A cell-associated protein complex of *Porphyromonas gingivalis* W50 composed of Arg- and Lys-specific cysteine proteinases and adhesins

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Porphyromonas gingivalis has been associated with the development of adult periodontitis and cysteine proteinases with trypsin-like specificity have been implicated as major virulence factors. We have extracted the major cell-associated trypsin-like proteolytic activity of *P. gingivalis* W50 using mild sonication. Anion-exchange and gel-filtration FPLC of the sonicate revealed that Arg- and Lys-specific proteinase activity was associated with a 300 kDa complex which could be dissociated into seven bands (48, 45, 44, 39, 27, 17 and 15 kDa) by SDS-PAGE with the 44 kDa band containing two different proteins as shown by N-terminal sequence analysis. On further chromatography of the 300 kDa complex on Arg-Sepharose the majority of the complex eluted from the affinity column as an undissociated complex. However, a small amount dissociated such that the Lys- and Arg-specific activities could be separated by eluting first with lysine then arginine, respectively. The 45 kDa protein of the complex was purified by further anion-exchange FPLC in the presence of octyl- β -D-glucopyranoside and was shown to be an Arg-specific, thiol-activated, calcium-stabilized cysteine proteinase. The 48 kDa protein was also further purified in a similar fashion and shown to be a Lys-specific cysteine proteinase that was not inhibited by EDTA. The two 44 kDa and the 39, 27, 17 and 15 kDa proteins of the complex exhibit amino acid sequence homology and are proposed to be haemagglutinins/adhesins. The 45 kDa Arg-specific proteinase and one of the 44 kDa adhesins as well as the 15, 17 and 27 kDa adhesins are processed from the single polyprotein encoded by the gene designated *prtR*, with all proteins preceded by an Arg or Lys residue within the polyprotein. Similarly, the 48 kDa Lys-specific proteinase, the 39 and 15 kDa adhesins as well as the other 44 kDa adhesin of the 300 kDa complex are encoded by a single gene designated *prtK*, with all proteins preceded by an Arg or Lys residue within the polyprotein. The 39, 15 and 44 kDa adhesins of *PrtR* all exhibit high homology with the 44, 15, 17 and 27 kDa adhesins encoded by *prtR*, particularly the 15 kDa proteins which are identical. The cell-associated proteinase–adhesin complex, designated *PrtR*–*PrtK*, is therefore composed of the two gene products, the mature *PrtR* (160 kDa) and mature *PrtK* (163 kDa) that are further proteolytically processed (most likely autolytically) to release proteinase and adhesin domains that remain non-covalently associated. The fully processed *PrtR*–*PrtK* complex comprises the cysteine proteinases *PrtR*45 and *PrtK*48 and seven sequence-related adhesin molecules, *PrtR*44, *PrtR*15, *PrtR*17, *PrtR*27 and *PrtK*39, *PrtK*15 and *PrtK*44. We propose that this proteinase–adhesin complex is a major virulence factor for *P. gingivalis*.

Abbreviations: TLCK, *N* α -p-tosyl-L-lysine chloromethyl ketone; Bz-L-Arg-pNA, benzoyl-L-Arg-p-nitroanilide; z-L-Lys-pNA, benzyloxycarbonyl-L-Lys-p-nitroanilide.

involved in the evasion of host defence and in the assimilation of haem and peptides.

Keywords: *Porphyromonas gingivalis*, cell-associated cysteine proteinases, adhesins

INTRODUCTION

Periodontal diseases are chronic inflammatory diseases of the supporting tissues of the teeth, associated with bacteria and range from the relatively mild form of gingivitis, the non-specific, reversible inflammation of gingival tissue, to the more aggressive forms of periodontitis which are characterized by the destruction of the tooth's supporting structures. Periodontitis is associated with a subgingival infection of a consortium of specific Gram-negative bacteria that leads to the destruction of the periodontium and is a major public health problem. One bacterium that has attracted considerable interest is *Porphyromonas gingivalis* as the recovery of this micro-organism from adult periodontitis lesions can be up to 50% of the subgingival anaerobically cultivable flora (Christersson *et al.*, 1989; Slots, 1982), whereas it is rarely recovered, and then in low numbers, from healthy sites (Moore *et al.*, 1991). A proportional increase in the level of *P. gingivalis* in subgingival plaque has been associated with an increased severity of periodontitis (Mayrand & Holt, 1988; Socransky *et al.*, 1991) and eradication of the micro-organism from the cultivable subgingival microbial population is accompanied by resolution of the disease (Loesche *et al.*, 1981; Van Dyke *et al.*, 1988). Holt *et al.* (1988) have demonstrated the progression of periodontitis lesions in non-human primates with the subgingival implantation of *P. gingivalis*. Further, immunization with killed whole *P. gingivalis* cells significantly reduced progression of periodontitis associated with the indigenous or *P. gingivalis*-superinfected subgingival flora in this non-human primate model (Persson *et al.*, 1994). These findings in both animals and humans suggest a major role for *P. gingivalis* in the development of adult periodontitis.

P. gingivalis is a black-pigmented, anaerobic, asaccharolytic, Gram-negative rod that obtains energy from the metabolism of specific amino acids. The micro-organism has an absolute growth requirement for iron, preferentially in the form of haem or its Fe(III) oxidation product haemin and when grown under conditions of excess haemin is highly virulent in experimental animals (Marsh *et al.*, 1994). A number of virulence factors have been implicated in the pathogenicity of *P. gingivalis* including the capsule, adhesins, cytotoxins and extracellular hydrolytic enzymes. In particular, proteases have received a great deal of attention for their ability to degrade a broad range of host proteins including structural proteins and others involved in defence. The proteins that have been shown to be substrates for *P. gingivalis* proteolytic activity include collagen types I and IV, fibronectin, fibrinogen, laminin, complement and plasma clotting cascade proteins, α_1 -antitrypsin,

α_2 -macroglobulin, antichymotrypsin, antithrombin III, antiplasmin, cystatin C, IgG and IgA, (Grenier, 1996; Pike *et al.*, 1996; Carlsson *et al.*, 1984; Fujimura *et al.*, 1993; Grenier *et al.*, 1989; Lantz *et al.*, 1991; Mayrand & Holt, 1988; Smalley *et al.*, 1989a; Sorsa *et al.*, 1987; Sundqvist *et al.*, 1985). The major proteolytic activities associated with this organism have been defined by substrate specificity and are 'trypsin-like', that is cleavage on the carboxyl side of arginyl and lysyl residues (Yoshimura *et al.*, 1984) and collagenolytic (Toda *et al.*, 1984) although other minor activities have been reported (Grenier & Mayrand, 1993).

P. gingivalis trypsin-like proteolytic activity has been shown to degrade complement, generating biologically active C5a (Wingrove *et al.*, 1992; Jagels *et al.*, 1996), to impair the phagocytic and other functions of neutrophils by modifying surface receptors (Jagels *et al.*, 1996; Cutler *et al.*, 1995; Kadowaki *et al.*, 1994), and to abrogate the clotting potential of fibrinogen thus prolonging plasma clotting time (Imamura *et al.*, 1995a). The trypsin-like proteolytic activity of *P. gingivalis* is also associated with vascular disruption and enhanced vascular permeation through the activation of the kallikrein-kinin cascade (Imamura *et al.*, 1994, 1995b). *P. gingivalis* spontaneous mutants with reduced trypsin-like activity as well as wild-type cells treated with the trypsin-like protease inhibitor *N* α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK) are avirulent in animal models (McKee *et al.*, 1988; Smalley *et al.*, 1989b; Kesavalu *et al.*, 1996). Further, Marsh *et al.* (1994) have shown that *P. gingivalis* grown under controlled, haemin-excess conditions expressed more trypsin-like and less collagenolytic activity and were more virulent in mice relative to cells grown under haemin-limited but otherwise identical conditions. The increased expression of the trypsin-like activity by the more virulent *P. gingivalis* led the authors to speculate that the trypsin-like proteolytic activity may be the major determinant for infection or disease.

These studies suggest a major role for the trypsin-like protease(s) of *P. gingivalis* in the pathogenesis of periodontitis and therefore have led to considerable interest and endeavour to purify and biochemically characterize these proteins. The most extensive work in characterization of these enzymes has been by Travis and co-workers who have purified Arg- and Lys-specific proteinases from the culture fluid of *P. gingivalis* H66 (Chen *et al.*, 1992; Pike *et al.*, 1994). The Arg-specific proteinase of the culture fluid was a 50 kDa, EDTA-sensitive, cysteine proteinase designated Arg-gingipain (Chen *et al.*, 1992). The Lys-specific enzyme of the culture fluid was proposed to be a 60 kDa cysteine proteinase designated Lys-gingipain and this enzyme

was not EDTA sensitive (Pike *et al.*, 1994). Notwithstanding this extensive characterization of the culture fluid trypsin-like proteinases, the cell-associated trypsin-like proteases of *P. gingivalis* remain to be comprehensively characterized. In this paper we characterize the cell-associated trypsin-like proteinases of *P. gingivalis* W50 as complexes composed of Arg-specific and Lys-specific cysteine proteinases and sequence-related adhesins encoded by the two genes *prtR* and *prtK*.

METHODS

Bacterial strain and growth conditions. Lyophilized cultures of *Porphyromonas gingivalis* W50 (McKee *et al.*, 1986) were kindly provided by Dr P. Marsh (PHLS Centre for Applied Microbiology and Research, Wiltshire, UK). *P. gingivalis* W50 was grown anaerobically at 37 °C on lysed horse blood agar and in modified BM medium containing 1 µg haemin ml⁻¹ (McKee *et al.*, 1986). Bacteria were maintained on lysed horse blood plates by routine passage (< 10 passages) and used to inoculate batch cultures. Batch culture growth was monitored at 650 nm using a spectrophotometer (Perkin-Elmer model 295E). Culture purity was checked routinely by Gram stain, microscopic examination and by a variety of biochemical tests according to Slots (1982). Stocks were maintained as lyophilized cultures.

Enzyme purification. *P. gingivalis* cells were grown in batch culture (5 l) to late exponential phase, harvested by centrifugation (5000 g, 20 min, 4 °C), and washed with TC buffer (20 mM Tris/HCl, pH 7.4, and 5 mM CaCl₂) containing 50 mM NaCl. The washed cells were resuspended in 160 ml TC buffer containing 50 mM NaCl and subjected to mild sonication for 15 min at 4 °C using a Branson Sonifier 250 with an output control of 3 and a 50% duty cycle. The sonicate was centrifuged (100000 g, 30 min, 4 °C) and the supernatant filtered (0.22 µm) prior to anion-exchange FPLC. The sonicate was applied to an anion-exchange column (Hiload XK 16/10 Q Sepharose, Pharmacia-LKB) cooled to 4 °C, in multiple injections using a 50 ml superloop (Pharmacia-LKB). The sample was eluted using a linear gradient of 0–100% buffer B over 90 min at a flow rate of 2.0 ml min⁻¹. The eluant was monitored at 280 nm and collected in 6 ml fractions using a Frac 100 fraction collector (Pharmacia-LKB). Buffer A was TC buffer containing 50 mM NaCl and buffer B was TC buffer containing 500 mM NaCl. Fractions were analysed for proteolytic and amidolytic activity using azocasein (A-2765, Sigma), benzoyl-L-Arg-p-nitroanilide (Bz-L-Arg-pNA, Sigma) and benzyloxycarbonyl-L-Lys-p-nitroanilide (z-L-Lys-pNA, Calbiochem), *vide infra*. Anion-exchange fractions containing the majority of proteolytic/amidolytic activity were pooled, washed and concentrated in TC buffer containing 150 mM NaCl using a Centricon-10 micro-concentrator (Amicon). The sample was divided into two aliquots and each was independently applied to a gel-filtration column (Superose 12, HR 10/30, Pharmacia-LKB) using TC buffer containing 150 mM NaCl at a flow rate of 0.3 ml min⁻¹. The eluant was monitored at 280 nm and peaks collected using a Frac 100 fraction collector. The molecular masses of eluant peaks were determined using molecular mass gel filtration standards (Pharmacia-LKB). The peak containing the majority of the proteolytic/amidolytic activity was applied at a flow rate of 0.1 ml min⁻¹ to an Arg-Sepharose column (5 ml arginine-Sepharose 4B beads, HR 5/5 column, Pharmacia-LKB). The column was washed with 500 mM NaCl and re-equilibrated with TC buffer containing 50 mM NaCl. The

column was first eluted with 200 mM lysine/HCl, pH 7.4, in TC buffer containing 50 mM NaCl at a flow rate of 0.1 ml min⁻¹. This was followed by 750 mM lysine/HCl, pH 7.4, in the same buffer. The column was then re-equilibrated with TC buffer containing 50 mM NaCl and then eluted with 200 mM arginine/HCl, pH 7.4, in TC buffer containing 50 mM NaCl at a flow rate of 0.1 ml min⁻¹. The eluant was monitored at 280 nm and peaks collected using a Frac 100 fraction collector. The peaks eluted from the Arg-Sepharose by 200 mM lysine and 200 mM arginine were equilibrated with TC buffer containing 50 mM NaCl and 1.0% octyl-β-D-glucopyranoside and then applied to a Mono Q (HR 5/5) anion-exchange column and eluted using a linear gradient of 0–100% buffer B at a flow rate of 1.0 ml min⁻¹. Buffer A was TC buffer containing 50 mM NaCl and 0.1% octyl-β-D-glucopyranoside and buffer B was TC buffer containing 500 mM NaCl and 0.1% octyl-β-D-glucopyranoside. The eluant was monitored at 280 nm and eluant peaks collected using a Frac 100 fraction collector.

Enzyme characterization. Azocasein, Bz-L-Arg-pNA and z-L-Lys-pNA were used to routinely assay FPLC fractions for proteolytic and amidolytic activity. A sample of each fraction (20–200 µl) was incubated at 37 °C with azocasein (5 mg ml⁻¹ final concentration) in TC buffer, pH 8.0, containing 150 mM NaCl and 10 mM cysteine. For azocasein the reaction was stopped by the addition of 30% trichloroacetic acid at 4 °C. After centrifugation and neutralization of the supernatant with 5 M NaOH, the absorbance was measured at 440 nm using a spectrophotometer (Perkin Elmer, model 552).

For the synthetic chromogenic substrates, samples of each chromatographic fraction (5–50 µl) were incubated at 25 °C with Bz-L-Arg-pNA or z-L-Lys-pNA (1.0 mM final concentration) in a total volume of 350 µl 100 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 10 mM cysteine and 5 mM CaCl₂. Inhibitors and activators were added to the purified enzymes in 100 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl and 1.0 mM 2-mercaptoethanol. Absorbance was measured at 410 nm using a Hewlett Packard 8452A Diode Array spectrophotometer and the amidolytic activity expressed in U, where U = µmol substrate converted min⁻¹ at 25 °C. Trypsin (EC 3.4.21.4, T 8253, Sigma) was used as a standard. The protein concentration of FPLC fractions and purified samples was determined using the Bradford protein assay (Bio-Rad) with BSA as a standard.

A sample of the gel-filtration chromatographic fraction (20 µl) exhibiting the major proteolytic and amidolytic activity was incubated for 4 h at 37 °C with 10 mg ml⁻¹ of pure α₁-casein (Reynolds, 1987) dissolved in TC buffer, pH 8.0, containing 150 mM NaCl and 50 mM 2-mercaptoethanol. Following incubation, the sample was equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA) dissolved in Milli Q water (buffer A). The sample was then applied to an HPLC reversed-phase analytical column (C8, 7 µm, 4.6 mm × 220 mm, Brownlee Aquapore RP300, Applied Biosystems) and peptides eluted using a linear gradient of 0–100% buffer B over 40 min at a flow rate of 1 ml min⁻¹ (140A solvent delivery system). Buffer B was 80% (v/v) acetonitrile in 0.1% (v/v) TFA in Milli Q water. The eluant was monitored at 214 nm using a 1000S diode array detector (Applied Biosystems). Peaks were collected manually and peptides identified using a combination of amino acid composition and sequence analyses as described previously (Reynolds *et al.*, 1994).

SDS-PAGE, protein transblot and N-terminal sequence analysis. SDS-PAGE was performed using a Mini protean II electrophoresis system (Bio-Rad) with 12% (w/v) 1 mm

separating gels, overlaid with 5% stacking gels (Laemmli, 1970). Each sample (20 μ l) was added to 10 μ l of sample buffer [0.5 M Tris/HCl, pH 6.8, 10.0% (w/v) SDS, 0.05% (w/v) bromophenol blue (75%, v/v) and glycerol (25%, v/v)]. In some cases the sample buffer also contained 5% (v/v) 2-mercaptoethanol or 3 mM TLCK. After mixing, the samples were either held at room temperature (non-boiled) or heated at 100 °C (boiled) for 4 min. SDS-PAGE was performed using a current of 30–50 mA and a potential difference of ≤ 200 V. For silver staining, gels were fixed in methanol/water/acetic acid (45:45:10, by vol.), washed in Milli Q water, reduced with 5 μ g dithiothreitol ml⁻¹ and then washed in Milli Q water, all for 30 min periods. Gels were then stained for 20 min with 0.1% (w/v) AgNO₃ and developed with 3% (w/v) sodium carbonate containing 0.1% (v/v) formaldehyde and development was stopped with glacial acetic acid according to the method of Merrill *et al.* (1982). For Coomassie blue staining, gels were fixed in 12% TCA and stained overnight using 0.1% (w/v) purified Coomassie brilliant blue G 250 in 2% (w/v) phosphoric acid, 6% (w/v) ammonium sulphate (Neuhoff *et al.*, 1985). Gels were destained with methanol/water/acetic acid (50:40:10, by vol.). Proteins were transferred onto a PVDF membrane [Problott, Applied Biosystems Inc. (ABI)] for sequence analysis using a transblot cell (Bio-Rad). PVDF membrane was wetted in 100% methanol and soaked in transfer buffer (10 mM CAPS/10% methanol, pH 11.5). Transfer was performed using a potential difference of 60 V (300 mA) for 90 min. Membranes were briefly stained using 0.1% (w/v) Coomassie brilliant blue R 250 in methanol/water/acetic acid (5:5:1, by vol.). Protein bands were excised, destained for 10–30 s in 50% methanol and N-terminal sequences determined using a Hewlett Packard 1005A protein sequencer or a modified ABI 471-02A protein sequencer fitted with a blott cartridge.

RESULTS

The ultrasonication procedure was effective in releasing the cell-associated Arg- and Lys-specific proteolytic activity of *P. gingivalis* W50 and 15 min was required for maximal release of activity. The sonicate of *P. gingivalis* W50 cells contained 0.30 mg protein ml⁻¹ and 2.6 and

Fig. 1. Anion-exchange FPLC of a *P. gingivalis* W50 sonicate. The sonicate in TC buffer containing 50 mM NaCl was applied to a HiLoad XK 16/10 Q-Sepharose column and eluted using a linear gradient of 0–100% buffer B over 90 min at a flow rate of 2.0 ml min⁻¹. Fractions (6 ml) were assayed for proteolytic and amidolytic activity using azocasein, Bz-L-Arg-pNA and z-L-Lys-pNA. The amidolytic activity of each 6 ml fraction with Bz-L-Arg-pNA is shown by the histogram.

Fig. 2. Gel-filtration FPLC of the pooled and concentrated fractions from Q-Sepharose anion-exchange FPLC containing proteolytic/amidolytic activity. Anion-exchange fractions containing the major peak of proteolytic/amidolytic activity were pooled, equilibrated in TC buffer containing 150 mM NaCl, concentrated and divided into two aliquots which were each independently applied to a gel-filtration column (Superose 12 HR 10/30) and eluted using the same buffer at a flow rate of 0.3 ml min⁻¹. Fractions (0.5 ml) were assayed for proteolytic and amidolytic activity. Bz-L-Arg-pNA amidolytic activity is shown by the histogram. V₀ and V_t indicate the void and total volumes of the column, respectively. The elution volumes of the standard proteins thyroglobulin (667 kDa), catalase (232 kDa) and aldolase (158 kDa) are marked.

1.3 μ mol min⁻¹ (mg protein)⁻¹ activity at 25 °C with 1.0 mM Bz-L-Arg-pNA and z-L-Lys-pNA as substrate, respectively. The crude sonicate was subjected to Q-Sepharose anion-exchange FPLC and a representative chromatogram is presented in Fig. 1. Proteolytic/amidolytic activity eluted as one major peak between 246–320 mM NaCl (Fig. 1) which was collected, concentrated using a Centricon-10 (Amicon) and then applied to the Superose 12 gel-filtration column (Fig. 2). Molecular mass gel filtration standards were used to determine the molecular masses of the peaks obtained and the major peak, which also exhibited the major proteolytic/amidolytic activity, corresponded to 300 kDa (Fig. 2). Proteolytic/amidolytic activity was also associated with the high-molecular-mass material (0.6 – $> 2.0 \times 10^6$ Da) eluted from the gel filtration column. The 300 kDa gel filtration peak contained seven bands at 48, 45, 44, 39, 27, 17 and 15 kDa on SDS-PAGE analysis (Fig. 3). The seven bands were transblotted and subjected to N-terminal sequence analysis (Table 1). This analysis revealed that the 44 kDa band contained two proteins and the N-terminal sequences of these two 44 kDa proteins were assigned after further purification. The N-terminal sequence of one of the 44 kDa proteins was identical to that of the 17 kDa protein and the 39 kDa and 27 kDa proteins also had identical N-termini (Table 1).

Repeated gel-filtration analyses of the Q-Sepharose-purified material or crude sonicates indicated that the

Fig. 3. SDS-PAGE (boiled-reduced conditions) of the 300 kDa peak from gel-filtration (Superose 12 HR 10/30) FPLC. Lane 1, Pharmacia molecular-mass standards (kDa); lane 2, 300 kDa peak from gel-filtration FPLC. Gel stained with Coomassie blue.

major proteolytic/amidolytic activity was associated with a peak corresponding to 300 kDa and higher molecular mass ($0.6 - > 2 \times 10^6$ Da) material that when boiled in SDS and subjected to SDS-PAGE analysis contained the same seven bands at 48, 45, 44, 39, 27, 17 and 15 kDa (Fig. 3). The 300 kDa complex was also observed when the sonication and chromatography were performed in the presence of reducing agent such that the association of the proteins in the complex was not by

Fig. 4. Specific cleavage sites (marked with arrows) of α_1 -casein by the proteolytic/amidolytic peak from gel-filtration FPLC corresponding to 300 kDa. The protein α_1 -casein was cleaved on the carboxyl side of arginyl and lysyl residues only.

intermolecular disulphide bond formation. SDS-PAGE without boiling of the 300 kDa complex produced bands corresponding to the relative molecular masses of approximately 300, 150, 104–115, 87, 76 and 66 kDa (data not shown).

The 300 kDa gel-filtration protein complex was incubated with α_{s1} -casein. The α_{s1} -casein peptides released by the action of the proteolytic activity of the 300 kDa complex were purified by RP-HPLC and identified by amino acid composition and sequence analyses. The sites of α_{s1} -casein cleavage by the 300 kDa complex were the carboxyl side of arginyl and lysyl residues only (Fig. 4). All arginyl and lysyl residues of α_{s1} -casein were

Table 1. N-terminal sequences of proteins in the 300 kDa complex separated by SDS-PAGE

Band (kDa)	N-terminal sequence
48*	DVYTDHGDLYNTPVRML
45†	YTPVEEKQNGRMIVIVAKKYEGLD
44†	SGQAEIVLEAHDVWNDGSGYQILLDADHDQYGQVIPSDTHFL
44*	PQSVWIERTVDLPAGTKYVAFR
39*	ANEAKVVLAADNVWGDNTGYQFLLDA
27†	ANEAKVVLAADNVWGDNTGYQFLLDA
17†	PQSVWIERTVDLPAGTKYVAFR
15*†	ADFTETTFESSTHGEAPAEWTTIDA

* Proteins eluted from Arg-Sepharose by 200 mM lysine.

† Proteins eluted from Arg-Sepharose by 200 mM arginine.

Fig. 5. Arg-Sepharose FPLC of the 300 kDa gel-filtration peak exhibiting Arg- and Lys-specific proteolytic activity. Gel-filtration fractions containing the major peak of proteolytic activity (300 kDa) were pooled and applied to an Arg-Sepharose column (5 ml Arg-Sepharose 4B) and washed with TC buffer containing 50 mM NaCl at 0.1 ml min^{-1} until the baseline returned to zero. The column was further washed with 500 mM NaCl and re-equilibrated with TC buffer containing 50 mM NaCl. The column was first eluted with 200 mM lysine in TC buffer containing 50 mM NaCl, followed by 750 mM lysine in the same buffer. The column was then re-equilibrated and eluted with 200 mM arginine in the same buffer at a flow rate of 0.1 ml min^{-1} . Peaks were collected and assayed for amidolytic and proteolytic activity. Bz-L-Arg-pNA amidolytic activity is shown by the histogram and the arrows indicate the start of each step gradient.

Fig. 6. SDS-PAGE (boiled/reduced conditions) of 200 mM lysine eluant from the Arg-Sepharose FPLC. Lane 1, Pharmacia molecular-mass standards (kDa); lane 2, 200 mM lysine eluant from Arg-Sepharose FPLC. Silver-stained gel.

cleaved except the N-terminal Arg and the Lys residues flanking the Ser(P) cluster sequence, presumably due to the high negative charge density (Fig. 4). The 300 kDa

Fig. 7. SDS-PAGE (boiled/reduced conditions) of the 750 mM lysine and 200 mM arginine eluants from the Arg-Sepharose FPLC and the purified 45 kDa Arg-specific proteinase. Lane 1, 750 mM lysine eluant; lane 2, 200 mM arginine eluant; lane 3, purified 45 kDa Arg-specific proteinase; lane 4, Pharmacia molecular-mass standards (kDa). Gel stained with Coomassie blue.

complex was then applied to an Arg-Sepharose column and washed with TC buffer containing 500 mM NaCl (Fig. 5). The Arg-Sepharose was eluted first with 200 mM lysine in TC buffer (Fig. 5) which eluted a small amount of the 48, 44, 39 and 15 kDa proteins of the 300 kDa complex as shown by SDS-PAGE (Fig. 6 and Table 1). N-terminal sequence analysis of these transblotted proteins revealed that only one of the 44 kDa proteins of the 300 kDa complex was eluted with 200 mM lysine (Table 1). This fraction eluted from Arg-Sepharose with 200 mM lysine contained only Lys-specific proteolytic/amidolytic activity. Next the Arg-Sepharose column was eluted with 750 mM lysine (Fig. 5) which removed the majority of the protein bound as the undissociated 300 kDa complex containing all seven bands (eight proteins) as shown by SDS-PAGE analysis (Fig. 7). The 750 mM lysine eluant exhibited both Arg- and Lys-specific proteolytic/amidolytic activity characteristic of the 300 kDa complex. The Arg-Sepharose column was then eluted with 200 mM arginine in TC buffer (Fig. 5). The 200 mM arginine eluant contained a small amount of the 45, 44, 27, 17 and 15 kDa proteins as shown by SDS-PAGE (Fig. 7). This fraction exhibited only Arg-specific proteolytic/amidolytic activity. N-terminal sequence analysis of these transblotted proteins eluted with 200 mM arginine revealed that only one of the 44 kDa proteins of the 300 kDa complex was eluted with 200 mM arginine and this 44 kDa protein was different to the 44 kDa protein eluted with 200 mM lysine (Table 1).

The proteins eluted from the Arg-Sepharose column

Table 2. Purification of the 45 kDa Arg-specific proteinase PrtR45

Purification step	Protein (mg)	Proteolytic activity (U)*	Specific activity (U mg ⁻¹)	Purification (-fold)	Yield (%)
Sonicate	48.0	124	2.6	1	100
Anion-exchange FPLC (Q-Sepharose)	8.2	64	7.8	3	52
Gel-filtration FPLC (Superose 12)	3.9	46	11.8	5	37
Affinity FPLC (Arg-Sepharose)	0.7	17	24.3	9	14
Anion-exchange FPLC (Mono Q)	0.2	13	65.0	25	11

* Amidolytic activity using 1.0 mM Bz-L-Arg-pNA; 1 unit (U) = 1 $\mu\text{mol min}^{-1}$ at 25 °C.

Table 3. Effects of various activators/inhibitors on the activity of the 45 kDa Arg-specific proteinase

Compound*	Concentration (mM)	Activity (%)
2-Mercaptoethanol	1.0	100
	10.0	158
	50.0	189
Dithiothreitol	1.0	109
	10.0	174
L-Cysteine	0.1	183
	1.0	320
	10.0	487
PMSF†	1.0	100
	10.0	90
AEBSF†	1.0	93
	10.0	80
TLCK†	1.0	0
Iodoacetic acid†	1.0	82
	10.0	19
PCMB†	1.0	100
	10.0	14
Leupeptin†	0.1	0
EDTA†	1.0	100
	10.0	4
	50.0	0
EDTA + Ca ²⁺ †	50.0	97
o-Phenanthroline†	10.0	100

* PCMB, *p*-chloromercuribenzoic acid; AEBSF, [4-(2-aminoethyl)-benzenesulfonylfluoride], TLCK, *N* α -*p*-tosyl-L-lysine chloromethyl ketone.

† These incubations also contained 1.0 mM 2-mercaptoethanol.

with 200 mM lysine and 200 mM arginine were washed, concentrated and equilibrated with TC buffer containing 50 mM NaCl and 1.0% octyl- β -D-glucopyranoside and applied independently to a Mono Q anion-exchange column. Elution from the Mono Q column

with a NaCl gradient associated the Arg-specific proteolytic activity with the 45 kDa protein with a 25-fold purification over the original crude sonicate (Table 2, Fig. 7). The specificity of the 45 kDa proteinase for arginyl residues was confirmed by the enzyme cleaving Bz-L-Arg-pNA but not z-L-Lys-pNA. The Arg-specific 45 kDa enzyme was activated by thiols (particularly cysteine), not inhibited by PMSF or 4-(2-aminoethyl)-benzenesulfonylfluoride but inhibited by SH-directed reagents, leupeptin and EDTA (Table 3). The inhibition by EDTA could be reversed by the addition of Ca²⁺ (Table 3). The pH optimum of the enzyme was 7.5–8.0 with activity declining markedly as the pH was lowered below 7.0. These results indicate that the 45 kDa enzyme is a calcium-stabilized, Arg-specific cysteine proteinase. The Lys-specific activity was characterized using the substrate z-L-Lys-pNA and was associated with the 48 kDa protein purified from the 200 mM lysine eluant by Mono Q FPLC. The Lys-specific enzyme was also activated by thiols and inhibited by SH-directed reagents but was not inhibited by leupeptin or EDTA.

DISCUSSION

We have purified and characterized a cell-associated 45 kDa, Arg-specific, Ca²⁺-dependent cysteine proteinase and a 48 kDa, Lys-specific cysteine proteinase from a sonicate of *P. gingivalis* W50 cells that are components of a 300 kDa complex of non-covalently associated proteins. The proteins of the 300 kDa complex were the 45 kDa and 48 kDa proteinases with six other proteins, two 44 kDa and 39, 27, 17 and 15 kDa proteins. The size of the complex (300 kDa) is consistent with the work of Bedi (1995) and Fujimura & Nakamura (1987) who have partially characterized a 300 kDa cysteine proteinase with trypsin-like specificity from the culture fluid of *P. gingivalis* strains FAY-19M-1 and ATCC 33277, respectively. The protease characterized by Bedi (1995), designated Ala, cleaved on the carboxyl side of both Arg and Lys residues in chromogenic substrates and was inhibited by SH-directed reagents and TLCK. The 300 kDa protease identified by Fujimura & Nakamura

Fig. 8. Schematic representation of the *prtR* gene. The PrtR nascent polypeptide is composed of a leader sequence, a prosequence followed by the Arg-specific cysteine proteinase PrtR45, and the adhesins PrtR44, PrtR15, PrtR17 and PrtR27 all preceded by an arginyl or lysyl residue (Slakeski *et al.*, 1996; GenBank accession no. L26341).

(1987) exhibited activity against Bz-L-Arg-pNA and was also inhibited by SH-directed reagents and TLCK. It would appear therefore that the 300 kDa cell-associated complex characterized in this present study has characteristics similar to those of the culture fluid enzyme(s) identified by Bedi (1995) and Fujimura & Nakamura (1987).

The 45, 27, 17, 15 kDa and one of the 44 kDa protein components of the 300 kDa complex are encoded by the recently reported gene, *prtR* (GenBank accession no. L26341; Slakeski *et al.*, 1996) as presented schematically in Fig. 8. The *prtR* gene is similar to the *rgp1* gene of *P. gingivalis* H66 reported by Pavloff *et al.* (1995). Each PrtR component is preceded by an arginyl or lysyl residue (Fig. 8), indicating that the polypeptide is processed by trypsin-like proteolytic activity. We have designated these component parts of the 300 kDa complex, by their relative molecular masses as determined by SDS-PAGE, as PrtR45, PrtR44, PrtR27, PrtR17 and PrtR15 which fit reasonably well with the predicted sizes from the deduced PrtR amino acid sequence (53.9, 44.8, 29.5, 17.5 and 14.3 kDa respectively). It is interesting to note that the 44 kDa protein, PrtR44, has been reported by previous workers as a culture fluid haemagglutinin/adhesin (Pike *et al.*, 1994; Curtis *et al.*, 1996). PrtR44 has homology with the other non-proteinase components of the multiprotein complex suggesting a similar role for PrtR27, PrtR17 and PrtR15 in interacting with the proteinase and/or in haemagglutination or adhesion (Slakeski *et al.*, 1996). The PrtR45 Arg-specific proteinase component of the PrtR complex has the same characteristics and N-terminal sequence as the 50 kDa Arg-specific proteinase identified in the culture supernatant of *P. gingivalis* H66 by Chen *et al.* (1992) designated Arg-gingipain and also has the same characteristics and N-terminal sequence as the 44 kDa enzyme purified from the culture supernatant of *P. gingivalis* 381 by Okamoto *et al.* (1995) designated Argingipain. The difference in size between the 44/45 and 50 kDa proteinases probably results from C-terminal truncation of the 50 kDa enzyme as suggested previously (Kirsbaum *et al.*, 1995).

Fig. 9. Schematic representation of the *prtK* gene. The PrtK nascent polypeptide is composed of a leader sequence, a prosequence followed by the Lys-specific cysteine proteinase PrtK48, and the adhesins PrtK39, PrtK15 and PrtK44 all preceded by an arginyl or lysyl residue (N. Slakeski and others, unpublished; GenBank accession no. U75366).

The other proteins of the 300 kDa complex, the 48 kDa Lys-specific proteinase, the other 44 kDa protein and the 39 kDa protein have recently been shown in this laboratory to be encoded by a single gene, *prtK* (unpublished; GenBank accession no. U75366) presented schematically in Fig. 9. *prtK* is similar to *prtR* in that it encodes a putative leader sequence, a prosequence followed by the proteinase domain which is then followed by sequence-related adhesins that have high homology with the C-terminal adhesins of *prtR*. We have designated the 48 kDa Lys-specific proteinase PrtK48 and its associated adhesins PrtK39, PrtK15 and PrtK44 (Fig. 9) based on the sizes measured by SDS-PAGE which fit reasonably well with the predicted sizes from the deduced PrtK amino acid sequence (55.9, 44.8, 14.3 and 47.9 kDa respectively). PrtK48 has the same enzyme characteristics as the 48 kDa proteinase purified from the culture supernatant of *P. gingivalis* 33277 by Fujimura *et al.* (1993). PrtK48 also has the same N-terminal sequence and enzyme characteristics as the 60 kDa Lys-specific proteinase previously purified from the culture fluid of *P. gingivalis* H66 by Pike *et al.* (1994) and designated Lys-gingipain. The difference in sizes of these two enzymes again may be attributed to C-terminal truncation. PrtK39, PrtK15 and PrtK44 are all sequence-related and have high homology with the PrtR haemagglutinins/adhesins particularly the 15 kDa protein which is identical in both gene products, suggesting that these proteins are also haemagglutinins/adhesins.

As the 300 kDa proteinase-adhesin complex and higher molecular mass forms are composed of proteins from the two genes, *prtR* and *prtK*, we suggest that they be designated PrtR-PrtK complexes. The deduced molecular mass of the mature PrtR is 160 kDa (Slakeski *et al.*, 1996) and mature PrtK is 163 kDa (unpublished) such that the mass of the PrtR-PrtK heterodimer would be 323 kDa. SDS-PAGE of the sample after boiling produced the seven bands of 48, 45, 44, 39, 27, 17 and 15 kDa corresponding to the domains of the two gene products, PrtR and PrtK. These domains were only seen when the sample was boiled, with or without reducing

agent, suggesting that the domains remain tightly non-covalently associated after proteolytic processing. The cell sonicate and the chromatographic fractions had minimal or no proteolytic activity in the absence of reducing agents thus ensuring minimal enzymic activity during the chromatographic purifications. Further, the components of the 300 kDa complex were not produced by proteolytic activation during sample preparation in the presence of reducing agent immediately prior to reducing SDS-PAGE as the same pattern of bands was observed on SDS-PAGE in the absence of reducing agent and in the presence of the inhibitor TLCK. The characterization of the 300 kDa cell-associated complex as being composed of processed domains of the two genes *prtR* and *prtK* suggests that the secreted, mature PrtR and PrtK proteins associate and are processed, perhaps autolytically to form the multiprotein complex. The identification of several of the domains of PrtR and PrtK in the culture supernatant by independent groups (Chen *et al.*, 1992; Pike *et al.*, 1994; Okamoto *et al.*, 1995; Fujimura *et al.*, 1993) is consistent with the proteolytic (autolytic) processing of these polyproteins.

The relative molecular mass of the processed PrtR–PrtK heterodimer is likely to be attributable to the composition of 1 PrtK48 + 1 PrtR45 + 1 PrtK44 + 1 PrtR44 + 1 PrtK39 + 1 PrtR27 + 1 PrtR17 + 1 PrtK15 + 1 PrtR15 = 294–323 kDa depending on C-terminal truncation, that is the 300 kDa complex would contain the five domains of *prtR* and the four domains of *prtK* gene products (Figs 8 and 9). This predicted relative molecular mass of 294–323 kDa is in good agreement with the molecular mass determined by gel filtration and non-boiling SDS-PAGE. As high-molecular-mass material ($0.6 - > 2 \times 10^6$ Da) on gel filtration (Fig. 2) was also composed of the seven PrtR–PrtK bands then this suggests that the 300 kDa PrtR–PrtK complexes may further associate to form larger cell-associated aggregates. The high amino acid sequence homology between PrtR44, PrtK44, PrtK39, PrtR27, PrtR17 and the 15 kDa protein of both PrtR and PrtK suggests that these adhesins are responsible for the non-covalent cohesive interactions between the components of the PrtR–PrtK complexes and between the complexes themselves in the larger aggregates. It is interesting to note that some dissociation of the 300 kDa PrtR–PrtK complex occurred during the affinity chromatography on Arg-Sepharose, although the majority of the protein eluted as the undissociated complex with 750 mM lysine. The partial dissociation of the complex on binding to substrate may be a mechanism by which the complex targets specific host macromolecules and cells releasing the proteinase/adhesin domains at the target site on binding.

Ciborowski *et al.* (1994) have extracted a cell-associated Arg- and Lys-specific proteinase from *P. gingivalis* W12 using the detergent CHAPS. The size of the proteinase, as determined by non-boiling SDS-PAGE, was 150 kDa. However, when the sample was boiled in SDS the proteinase separated into smaller components of 20–50 kDa on SDS-PAGE with one of the components being of similar size and having the same N-terminal sequence

as PrtK39. Furthermore, the authors claimed that the enzyme contained two distinct active sites, one for arginyl and one for lysyl residues, based on differential inhibition by leupeptin. As the PrtR–PrtK complex also exhibits a 150 kDa band on non-boiling SDS-PAGE, presumably corresponding to the unprocessed, dissociated forms of the mature PrtR (160 kDa) and mature PrtK (163 kDa) or the partially dissociated complex of processed domains composed of the two proteinase domains and some of the adhesins, then it is likely that the proteinase characterized by Ciborowski *et al.* (1994) was PrtR–PrtK.

Recently Potempa *et al.* (1995) using SDS-PAGE (non-boiling) and Western blotting with antibodies against the 50 kDa Arg-specific *P. gingivalis* proteinase from the culture fluid designated Arg-gingipain, suggested that Arg-gingipain exists as a 110 kDa heterotrimer or 95 kDa heterodimer of the proteinase and the 44 kDa haemagglutinin depending on the presence of a 15 kDa protein which was speculated to be a membrane anchor protein. In the present study the 300 kDa PrtR–PrtK on SDS-PAGE without boiling ran approximately as 66 kDa, 76 kDa, 87 kDa, 104–115 kDa, 150 kDa and 300 kDa bands presumably corresponding to dimers and trimers as well as the intact mature PrtR, PrtK and complex. For example, the fully processed PrtR–PrtK on partial dissociation could give rise to heterotrimers PrtR45 + PrtR44 + PrtR15 = 104 kDa, PrtK48 + PrtK39 + PrtK15 = 102 kDa, heterodimers PrtK48 + PrtK39 = 87 kDa, PrtR45 + PrtR44 = 89 kDa and other similar dimer and trimer combinations of processed domains. Alternatively, the 110–115 kDa forms could arise from incomplete processing of the mature PrtR and PrtK polyproteins. For example, C-terminal truncation of both the mature PrtR and PrtK by loss of the C-terminal PrtR27 + PrtR17 adhesins and PrtK44 adhesin respectively would result in a 113 kDa, truncated PrtR and 115 kDa, truncated PrtK.

It is apparent therefore that SDS-PAGE without boiling will dissociate the 300 kDa PrtR–PrtK complex into dimers and trimers similar to the dimers and trimers found in the culture supernatant of *P. gingivalis* strain H66 by Potempa *et al.* (1995). It is interesting to speculate that the 15 kDa membrane anchor protein suggested by Potempa *et al.* (1995) is PrtR15. However, this protein as well as the other components of the PrtR–PrtK complex (Slakeski *et al.*, 1996; N. Slakeski and others, unpublished) do not appear to contain a membrane-spanning hydrophobic sequence using the methods of Kyte & Doolittle (1982) and Engelman *et al.* (1986). It is possible that the PrtR–PrtK complex is attached at the cell surface by association with LPS. Boyd & McBride (1984) have reported the presence of LPS in a crude haemagglutinin preparation from *P. gingivalis*. Furthermore, Okuda *et al.* (1986) purified a 40–60 kDa haemagglutinin from *P. gingivalis* 381 that was inhibited by arginine and lysine and was composed of 73% protein, 12% sugar and 6% phosphorus possibly suggesting the presence of bound LPS. Okuda *et al.* (1986) also observed that the haemagglutinin

aggregated to form large complexes which is consistent with the results of the present study.

In conclusion, we have characterized the major cell-associated Arg-specific and Lys-specific proteolytic activity of *P. gingivalis* as a complex of two proteinases and sequence-related adhesins encoded by the two genes, *prtR* and *prtK*. It is proposed that this proteinase-adhesin complex is a major virulence factor for *P. gingivalis* involved in the evasion of host defence and in the assimilation of haem and peptides.

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