorogenic or chromogenic substrates.

Protease purification

Purification of the predominant protease yielded material with a 14-fold increase in purity and a 10% yield. This purified material readily degraded thiol-containing substrate (previously untested in this study), trypsin substrates and, to a lesser degree, the subtilisin substrate; other substrates were unaffected. Activity against the trypsin substrate, BAPNA, was inhibited by ethylamine, butylamine, ethylguanidine and TLCK, but not by TPCK or soyabean trypsin inhibitor. The optimal pH and temperature of this enzyme activity were 7.5 and 37°C respectively (figure).

Discussion

A wide range of host, dietary and microbial proteins are potentially available as substrates for bacteria in

the human colon. Recent investigations have revealed the asaccharolytic nature of *C. difficile* and the possible requirement for small peptides for metabolism.¹²

Our results show clearly that *C. difficile* has some proteolytic activity. Generally, protease activity appeared to be cell associated with only limited amounts detectable in culture supernates. The most commonly produced protease has substrate specificities similar to mammalian trypsin and bacterial subtilisin enzymes, with seven of the 10 test isolates degrading, to some degree, both BAAMC and BAPNA substrates.

The production of proteases by this organism could be important in releasing suitable substrates from available protein sources for metabolism. Furthermore, proteolytic activity has been linked to pathogenicity in other clostridia.¹⁰ These proteolytic enzymes, together with other hydrolytic enzymes already described for this organism,⁹ may be involved in host

Figure. Temperature and pH optima of purified protease activity. One Unit (U) = amount of enzyme required to release 1 μ mol of trypsin substrate (BAPNA) /ml /min.

tissue breakdown. Collagenase activity could clearly be a major contributory factor to such tissue breakdown, and results obtained in this study with a synthetic collagenase substrate confirm results, already published, obtained with native collagen.⁹ That the organism appears to be incapable of breaking down the more globular protein albumin is of interest, as it has been suggested that serum albumin accumulating in the gut due to the activity of *C. difficile* toxin A may compete with toxins A and B for host proteases and thus protect the toxins from degradation.¹⁸

Recent evidence indicates that toxin A of *C. difficile* is produced in a partially inactive form that has cytotoxic activity but lacks haemagglutinating activity.^{8,19} The possession of trypsin-like enzymes by *C. difficile* could account for this activation. It is also of interest that none of the strains demonstrated any chymotrypsin activity which would degrade toxins A and B.²⁰ It is possible that the expression of these proteases and the degree of toxin production, or protoxin activation, or both, might be linked, as has been speculated for *C. botulinum*.²¹ Furthermore, whilst no relationship was apparent between toxigenic status and protease expression by the test isolates, those strains known to be more virulent in the hamster model¹⁷ were the most proteolytic. These results are

in keeping with observations previously reported for other hydrolytic enzymes produced by these isolates.⁹

The substrate specificity of the partially purified protease confirmed the trypsin-like nature of the most prominent protease. However, the inhibition studies did not confirm this hypothesis but suggested, instead, that the protein was a sulph-hydrol enzyme. Therefore, it is possible that this enzyme could be similar to the clostridio-peptidase, clostripain, produced by *C. histolyticum*.²² This enzyme cleaves the carboxy peptide bond of arginine, similar to the action of trypsin, but is a sulph-hydrol protease rather than a serine protease, and, as such, is not inhibited by soyabean trypsin inhibitor or TPCK, but is inhibited by TLCK, ethylamine, butylamine and ethylguanidine.²³ The *C. difficile* protease displays a very similar inhibition pattern. Furthermore, the temperature and pH optima are similar to those reported for clostripain.²³ Clearly, further work is necessary to fully purify and characterise this enzyme.

In view of the role in the aetiology of disease of proteolytic enzymes produced by other clostridia, it is possible that the proteases produced by *C. difficile* may similarly be a contributory factor to their virulence in man.

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