Characterization of the trypsin-like activity of *Bacteroides forsythus*

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***Bacteroides forsythus*, a bacterial species frequently associated with diseased periodontal sites, is known to possess trypsin-like activity. The present study was undertaken to determine the major characteristics of this activity. The trypsin-like activity was mainly found on the surface of the bacteria and could be solubilized with a zwitterionic detergent (Zwittergent 3-14). Using *N*- α -benzoyl-DL-arginine-*p*-nitroanilide as substrate, the optimum pH was between 7.5 and 8.5 and the optimum temperature was 35 °C. The evidence suggests that the enzyme is a serine protease since it was strongly inhibited by diisopropylfluorophosphate (DFP), *N*- α -*p*-tosyl-L-lysine chloromethyl ketone hydrochloride, leupeptin and antipain. The *B. forsythus* trypsin-like enzyme cleaved numerous chromogenic synthetic peptides containing either an arginine or lysine bond, but could not hydrolyse native proteins including casein, gelatin and BSA. Incubation of a cell envelope extract of *B. forsythus* in the presence of [³H]DFP, which is known to bind irreversibly to serine proteases, labelled two bands at 70 and 81 kDa following SDS-PAGE (under reducing conditions) and fluorography. It is suggested that the *B. forsythus* trypsin-like enzyme may be mainly involved in the degradation of small peptides resulting from hydrolysis of larger proteins by other oral bacteria.**

Keywords: *Bacteroides forsythus*, trypsin-like activity, protease, periodontal disease

INTRODUCTION

The term periodontitis refers to a group of diseases affecting the underlying structures of the periodontium which are characterized by a significant breakdown of connective tissue (Williams, 1990), and which are thought to be initiated by an overgrowth of specific bacterial species found at the gingival margin (Socransky & Haffajee, 1992). *Bacteroides forsythus* is one of a number of bacterial species frequently associated with advanced periodontitis as well as recurrent periodontitis (Lai *et al.*, 1987; Dzink *et al.*, 1988). However, it is still not clear whether these bacteria play a major role in the pathogenic process of periodontitis or are secondary colonizers of diseased periodontal sites.

A characteristic common to most bacteria associated with periodontal disease is their ability to produce hydrolytic

enzymes, such as proteases, as virulence factors (Holt & Bramanti, 1991; Socransky & Haffajee, 1991) which may play an important role in disease progression. In addition to *Treponema denticola* (Laughon *et al.*, 1982; Ohta *et al.*, 1986) and *Porphyromonas gingivalis* (Slots, 1981; Laughon *et al.*, 1982; Grenier & Mayrand, 1993), *B. forsythus* also produces hydrolytic activity towards the synthetic substrate for trypsin-like enzymes (Tanner *et al.*, 1985, 1986). As these three species are associated with diseased periodontal sites, the determination of trypsin-like activity has been proposed as a diagnostic test to evaluate clinical disease of patients (Loesche *et al.*, 1990). Trypsin-like enzymes produced by *T. denticola* and *P. gingivalis* have been purified and fully characterized (Ohta *et al.*, 1986; Grenier & Mayrand, 1993). No such data are currently available concerning the nature and properties of the trypsin-like enzyme produced by *B. forsythus*. The aim of this study was to characterize the trypsin-like activity of *B. forsythus*.

METHODS

Bacteria and growth conditions. *B. forsythus* ATCC 43037 was used throughout the study. Four additional strains of *B.*

Abbreviations: BAPNA, *N*- α -benzoyl-DL-arginine-*p*-nitroanilide; DFP, diisopropylfluorophosphate; pNa, *p*-nitroanilide; TLCK, *N*- α -*p*-tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone hydrochloride.

*for*sythus (JL142684, 4019M13, 4067M27, 4090M22), obtained from Dr C. Mouton (Université Laval, Québec), were also tested for their trypsin-like activity. Bacteria were grown anaerobically [$\text{N}_2/\text{H}_2/\text{CO}_2(80:10:10)$] at 37 °C in brain heart infusion broth (BBL Microbiology Systems) supplemented with 5% (v/v) heat-inactivated calf serum and 0.001% *N*-acetylmuramic acid (Wyss, 1989). Unless otherwise indicated, bacteria were cultivated for 5 d. Samples were also taken periodically during batch culture for the determination of cell-associated trypsin-like activity. Cells were harvested by centrifugation (10000 *g* for 30 min) and suspensions were prepared in distilled water to an OD₆₆₀ of 1.0.

Assay for trypsin-like activity. Trypsin-like activity was measured by monitoring hydrolysis of the chromogenic synthetic peptide *N*- α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). Bacterial samples (12.5 μl) were incubated in the presence of 150 mM Tris/HCl buffer pH 7.8 (125 μl), 4 mM BAPNA (50 μl) and distilled water (12.5 μl). The assay mixtures were then incubated at 37 °C for 2 h. The release of *p*-nitroaniline was determined by reading the A_{405} using an ELISA microtitre plate reader (Easy Beam, SLT Labinstruments). Boiled (10 min) samples were used as controls.

Cell fractionation. *B. for*sythus ATCC 43037 was subjected to a cell fractionation procedure in order to investigate the distribution of trypsin-like activity in the different cellular fractions. Cells from a 21 culture (5 d) were collected by centrifugation (10000 *g* for 30 min). The culture supernatant was concentrated 20-fold by ultrafiltration at 4 °C through a membrane with a molecular mass cut-off of 5000 Da. This fraction (100 ml) was referred to as concentrated culture supernatant. The bacterial cells (2.6 g, wet weight) were washed twice in 0.05 M Tris/HCl buffer, pH 7.8, containing 0.03 M NaCl, and resuspended in 25 ml 0.05 M Tris/HCl, pH 7.8, containing 30% (w/v) sucrose and 0.001 M EDTA. After allowing plasmolysis for 15 min at room temperature, the cells were collected by centrifugation (8000 *g* for 30 min). The bacterial cells were then submitted to osmotic shock by dispersion of the pellet in 50 ml ice-cold distilled water. After 5 min, the cells were removed by centrifugation (8000 *g* for 30 min), and the supernatant, which represented the periplasmic material, was concentrated 10-fold by ultrafiltration at 4 °C using a membrane with a molecular mass cut-off of 5000 Da. The bacterial pellet was suspended in 30 ml 0.05 M Tris/HCl, pH 7.8, containing 10% (v/v) glycerol, 0.002 M MgCl_2 , deoxyribonuclease (Sigma) at 0.2 mg ml⁻¹ and ribonuclease (Sigma) at 0.2 mg ml⁻¹. This suspension was submitted to an ultrasonic treatment (10 $\times$ 1 min; energy level 7; Sonic Dismembrator model 150, Artek Systems) in the presence of 15% (v/v) glass beads (100 μm diameter; Sigma) in an ice bath. The glass beads and the unbroken cells were then removed by two consecutive centrifugations at 6000 *g* for 15 min, and discarded. The crude cell envelope fraction was pelleted by centrifugation for 2 h at 200000 *g*, and the supernatant, which was dialysed overnight at 4 °C against distilled water, was designated as the cytoplasmic material. The crude cell envelope fraction containing outer and cytoplasmic membranes was suspended in 10 ml distilled water. All fractions were stored at -20 °C until used. Protein concentrations were estimated by a protein assay kit (Bio-Rad) using BSA as the standard. Malate dehydrogenase activity, a cytosol marker, and alkaline phosphatase activity, a periplasm marker, were determined as previously described (Shah & Williams, 1982; Yamashita *et al.*, 1990). Each of the bacterial fractions was tested for trypsin-like activity, and one unit was arbitrarily defined as the amount of enzyme that released 0.05 μmol nitroaniline from BAPNA under the conditions described above.

Extraction of trypsin-like activity from the bacterial cell envelope. Trypsin-like activity of *B. for*sythus ATCC 43037 was extracted by suspending whole cells (1.2 g, wet weight) from a 1 litre culture in 10 ml 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM EDTA and 0.15% Zwittergent 3-14 (Calbiochem). After shaking for 1 h at room temperature, the suspension was centrifuged (10000 *g* for 30 min) and the supernatant, referred to as the cell envelope extract, was collected. The bacterial cells were treated three additional times and the extracts were pooled. The Zwittergent 3-14 was removed from the extract by ultrafiltration through a membrane with a molecular mass cut-off of 10000 Da. The protein concentration in the extract was determined as described above.

Determination of optimum pH. Trypsin-like activity present in the cell envelope extract of *B. for*sythus was measured at different pH values using the following buffers: 0.5 M citrate buffer (pH 4, 5 and 6), 0.5 M Tris/HCl buffer (pH 7, 7.5, 8, 8.5 and 9), and 0.5 M carbonate buffer (pH 10 and 11). Assays were run in triplicate to ensure reproducibility.

Determination of optimum temperature and heat stability. The optimum temperature and heat stability of the trypsin-like activity of *B. for*sythus was determined using the cell envelope extract. Assays for trypsin-like activity were carried out at 25, 30, 35, 40, 45 and 50 °C. The heat stability was measured by preincubating (30 min) the cell envelope extract at the above temperatures prior to performing the assay at 37 °C. Assays were run in triplicate to ensure reproducibility.

Effect of various compounds on trypsin-like activity. Trypsin-like activity present in the cell envelope extract was measured in the presence of various compounds: 1,10-phenanthroline, EDTA, iodoacetamide, epoxysuccinyl-1-leucylamido(4-guanidino)-butane (E-64), pepstatin A, PMSF, diisopropylfluorophosphate (DFP), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), *N*- α -*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), leupeptin,

antipain, dithiothreitol, SDS, CaCl_2 , MgCl_2 and ZnCl_2 . The cell envelope extract was preincubated for 30 min at room temperature in the presence of the compound before assessment of the trypsin-like activity as described earlier. Assays were run in triplicate and the mean $\pm$ standard deviation (SD) was calculated.

Degradation of synthetic peptides and native proteins. Synthetic peptides, including a variety containing either an arginine or lysine residue, were tested for their susceptibility to hydrolysis by the cell envelope extract. The assay was carried out essentially as for the determination of trypsin-like activity. Assays were run in triplicate and the mean $\pm$ SD was calculated. The hydrolysis of the chromogenic substrate azocoll was measured as previously described by Mayrand & McBride (1980). The ability of the cell envelope extract to hydrolyse a variety of native proteins was determined by assaying for the production of lower-molecular-mass fragments in SDS-PAGE (11.5%, w/v, gels) using the buffer system of Laemmli (1970). Briefly, the extract (50 μl) was incubated in the presence of 100 mM Tris/HCl buffer (50 μl), pH 7.8, and the test protein (50 μl ; 2 mg ml⁻¹). Proteins tested were IgG, BSA, casein, fibronectin, gelatin and type I collagen. After 16 h at 37 °C, the assay mixtures were boiled for 10 min in the presence of an equal volume of solubilization buffer (62.5 mM Tris/HCl, pH 6.8, containing 2%, w/v, SDS, 20%, v/v, glycerol, 2%, w/v, 2-mercaptoethanol and 0.01% bromophenol blue) and run on SDS-PAGE gels. The proteins were stained with Coomassie blue.

[³H]DFP-binding assay. Samples of the cell envelope extract

from *B. forsythus* were treated with [^3H]DFP essentially as described by Aphale & Strohl (1993). Briefly, the extract (50 μl ; 2.2 mg protein ml^{-1}) was incubated at 37 °C for 30 min with [^3H]DFP [Dupont-New England Nuclear; 6.0 Ci mmol^{-1} (222 GBq mmol^{-1} , 1 μCi μl^{-1} (37 kBq μl^{-1})] at a final concentration of 50 μM . The proteins were then precipitated with 10% (v/v) ice-cold trichloroacetic acid for 30 min at room temperature. The precipitate was harvested by centrifugation (10 000 g for 10 min), resuspended in the original volume of solubilization buffer (62.5 mM Tris/HCl, pH 6.8, containing 2% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.01% bromophenol blue), boiled for 10 min, and the proteins were separated by SDS-PAGE according to the method of Laemmli (1970). The gels were soaked in 20% (v/v) methanol/10% (v/v) acetic acid for 20 min and then treated with the Entensify Universal (Dupont-NEN) according to the manufacturer's instructions. The gels were dehydrated, and the DFP-binding proteins were detected by fluorography after an exposure time of 72 h on X-ray film (XAR; Kodak).

RESULTS

During growth in batch culture, the trypsin-like activity of *B. forsythus* ATCC 43037 was found to be cell-associated. The time-course of production of activity indicated that the amount of cell-bound activity was high even on bacterial cells from early exponential growth phase (Fig. 1). The maximum level of trypsin-like activity was found on cells obtained from the stationary phase. Four additional strains of *B. forsythus* were tested and found to possess cell-associated trypsin-like activity. When a value of 100% was given to the activity of strain 43037, the activity of the other strains ranged from 39% $\pm$ 8 (strain 4019M13) to 118% $\pm$ 6 (strain 4067M27). Results of the cell fractionation procedure showed that approximately 61% of the trypsin-like activity was membrane-bound (Table 1). A significant proportion of the activity was also found to be cytoplasmic (29%) and to a lesser extent periplasmic (10%). No trypsin-like

Fig. 1. Time-course of production of cell-associated trypsin-like activity by *B. forsythus* ATCC 43037. Cells were harvested at various times, suspended in distilled water ($\text{OD}_{660} = 1.0$) and incubated with 150 mM Tris/HCl and 50 mM BAPNA at 37 °C for 2 h. The A_{405} was then recorded. The trypsin-like activity was expressed as a percentage of the maximum activity. (■), Trypsin-like activity; (□), bacterial growth.

Table 1. Cellular distribution of the trypsin-like activity of *B. forsythus* ATCC 43037

Cellular fraction*	Total units†	Total activity (%)	Specific activity [U (mg protein) $^{-1}$]
Concentrated culture supernatant	0	0	0
Periplasm	7145	10	1786
Cytoplasm	20010	29	914
Cell envelope	42668	61	1905

* Cells from a 2 l culture were fractionated.

† One unit of trypsin-like activity was arbitrarily defined as the amount of enzyme that released 0.05 μmol nitroaniline from BAPNA under the conditions described in Methods.

activity was found to be secreted in the culture environment. The cell fractionation procedure was found to be efficient as alkaline phosphatase activity was detected in high amount in the periplasmic fraction and in low amount in the cytoplasmic fraction.

The trypsin-like activity could be released from *B. forsythus* cells by treatment with the zwitterionic detergent Zwittergent 3-14. After three extractions, no trypsin-like activity remained associated with the treated bacterial cells. The trypsin-like activity present in this cell envelope extract was retained by filters with molecular mass cut-off of 10 kDa and 30 kDa. However, more than 80% of the activity was not retained by a molecular mass cut-off filter of 100 kDa.

The optimum pH for the trypsin-like activity was found to be between pH 7.5 and 8.5 (in Tris/HCl buffer). More than 70% of the activity was detected at pH 6 and 9 whereas about 20% of the activity was still present at pH 5 and 10. No activity was observed at pH 4 and 11. The optimum incubation temperature for the activity was 35 °C. More than 80% of the activity was detected following incubation at 25 °C. Treatment of the bacterial cells at 45 °C for 30 min resulted in a residual activity of 15%. No activity remained after treatment at 50 °C for 30 min.

Inhibitor studies showed that a variety of serine protease inhibitors, including DFP, TLCK, leupeptin and antipain, were strongly effective in reducing the trypsin-like activity present in the cell envelope extract (Table 2). Activity was also inhibited by ZnCl_2 and SDS. The reducing agent dithiothreitol had no effect on the activity. Cysteine-, acidic- and metallo-protease inhibitors did not significantly affect the trypsin-like activity.

The chymotrypsin and elastase synthetic substrates [*N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (*p*Na) and *N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*Na, respectively] were not degraded by the cell envelope extract. The extract hydrolysed a wide range of

Table 2. Effect of various compounds on trypsin-like activity of the cell envelope extract from *B. forsythus* ATCC 43037

Assays were run in triplicate and the mean $\pm$ SD was calculated.

Inhibitor	Amount (final)	Protease specificity	Residual activity (%) $\pm$ SD
None	—	—	100
1, 10-Phenanthroline	10 mM	Metallo-	102 $\pm$ 3
EDTA	10 mM	Metallo-	95 $\pm$ 7
Iodoacetamide	10 mM	Cysteine	100 $\pm$ 2
E-64	0.1 mM	Cysteine	102 $\pm$ 5
Pepstatin A	0.1 mM	Acidic	105 $\pm$ 8
PMSF	10 mM	Serine	69 $\pm$ 5
PMSF	1 mM	Serine	97 $\pm$ 4
DFP	10 mM	Serine	11 $\pm$ 6
DFP	1 mM	Serine	37 $\pm$ 5
TPCK	10 mM	Serine (chymotrypsin)/cysteine	101 $\pm$ 2
TLCK	10 mM	Serine (trypsin)	6 $\pm$ 3
TLCK	1 mM	Serine (trypsin)	9 $\pm$ 4
Leupeptin	0.01 mM	Serine (trypsin)/cysteine	30 $\pm$ 2
Leupeptin	0.002 mM	Serine (trypsin)/cysteine	60 $\pm$ 4
Antipain	0.01 mM	Serine (trypsin)/cysteine	12 $\pm$ 6
Antipain	0.002 mM	Serine (trypsin)/cysteine	23 $\pm$ 1
CaCl ₂	10 mM	—	110 $\pm$ 6
MgCl ₂	10 mM	—	114 $\pm$ 7
ZnCl ₂	10 mM	—	22 $\pm$ 3
Dithiothreitol	10 mM	—	108 $\pm$ 5
SDS	10 mM	—	2 $\pm$ 2

peptides containing arginine bonded to *p*Na (Table 3). Only one, *N*-benzoyl-Phe-Val-Arg-*p*Na, was not cleaved. In addition to being active on bonds involving arginine, the enzyme present in the extract was found to hydrolyse lysine bonded to *p*Na. The extract did not show any activity against L-Arg-*p*Na and L-Lys-*p*Na, the simplest aminopeptidase substrates. No proteolytic activity against a wide array of native proteins could be demonstrated in the cell envelope extract.

The cell envelope extract of *B. forsythus* was incubated in the presence of [³H]DFP, which is known to bind irreversibly to serine proteases. After electrophoresis under reducing conditions and fluorography, two bands at 70 and 81 kDa were revealed (Fig. 2).

DISCUSSION

As trypsin-like activity is elaborated by suspected periodontopathogens such as *T. denticola*, *P. gingivalis* and *B. forsythus*, this activity has been suggested as a marker for periodontal disease (Loesche *et al.*, 1990). It is also thought that trypsin-like enzymes from these bacteria may actively participate in the progression of periodontal disease. Indeed, these enzymes may play numerous roles in pathogenesis, for example, in bacterial invasion of the host tissues, in countering host defence mechanisms, and

in bacterial nutrition during infections (Grenier & Mayrand, 1993; Holt & Bramanti, 1991). The present study was undertaken to determine the major characteristics of the trypsin-like activity of *B. forsythus*.

The trypsin-like enzyme of *B. forsythus* was found to be associated with the surface of the bacteria. The activity could be effectively solubilized by treatment of the bacterial cells with a zwitterionic detergent (Zwittergent 3-14). The evidence suggests that the enzyme is a serine protease since it was strongly inhibited by DFP, TLCK, leupeptin and antipain. The inhibition by DFP and TLCK indicates that the catalytic site of the enzyme involves a serine and a histidine residue, respectively. The detection of two bands after labelling with [³H]DFP suggests that (i) *B. forsythus* produces two forms of trypsin-like enzymes, or (ii) the trypsin-like enzyme (81 kDa) had undergone proteolysis and that the 70 kDa protein is a degradation product. The possibility that the extract contained another serine protease whose activity is not trypsin-, chymotrypsin- or elastase-like should not be ruled out.

Trypsin-like enzymes from *T. denticola* and *P. gingivalis* have been previously characterized. The trypsin-like enzyme of *T. denticola* is cell-associated and has a molecular mass of approximately 69 kDa, as determined by SDS-PAGE (Ohta *et al.*, 1986). This enzyme is completely

Table 3. Degradation of synthetic peptides by the cell envelope extract from *B. forsythus* ATCC 43037

A value of 100 % was assigned to the A_{405} obtained following incubation (2 h at 37 °C) of the cell envelope extract with BAPNA. Assays were run in triplicate and the mean $\pm$ SD was calculated.

Synthetic peptide	Activity (%) $\pm$ SD
BAPNA	100
Aminopeptidase substrates	
L-Arg- <i>p</i> Na	0
L-Lys- <i>p</i> Na	0
Endopeptidase substrates	
Gly-Arg- <i>p</i> Na	110 $\pm$ 9
DL-Val-Leu-Arg- <i>p</i> Na	40 $\pm$ 4
N-Benzoyl-Phe-Val-Arg- <i>p</i> Na	0
N-Benzoyl-Pro-Phe-Arg- <i>p</i> Na	32 $\pm$ 5
N-Benzoyl-Val-Gly-Arg- <i>p</i> Na	161 $\pm$ 12
N- <i>p</i> -Tosyl-Gly-Pro-Arg- <i>p</i> Na	51 $\pm$ 5
N- <i>p</i> -Tosyl-Gly-Pro-Lys- <i>p</i> Na	122 $\pm$ 11
N-Tosyl-BOC-Leu-Gly-Arg- <i>p</i> Na	114 $\pm$ 9
N-Tosyl-BOC-Leu-Ser-Thr-Arg- <i>p</i> Na	118 $\pm$ 16
N-Tosyl-BOC-L-Val-Leu-Gly-Arg- <i>p</i> Na	119 $\pm$ 14
N-Tosyl-BOC-benzoyl-Ser-Gly-Arg- <i>p</i> Na	164 $\pm$ 12

Fig. 2. Fluorogram of SDS-PAGE for boiled (A) and non-boiled (B) cell envelope extract of *B. forsythus* ATCC 43037 labelled with [3 H]DFP. Molecular mass markers were (from top to bottom): myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (69.8 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

inhibited by serine protease inhibitors (DFP and leupeptin) but is not affected by metal chelators or sulphhydryl reagents. The *T. denticola* enzyme hydrolyses synthetic trypsin substrates containing either an arginine or lysine bonded to *p*Na but does not cleave natural proteins such as casein and gelatin. The *B. forsythus* trypsin-like activity described in the present study appears to share numerous properties with the *T. denticola* enzyme. On the other hand, several trypsin-like enzymes having molecular masses ranging from 35 to 300 kDa were purified from *P. gingivalis*. These BAPNA-hydrolysing enzymes are activated by reducing agents, inhibited by serine and thiol protease inhibitors and are active on a variety of natural substrates (Grenier & Mayrand, 1993). Therefore, they differ considerably from the activity produced by *B. forsythus*.

The trypsin-like activity of *B. forsythus* cleaved numerous chromogenic synthetic peptides containing either an arginine or lysine bonded to *p*Na, but could not hydrolyse native proteins. This suggests that the *B. forsythus* enzyme may be mainly involved in the degradation of small peptides resulting from hydrolysis of larger proteins by other oral bacteria. The role of the *B. forsythus* trypsin-like activity in the pathogenesis of periodontal disease thus appears to be minimal compared to the trypsin-like enzymes produced by *T. denticola* and *P. gingivalis*.

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