Characterization of the trypsin-like enzymes of *Porphyromonas gingivalis* W83 using a radiolabelled active-site-directed inhibitor

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The trypsin-like enzyme activity of *Porphyromonas gingivalis* is an important virulence determinant of this organism in destructive periodontitis. An active-site-directed inhibitor, tyrosyl-alanyl-lysyl-arginine chloromethyl ketone (YAKR-CK) was radio-iodinated and used with SDS-PAGE and autoradiography to determine the number and molecular masses of enzymes with trypsin-like specificity produced by *P. gingivalis* W83. Two forms (I & II) were detected in both crude culture supernatant and whole cell sonicates. Protease I was a sharp band (47 kDa) on reducing SDS-PAGE; Protease II electrophoresed as a diffuse band in the range 70–90 kDa. The specificity with which the inhibitor bound to Protease I was established in competition experiments using other active-site-directed agents. YAKR-CK inhibited *P. gingivalis* whole cell haemagglutination, supporting the possible role of trypsin-like proteases of this organism in adhesion mechanisms.

Introduction

The anaerobic Gram-negative rod *Porphyromonas* (formerly *Bacteroides*) *gingivalis* is strongly associated with certain forms of periodontitis (destructive inflammatory conditions of the tooth-supporting tissues), particularly adult periodontal disease (Slots, 1982; Slots & Listgarten, 1988). Compared with quiescent sites, sites in the periodontium which show progressive disease contain elevated numbers of cultivable *P. gingivalis* (Slots *et al.*, 1986), and the serum IgG antibody response to this organism is consistently elevated in cases with a history of destructive disease when compared with controls (Genco & Slots, 1984; Ebersole *et al.*, 1986; Wilton *et al.*, 1991). However, the precise role of the organism in initiation and progression of the disease is unclear. Analysis of potential virulence determinants of *P. gingivalis* and subsequent interference in their function may help determine which of these components are most closely associated with pathogenesis.

Candidate virulence factors of *P. gingivalis* include its many hydrolytic enzyme activities (Mayrand & Holt, 1988) and much attention is being directed towards thiol-activated trypsin-like enzyme activity, often used in taxonomy to distinguish *P. gingivalis* from other black-pigmenting species in the subgingival microbial plaque

(Laughon *et al.*, 1982). Both whole cells and culture supernatant of *P. gingivalis* can hydrolyse and inactivate human plasma proteins involved in the host defence of the periodontal tissues, including immunoglobulins (Sundqvist *et al.*, 1985; Grenier *et al.*, 1989a; Kilian, 1989), iron-binding proteins (Carlsson *et al.*, 1984a) and protease inhibitors (Carlsson *et al.*, 1984b; Nilsson *et al.*, 1985), and this ability correlates with the thiol-activated trypsin-like enzyme activity (Fishburn *et al.*, 1991). Hence this activity is potentially an important virulence determinant of *P. gingivalis*, but its actual importance in the overall virulence of *P. gingivalis* cannot be established until the enzyme(s) in question have been thoroughly characterized.

Trypsin-like enzyme activity is found, using appropriate chromogenic substrates such as nitroanilide derivatives of C-terminal arginyl peptides, in the culture supernatant and associated with whole cells and vesicles of *P. gingivalis*. How many different trypsin-like enzymes are produced by this organism is not known. Many reports in the literature concern the purification or partial purification of supernatant-derived trypsin-like enzymes from different strains of this organism but there is little agreement as yet on either the total number or molecular masses of individual enzymes. For example, using BSA-polyacrylamide conjugate electrophoresis, Grenier *et al.* (1989b) described the presence of six thiol-activated enzymes with specificity for benzoyl-arginyl-*p*-nitroanilide ranging in molecular mass from 50 to

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100 kDa in the culture supernatant of *P. gingivalis*. A 300 kDa trypsin-like enzyme was reported by Fujimura & Nakamura (1987) and a 17 kDa enzyme by Chen *et al.* (1991). Thiol-activated trypsin-like enzymes with molecular masses between these two extremes have been partially purified by a number of different workers (Yoshimura *et al.* 1984; Ono *et al.*, 1987; Sorsa *et al.*, 1987; Tsutsui *et al.*, 1987; Uitto *et al.*, 1989).

In the present investigation we sought to label specifically the cell-associated and extracellular trypsin-like enzymes of this organism in order to determine their number and molecular masses. We describe the application of an affinity-labelling technique for trypsin-like protease active sites based on a chloromethyl ketone derivative of radio-iodinated tyrosyl-alanyl-lysyl-arginine.

Methods

Bacterium and growth conditions. *P. gingivalis* W83, a highly proteolytic strain, was inoculated from a 48 h horse blood agar plate into brain-heart infusion medium supplemented with 5 mg haemin l⁻¹. The inoculated broths were incubated anaerobically (80% N₂, 10% H₂, 10% CO₂, by vol.) for 6 d at 37 °C.

Enzyme preparations. Extracellular enzyme activity, representing both vesicle-associated and freely soluble enzymes, was harvested by centrifugation (18000 g, 30 min, 4 °C) of the 6 d culture. The supernatant fluid was then filter-sterilized (0.22 µm filter) and is referred to throughout the manuscript as crude supernatant. For the preparation of whole cell sonicate, bacterial cells were pelleted by centrifugation and washed once in Tris/HCl (50 mM, pH 7.2). Following resuspension to 1/5 vol. in HEPES (10 mM, pH 7.2), they were sonicated on ice for 10 s periods (×5) with intermittent cooling at maximum power using a Soniprep 150 (MSE Scientific Instruments). The sonicate was then centrifuged (40000 g, 60 min, 4 °C) to remove unbroken cells and cell membranes.

Protease I from crude supernatant was prepared via an isoelectric focussing procedure. Vesicles were precipitated with 40% saturated ammonium sulphate and discarded. Soluble proteins were precipitated with 70% saturated ammonium sulphate and then dissolved in, and dialysed against, Tris/HCl (50 mM, pH 7.2). The dialysate was then made to 1/10 the original culture volume, filter sterilized (0.22 µm pore size) and stored at -70 °C. Samples of the dialysate were isoelectrically focussed in preparative flatbeds (pH 3.5–10; Application Note 198, LKB Instruments). A trypsin-like enzyme activity, free from contaminating protease activity of differing specificity, was found in fractions of pI 7.5 and pI 4.6 (Harper *et al.*, 1990). The pI 4.6 value, agrees with published estimates for the pI of a trypsin-like enzyme of this organism (Fujimura & Nakamura, 1987; Ono *et al.*, 1987), and the corresponding material was therefore dialysed overnight against Tris/HCl to remove ampholytes and stored at -70 °C until required. This material is referred to throughout as the Protease I preparation.

Protein determinations were done by the method of Simpson & Sonne (1982) using bovine serum albumin as the standard.

Enzyme assays. Trypsin-like enzyme activity was assessed using the chromogenic substrate Chromozym TRY (Boehringer Mannheim) at 150 µM final concentration. Assays were performed in Tris/HCl (50 mM, pH 7.2, 10 mM-CaCl₂, 10 mM-cysteine.HCl), final volume 1.01 ml, and the rate of change of absorbance was measured at 405 nm.

The effects of the following inhibitors were examined: tyrosyl-alanyl-lysyl-arginyl-chloromethyl ketone (YAKR-CK), tosyl-lysyl-chloro-

methyl ketone (TLCK), antipain, chymostatin, leupeptin and mercuric chloride. Inhibitor and enzyme preparation were pre-incubated for 15 min prior to addition of the chromogenic substrate and measurements of residual enzyme activity. All experiments were performed in duplicate on at least two separate occasions and the results are presented as mean data. The standard deviations of the means did not exceed ±5% in each case. Total protease activity was determined by assessment of the rate of degradation of fluorescein-isothiocyanate-conjugated bovine serum albumin, using the method of Homer & Beighton (1990).

Preparation of ¹²⁵I-tyrosine-alanine-lysine-arginine chloromethyl ketone (YAKR-CK). YAKR-CK was a generous gift from Professor E. Shaw (Friedrich Miescher Institute, Basel, Switzerland). The peptide derivative was radio-iodinated using the method of Fraker & Speck (1978) using 3,4,6-tetrachloro-3α,6α-diphenylglycouril which was dried onto the surface of a 1 ml glass reaction vial from chloroform solution (25 µg ml⁻¹). Peptide derivative (50 µg, 1 mg ml⁻¹) was radiolabelled in the vial using 5 µl [¹²⁵I]sodium iodide (100 mCi ml⁻¹, 3.7 GBq ml⁻¹, Amersham) for 15 min before removal to an untreated container. Sodium iodide (50 µl, 10 mM) was then added and the total reaction mixture was layered on to a Sep-Pack C₁₈ cartridge (Millipore) which had been prewashed with 20 ml acetonitrile followed by 20 ml distilled water. Unbound ¹²⁵I was then eluted with distilled water, and labelled peptide eluted using 50% (v/v) acetonitrile in distilled water. The eluted fractions were allowed to evaporate overnight.

Affinity-labelling of *P. gingivalis* enzyme preparations. All labelling procedures were performed in 50 mM-Tris/HCl, pH 7.4 (1 mM-L-cysteine, 10 mM CaCl₂) except where indicated. (A 10-fold lower cysteine concentration than that used in the spectrophotometric enzyme assay was used to reduce the free thiols in the system, which would also react with the chloromethyl ketone.) Crude supernatant, whole cell sonicate or the Protease I preparation was incubated with excess [¹²⁵I]YAKR-CK for 10 min at room temperature. The samples were then heated at 100 °C for 5 min prior to addition of reducing SDS/PAGE sample buffer and further 100 °C treatment for 10 min. The effect of pretreatment with other active-site-directed inhibitors on the labelling efficiency was examined by preincubation of the appropriate inhibitor with the Protease I preparation for 15 min prior to addition of the [¹²⁵I]YAKR-CK. SDS-PAGE was performed essentially according to Laemmli (1970) on 10% (w/v) acrylamide/bisacrylamide separating gels incorporating a 4% (w/v) stacking gel, and proteins were visualized using Coomassie blue. Autoradiography of dried, stained gels was performed using Hyperfilm-ECL (Amersham).

Haemagglutination assay. Haemagglutinating activity was assayed using washed normal human red blood cells in complement fixation diluent (CFD, Oxoid). Bacterial suspensions were washed three times in CFD, and resuspended to A₆₄₀ = 1.0. Doubling dilutions of bacterial suspension or the Protease I preparation were made in CFD from 1/2–1/256 in a total volume of 50 µl in 96-well microtitre plates. To each well, a further 50 µl CFD and 50 µl 0.5% human red blood cells were added, and the plates were incubated at room temperature for 2 h. When an agglutinating titre had been established, the effect of a range of protease inhibitors was examined. Solutions of each inhibitor were double-diluted in CFD and incubated with four times the minimum haemagglutinating dose of Protease I or bacterial suspension for 20 min at room temperature. Human red blood cells were added and incubation continued for a further 2 h.

Results and Discussion

The concept of affinity labelling was first demonstrated by active-site alkylation of the His-57 in chymotrypsin by

Fig. 1. Effect of YAKR-CK on trypsin-like enzyme activity of crude supernatant (■), cell sonicate (●) and the Protease I preparation (▼) from the crude supernatant of *P. gingivalis* W83. Insert shows the % residual total protease activity in each preparation following treatment with 5 μM-YAKR-CK, measured using FITC-BSA: Protease I, solid bar; cell sonicate, open bar; culture supernatant, hatched bar.

Fig. 2. Affinity labelling of the trypsin-like enzymes in crude supernatant *P. gingivalis* W83 using ^{125}I -YAKR-CK. Lanes 1 and 2, Coomassie-blue-stained SDS-PAGE gel of 2.5 and 5.0 μg crude supernatant protein pretreated with labelled inhibitor; lanes 3 and 4, autoradiogram of lanes 1 and 2. Numbers in left margin refer to positions of molecular mass markers: ovotransferrin (77 kDa); albumin (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (29 kDa). I and II, bands discussed in text.

a chloromethyl ketone (Tos-Phe-CH₂Cl), an alkylating derivative of a good substrate for this enzyme (Schoellmann & Shaw, 1973; Singh *et al.*, 1962; Shaw, 1970). We had observed that all the trypsin-like enzyme activity of crude culture supernatant and whole cell sonicates of *P. gingivalis* was fully inhibited by certain chloromethyl ketone derivatives of substrates of trypsin-like enzymes, particularly TLCK. This led us to reason that the use of a radio-labelled chloromethyl ketone derivative of a peptide, preferably with a C-terminal arginine or lysine residue, would be a suitable reagent for affinity-labelling all the trypsin-like enzymes of *P. gingivalis*. Coupled to SDS-PAGE and autoradiography, this would then allow

us to quantify each enzyme and determine molecular mass. An identical procedure had been employed for the demonstration of multiple trypsin-like proteases in rat islets of Langerhans using YAKR-CK, in which the tyrosine residue proved an ideal vehicle for radioiodination (Docherty, 1982). The trypsin-like enzyme activity in crude supernatants and whole cell sonicates of *P. gingivalis* W83, adjusted to the same initial activity, was abolished by YAKR-CK (Fig. 1), demonstrating the suitability of this reagent for the quantification of these enzymes. However, although this inhibitor was effective against the trypsin-like activity, based on the synthetic chromogenic substrate assay, the total protease assay using FITC-BSA demonstrated that a proportion of the proteolytic enzyme activity was still active in the crude preparations (22% for crude supernatant and 14% for sonicated whole cells) following treatment with excess YAKR-CK (Fig. 1, inset). The trypsin-like activity and total protease activity of the Protease I preparation were both reduced to < 5% following treatment with excess YAKR-CK. Thus non-trypsin-like enzyme(s) contribute to the total protease activity of this organism.

Affinity labelling of *P. gingivalis* W83 culture supernatant, cell sonicates and 47 kDa enzyme preparation

Autoradiograms of SDS-PAGE gels of crude supernatant labelled with [^{125}I]YAKR-CK demonstrated two distinct activities in the total extracellular fraction (Fig. 2). First, a well defined band with a mobility corresponding to a molecular mass of about 47 kDa (labelled I in Fig. 2). This coincided in position with a major protein on Coomassie-blue-stained gels of culture supernatant (lanes 1 & 2, Fig. 2). The specificity of the labelling procedure was demonstrated by the absence of radioactivity associated with a protein of slightly higher molecular mass and similar staining intensity to the 47 kDa protein on the Coomassie-blue-stained gel. A second, less intense, and very diffuse activity was located in the 70–90 kDa area of the gel (labelled II in Fig. 2). Repeated attempts to improve the resolution of this band were unsuccessful. The reason for the poor electrophoretic behaviour of this species is unknown at present. Possible explanations may involve either high concentrations of associated carbohydrate or autolysis prior to inactivation. Sonicates of whole cells showed a similar pattern of labelling with YAKR-CK, although the relative proportion of Protease II in these preparations appeared higher than in culture supernatant (Fig. 3). SDS-PAGE analysis of the Protease I preparation demonstrated a predominant 47 kDa component, with small amounts of 43, 40 and 30 kDa contaminant proteins. Autoradiography of [^{125}I]YAKR-CK-treated

Fig. 3. Affinity labelling of the trypsin-like enzymes in whole cell sonicates of *P. gingivalis* W83 using ^{125}I -YAKR-CK. Lanes: 1-3, Coomassie-blue-stained SDS-PAGE of sonicated whole cells (0.8, 1.6 and 4 μg protein, respectively) pretreated with labelled inhibitor; 4-6, autoradiogram of lanes 1-3. I and II, bands discussed in text.

Fig. 4. Affinity labelling of an iso-electric-focussing preparation of Protease I of *P. gingivalis* W83 using ^{125}I -YAKR-CK. Lane 1, Coomassie-blue-stained SDS-PAGE gel of enzyme (0.5 μg) pretreated with labelled inhibitor; lane 2, autoradiogram of lane 1.

Fig. 5. Effect of active-site-directed inhibitors on the trypsin-like enzyme activity of Protease I: $\times$, YAKR-CK; $\blacksquare$, antipain; $\bullet$, chymostatin; $\blacktriangledown$, TLCK; $\blacktriangle$, HgCl_2 .

Protease I preparation gave a single strong band at 47 kDa, indicating the separation of Protease I from Protease II by the iso-electric focussing procedure (Fig. 4). This material was then used to examine the specificity and blocking of YAKR-CK-binding to Protease I.

Fig. 6. Effect of protease inhibitors on ^{125}I -YAKR-CK labelling of Protease I (0.5 μg). The enzyme was pretreated with each of the inhibitors/modifiers for 15 min prior to labelling with ^{125}I -YAKR. (a) Lanes: 1, L-cysteine (10 mM); 2, Tris/HCl, pH 7.2 (50 mM); 3 and 4, HgCl_2 (5 and 25 mM); 5 and 6, TLCK (0.01 and 1.0 mM). (b) Lanes: 1, L-cysteine (10 mM); 2 and 3, chymostatin (0.01 and 1.0 mM); 4 and 5, antipain (0.01 and 1.0 mM); 6, heat-inactivated enzyme (100 $^{\circ}\text{C}$, 10 min).

Inhibitors of YAKR-CK-labelling of Protease I

The specificity of binding of YAKR-CK to Protease I was examined by preincubation of the enzyme with other inhibitors which interact with the active site of serine/cysteine enzymes. Antipain and chymostatin are peptide aldehydes which inhibit members of both classes of enzyme through the formation of a hemiacetal or hemithio-acetal linkages between the aldehyde carbonyl of the inhibitor and the active site serine or cysteine of the enzyme. Both of these aldehyde peptides inhibited the hydrolysis of Chromozym TRY by Protease I (Fig. 5). The apparent discrepancy in the effectiveness of the two inhibitors most likely reflects the relative purity/activity of these two commercial preparations rather than absolute differences in the stoichiometry of the respective interactions. Preincubation of the Protease I preparation with these inhibitors, at concentrations which achieved full enzyme inhibition, eliminated the binding of YAKR-CK to the enzyme (Fig. 6a), indicating the requirement for an unblocked serine/cysteine

active site residue for YAKR-CK binding. Similarly, TLCK which shares the same mechanism of inhibition as YAKR-CK, inhibited enzyme activity and blocked the binding of the radiolabelled inhibitor (Figs 5 and 6b).

In an attempt to probe the identity of the nucleophilic residue at the active site of Protease I, we examined the binding of YAKR-CK in the presence and absence of activating concentrations of thiol groups in the buffer and of mercuric ions, which inhibit cysteine enzymes through non-specific interaction with free thiol groups on the protein. No hydrolysis of Chromozym TRY by Protease I was observed in the absence of exogenously added thiols. Maximal activity was obtained at cysteine. HCl concentrations greater than 1 mM (data not shown). This is in agreement with several reports in the literature on the thiol-activated nature of the trypsin-like enzyme activity of this organism. However, absence of cysteine in the labelling buffer had no effect on the binding of YAKR-CK to the enzyme (Fig. 6b). An incidental finding upon inclusion of cysteine was the occasional generation of a minor component of lower molecular mass which was also labelled with YAKR-CK (Fig. 6b, lane 1). An explanation for the appearance of this component may be the autolytic modification of Protease I in the presence of activating concentrations of thiol residues prior to full inhibition by YAKR-CK. Autolytic degradation of the trypsin-like proteases of this organism may account for the multiple molecular masses reported for these enzymes (Grenier *et al.*, 1989b). Mercuric ions, above 10 mM, fully inhibited Protease I in the chromogenic substrate assay but only partially reduced the binding of YAKR-CK when present at 25 mM. This discrepancy may be explained by the mercuric ion inhibition in the enzyme assay being a result of the consumption of free thiols from the buffer rather than reaction with an active site cysteine residue, which would be expected to cause a concomitant inhibition of labelling with YAKR-CK. Alternatively, inhibition of activity by mercuric ions may be mediated through reaction with a cysteine residue not directly involved in the catalytic process: there are some members of the subtilisin family of serine proteases which have a free -SH group, apparently not catalytically functional, whose reaction with mercury causes inactivation, possibly because of proximity to essential residues (Betz *et al.*, 1986; Mizuno *et al.*, 1988). Shah *et al.* (1991) have isolated a cysteine protease from *P. gingivalis*, although a full characterization of the enzyme has not been reported. However on the basis of an approximate molecular mass of 95–100 kDa, it appears to be distinct from Protease I and II in the present report. Unequivocal classification of Protease I must await determination of the primary sequence of the active site residues. However, use of YAKR-CK may permit a more targeted approach to

Table 1. Minimum inhibitory concentration of inhibitors of agglutination of human red blood cells by *P. gingivalis* whole cells and Protease I

Numbers in parentheses refer to maximum concentration used.

Inhibitor	Minimum inhibitory concn (mM) for:	
	Whole cells	Protease I
YAKR-CK	< 0.002	0.25
HgCl ₂	nil (12.5)	nil (12.5)
TLCK	0.13	< 0.040
Chymostatin	nil (0.4)	nil (0.4)
Antipain	nil (0.25)	nil (0.25)

this analysis via N-terminal sequence analysis of peptide maps of the labelled enzyme.

Inhibition of haemagglutination

There is increasing evidence that the trypsin-like enzyme activities have a role in the adherence of *P. gingivalis* to a variety of surfaces in the sub-gingival environment. Attachment of *P. gingivalis* whole cells to collagenous substrata (Naito & Gibbons, 1988) and haemagglutination of human red blood cells by this organism (Inoshita *et al.*, 1986; Okuda *et al.*, 1986) are both blocked by protease inhibitors, and the co-aggregation of *P. gingivalis* with *Actinomyces viscosus* is similarly disrupted by agents capable of inhibiting the cell-associated trypsin-like enzyme activity (Li *et al.*, 1991). In the current investigation, the Protease I preparation was able to agglutinate human red blood cells, and this was inhibited by YAKR-CK (Table 1). Although other minor protein components are present in this preparation, YAKR-CK bound only to the 47 kDa moiety and hence it is reasonable to conclude that the protease itself is involved in the haemagglutination reaction. Haemagglutination was also inhibited by TLCK, but not by chymostatin nor antipain, both of which could fully inhibit Chromozym TRY hydrolysis at much lower concentrations (Fig. 5). Similar data were obtained using *P. gingivalis* whole cells. The discrepancy between the data for inhibition of haemagglutination compared to those for inhibition of protease activity indicates that simply blocking of the active site of the protease may be insufficient to prevent binding to the erythrocyte membrane surface: other sub-sites on the molecule may be involved, or the haemagglutinating molecule may represent a multicomponent system incorporating a proteolytic element(s) and adhesion polypeptide(s). The present data do not therefore enable the distinction between (1) a direct role for the trypsin-like proteases of this organism in haemagglutination via binding to the

erythrocyte surface through the active site(s), and (2) an indirect role via proteolytic unmasking of epitopes which may act as targets for *P. gingivalis* adhesins on the erythrocyte membrane.

We conclude that *P. gingivalis* W83 expresses two distinct proteases with trypsin-like specificity. Both enzymes are cell-associated and released into the supernatant of batch cultures of the organism. We cannot yet state whether they are both components of the extra-cellular vesicle trypsin-like activity (Smalley & Birss, 1987). These data are complementary to those recently presented by Hinode *et al.* (1991) who described the purification of three proteases from the culture supernatant of *P. gingivalis* 381. Two of these were active against benzoyl-arginyl-*p*-nitroanilide and had similar electrophoretic mobilities on SDS-PAGE to Proteases I and II as described in this investigation. Given the wide disparity of molecular masses in the literature for the trypsin-like enzymes produced by this organism, it is possible that the enzymes exist in polymeric forms and/or undergo proteolytic modification when in solution. However, the relative ease of separation of the two activities in culture supernatant by iso-electric focussing suggests that they do not co-polymerize. The extent of monomer association and autolysis is currently under investigation.

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