Altered expression and modification of proteases from an avirulent mutant of *Porphyromonas gingivalis* W50 (W50/BE1)

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Proteases of *Porphyromonas gingivalis* are considered to be important factors in the virulence of this organism. A non-pigmenting mutant of *P. gingivalis* W50 (W50/BE1) has been shown to be less virulent in animal models and to produce significantly less Arg-specific protease activity than the parent strain. Three proteases are present in the culture supernatant of *P. gingivalis* W50: RI, RIA and RIB. All three proteases are derived from *prpR1*, which encodes a polypeptide of 1706 amino acids that is organized into distinct domains (pro, α , β and γ). The aim of the present investigation was to purify and characterize the Arg-specific proteases produced by the avirulent W50/BE1 strain. Significant differences were observed between the proteases of *P. gingivalis* W50 and W50/BE1. The levels of RI present in the culture supernatant of W50/BE1 were lower than those present in W50, and RIA and RIB were absent. RI from W50/BE1 was composed of three polypeptide chains, unlike the enzyme from W50, which is a heterodimer. The remainder of the Arg-specific protease activity in W50/BE1 was derived from a second gene, *prR2*, and was present in two fractions, RIIA^S/BE (soluble) and RIIA^V/BE (vesicle-bound). This activity contained two peptide chains: a ~ 54 kDa chain corresponding to the protease domain and a ~ 26 kDa chain, derived from the propeptide domain of the PrRII precursor. No enzyme with large glycan additions, equivalent to RIB in the vesicle fraction of the wild-type W50, was present. These data indicate that the reduced level of extracellular protease activity in W50/BE1 reflects reduced synthesis and/or export of *prpR1* enzymes, which is only partially compensated by synthesis of *prR2*-derived enzymes, and that all of these proteases undergo altered post-translational modification compared to the parent strain.

Keywords: *Porphyromonas gingivalis*, avirulent, extracellular arginine-specific proteases, altered modification

INTRODUCTION

Porphyromonas gingivalis has been implicated as one of the causal organisms in chronic adult periodontal disease. Virulence factors of this organism include haemagglutinins (Han *et al.*, 1996; Hinode *et al.*, 1995), fimbriae (Chandad & Mouton, 1995) and extracellular proteases, of which those specific for Arg-x and Lys-x peptide bonds (Okamoto *et al.*, 1996; Aduse-Opoku *et al.*, 1995; Pike *et al.*, 1994) have received the greatest

attention. Three forms of Arg-x proteases have been isolated, purified and characterized from *P. gingivalis* W50 culture supernatant in our laboratory (Rangarajan *et al.*, 1997a). These are referred to as RI, RIA and RIB. RI is a heterodimer composed of a catalytic ~ 54 kDa α -chain and an ~ 58 kDa adhesin β -chain, whereas RIA consists of the ~ 54 kDa catalytic α -chain, and RIB consists of the α -chain covalently modified with carbohydrate (70–80 kDa). All three enzymes are derived from *prpR1* (Aduse-Opoku *et al.*, 1995). More recently, a second gene (*rgpb/prR2*) has been identified which also encodes Arg-specific protease activity (Nakayama *et al.*, 1995; Rangarajan *et al.*, 1997b). Construction of a *prpR1* isogenic mutant allowed analysis of the proteases from this second gene. Two enzymes, RIIA and RIIB,

Abbreviations: DL-BRpNA, *N* α -benzoyl-DL-arginine *p*-nitroanilide; L-BRpNA, *N* α -benzoyl-L-arginine *p*-nitroanilide; DNS-EGRCK, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone, dihydrochloride; HMG, high-molecular-mass gingipain.

Fig. 1. Generation of Arg-specific proteases from PrpRI and PrRII in *P. gingivalis*. PrpRI (1706 amino acids) is organized into four domains: pro, α , β and γ . PrRII is organized into two domains: pro and α . Vertical arrowheads above PrpRI indicate the positions of the proteolytic processing sites which generate the heterodimer RI. Arrows above PrRII indicate the position of the processing site which generates RIIA and RIIB. The resulting N-termini are shown below PrpRI and PrRII. (Note that the sequence at the junction of the β - and γ -regions is in italics to indicate that this processing site is an assumption based on the molecular size of the β -component.) The open arrowhead marks the position of the putative signal peptidase cleavage site. The monomeric RIA and RIB may be derived by either processing of PrpRI or truncated transcription of the gene.

were isolated which differed from RIA and RIB by two amino acid changes within the first 20 amino acids from the N-terminus, Gln₈ → Glu₈ and Ala₁₇ → Pro₁₇, as shown in Fig. 1.

A spontaneously arising mutant with weakly pigmented colony forms was isolated from cultures of *P. gingivalis* W50 grown in the chemostat by McKee *et al.* (1988). This mutant was named W50/BE1 due to the beige colour of the colonies after 21 d, in contrast to the wild-type, which pigments black by 7 d. The wild-type and the mutant were tested for virulence in a mouse model of pathogenicity based on the method of van Steenberg *et al.* (1982). *P. gingivalis* W50 was injected subcutaneously into the mouse at an infective dose of 1×10^9 viable cells, which caused a spreading inflammation characterized by necrosis of tissues from the epidermis to the abdominal muscle layers, localized abscess formation, diffuse accumulation of polymorphonuclear leucocytes, oedema and death of all the mice. W50/BE1 killed no animals and caused either a mild inflammatory reaction or microscopic foci of dermal inflammation and remained avirulent at all infective doses tested (McKee *et al.*, 1988).

W50/BE1 lacked gelatinase, collagenase and dipeptidyl aminopeptidase activities compared to the parent strain, and had reduced hydrophobicity, haemagglutinating activity, fimbriation and extracellular vesicle production (Shah *et al.*, 1989). In particular, the level of extracellular Arg-x protease activity in W50/BE1 was significantly lower than in the parent strain *P. gingivalis* W50. These enzymes are considered to play an important role in virulence, supported by evidence from several studies on spontaneously arising and transposon-induced mutants lacking Arg-x activity. Kesavalu *et al.* (1996) showed that *P. gingivalis* W50 treated with an active-site inhibitor (*N* α -*p*-tosyl-L-lysine chloromethyl ketone; TLCK) specific for the Arg-x and Lys-x proteases prior to subcutaneous injection into the mouse failed to give the necrotic reaction observed when untreated cells were

injected. In the same study, the protease-deficient mutant NG4B19 derived from *P. gingivalis* strain 3079.03 was found to be completely avirulent. Similarly, mutant V2296 derived from insertional inactivation of the *prtH* gene encoding Arg-x protease activity from *P. gingivalis* strain W83 also showed a corresponding reduction in virulence in the mouse model (Fletcher *et al.*, 1995).

The aim of this study was to isolate and characterize the enzymes from W50/BE1 to determine whether the reduced level of activity in the mutant represents reduced synthesis of all of the wild-type enzymes, or production of differing amounts of the three forms. This information will lead to a better understanding of the role of these proteases in the virulence of the organism.

METHODS

To distinguish the proteases of *P. gingivalis* W50 from those of the mutant *P. gingivalis* W50/BE1, enzymes derived from the mutant will be given the suffix '/BE'.

Materials. DEAE-Sephacel and Sephacryl S-200 HR were obtained from Pharmacia Biotechnology. L-Arg-4% beaded agarose, DL-BRpNA, L-BRpNA and leupeptin were purchased from Sigma or Bachem Feinchemikalien. Zwittergent 3-14 detergent and DNS-EGRCK were obtained from Calbiochem Novabiochem (UK). All other chemicals and reagents were the purest grades available from Sigma or BDH.

Bacterial strains and growth conditions. *P. gingivalis* W50/BE1 was obtained from P. Marsh (PHLS, Porton Down, UK). Strains were maintained on blood agar plates [2.5%, v/v, horse blood (TCS) added to 4%, w/v, blood agar base no. 2 (Oxoid) in distilled water] at 37 °C in an anaerobic cabinet (Don Whitley Scientific) equilibrated in nitrogen/hydrogen/carbon dioxide (80:10:10, by vol.). Batch culture was carried out in 3.7% (w/v) brain heart infusion broth (Oxoid) containing 0.0005% (w/v) haemin for 6 d in the anaerobic cabinet.

Enzyme assays. Measurement of Arg-specific enzyme activity was carried out as described by Rangarajan *et al.* (1997a). In general, 0.3–0.5 μ g enzyme was used per assay. CAPS, CHES,

HEPES, MES, MOPS and Tris/HCl were used as buffers in the determination of pH optima. The pH of the buffers was adjusted at room temperature; the actual pH at 30 °C was calculated from the heats of ionization of the buffers: $\Delta\text{pH per } ^\circ\text{C} = -0.011$ for MES, -0.013 for MOPS, -0.014 for HEPES, -0.028 for Tris/HCl (Good *et al.*, 1966), -0.011 for CHES and -0.012 for CAPS (Dawson *et al.*, 1986). The buffers were then adjusted to an ionic strength of 12 mM by addition of NaCl. To avoid affecting the pH for measurement of the pH optimum, DTT was used as the reducing agent in the assay buffer in place of cysteine (HCl). One unit of enzyme activity is defined as that amount of enzyme which liberates 1 $\mu\text{mol p-nitroaniline min}^{-1}$ at 30 °C.

Values of maximum velocity, V_{max} (units mg^{-1}), and apparent K_m (μM) were derived by non-linear regression analysis of the data using the ENZFITTER Data Analysis program (Biosoft) with 2.5–250 μM L-BRpNA at 30 ± 0.1 °C.

Determination of protein concentrations. Protein concentrations were determined by A_{280} and A_{260} measurements (Dawson *et al.*, 1986) in a Kontron Uvicon 930 spectrophotometer. Protein concentration was then calculated according to the formula $[1.55 (A_{280}) - 0.76 (A_{260})]$.

Purification of Arg-specific proteases. *P. gingivalis* W50/BE1 6 d culture (6–8 l) was centrifuged and the supernatant used as the source of the Arg-specific proteases. Enzymes were purified using the techniques developed for the wild-type *P. gingivalis* W50 as described by Rangarajan *et al.* (1997a). Following ammonium sulphate precipitation of the culture supernatant, Arg-specific activity of *P. gingivalis* W50 is separated into two fractions, soluble (S) and pellet (P), by differential detergent solubilization of the ammonium sulphate pellet. Fraction S contains RI and RIA, which are soluble in low-detergent buffer (0.0055%) and are separated from each other by Arg-agarose affinity chromatography. Fraction P contains RIB, which is membrane/vesicle-associated and requires high-detergent buffer (0.05%) for solubilization.

SDS-PAGE of purified proteins. Protein samples were run on 12.5% polyacrylamide slab gels (10 cm $\times$ 7 cm $\times$ 0.15 cm) containing SDS (Laemmli, 1970) at 5 °C. In general, 20 μg protein was used per sample, which in each case was first treated with an equal volume of 1 mM leupeptin at 22 °C for 20 min and heated at 100 °C for 5 min to inhibit autolytic activity. Samples containing high salt were then dialysed against 5% (v/v) acetic acid/0.0055% Zwittergent and dried *in vacuo* before being treated with SDS sample buffer. Proteins were transferred to nitrocellulose membranes in a CAPS buffer system (10 mM CAPS, pH 11; 10%, v/v, methanol) at a constant current of 400 mA for 30 min. After blotting, the membranes were blocked in 5% (w/v) BSA and incubated overnight in primary antibody in 1% (w/v) BSA. Antibody binding was detected using horseradish-peroxidase-conjugated anti-species immunoglobulins and diaminobenzidine (0.05%, w/v).

N-terminal sequencing. Proteins were transferred to PVDF Immobilon membranes (Millipore) after SDS-PAGE. N-terminal amino acid sequencing was carried out at the Haemostasis Research Group, Royal Postgraduate Medical School, London, UK.

Active-site titration. Active-site titration was used to determine the proportion of active protease present in the purified preparations, based on the method of Potempa *et al.* (1997). A series of standard solutions of the fluorescent active-site inhibitor DNS-EGRCK at concentrations of 0.1–1 μM was made in 1 mM HCl. Enzyme (0.2–0.6 mg ml^{-1}) was incubated

in 0.125 M Tris/HCl/12.5 mM CaCl_2 /12.5 mM L-cysteine, pH 8.0, at 22 °C for 30 min to activate the protease. Aliquots (20 μl) of activated protease were measured into 1.5 ml disposable cuvettes (Elkay Products) and 20 μl of each standard solution of inhibitor was added. The solutions were mixed thoroughly and incubated for 30 min at 30 °C. The reaction was stopped by the addition of 0.8 ml of assay buffer maintained at 30 °C and enzyme activity remaining was measured by the addition of 0.2 ml 500 mM DL-BRpNA substrate. All assays were performed in duplicate.

RESULTS

Arg-x enzyme activity of *P. gingivalis* W50/BE1

Total Arg-x specific enzyme activity in the 6 d culture was 0.16 U (ml culture^{-1}) as compared with 0.48 U ($\text{ml W50 culture}^{-1}$). Arg-x specific enzyme activity in the 6 d culture supernatant of *P. gingivalis* W50/BE1 was approximately 25–30% of the activity of the parent strain [0.11 U ($\text{ml W50/BE1 culture}^{-1}$) as compared with 0.37 U ($\text{ml W50 culture}^{-1}$)]. The mutant also showed a corresponding reduction in the Lys-x specific activity to approximately 20% of the level in the parent strain. Details of purification of the Arg-specific enzymes from the culture supernatant are shown in Table 1. Greater than 95% of the Arg-x enzyme activity was precipitated from the culture supernatant using 85% ammonium sulphate. Approximately 70% of enzyme activity could be solubilized from the ammonium sulphate pellet using low-detergent (0.0055%) buffer. In keeping with the nomenclature used for the parent strain enzymes, the portion of the enzyme activity in this soluble fraction which was bound on the Arg affinity chromatography resin was designated RI/BE and activity not retained was referred to as RIA/BE. RI/BE was isolated with relatively high purity due to the specificity of the affinity chromatography step. In the residual ammonium sulphate pellet, a further 14% of total enzyme activity could be solubilized using high-detergent (0.05%) buffer. This was designated RIB/BE. The bulk of the remaining activity could only be extracted with large volumes of high-detergent buffer following repeated extractions and was therefore not routinely attempted. RIA/BE and RIB/BE required several ion-exchange chromatography steps to achieve pure enzyme. This is reflected in the low values of yield and specific activity (Table 1) compared to enzymes from *P. gingivalis* W50, which suggests that some inactivation could have occurred (see later).

Genetic origin of purified proteins

Purity of the three enzymes was assessed by SDS-PAGE (Fig. 2). Comparison of RI from the wild-type versus RI/BE showed major differences. Whereas RI gave only one band on SDS-PAGE, which contained both α - and β -subunits (Rangarajan *et al.*, 1997a), RI/BE showed the presence of three strong bands of approximately 54, 36 and 30 kDa. N-terminal amino acid sequencing of these bands indicated that the 54 kDa protein corresponds to the α -catalytic domain, the 36 kDa protein represents a

Table 1. Purification and yields of the three forms of Arg-x protease from *P. gingivalis* W50/BE1

For experimental details see text. The values are from 7 l culture supernatant. Bold typeface indicates data for pure protease samples.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)		Purification (-fold)
				Step	Total	
1. Culture supernatant	1245	117 600	0.010	100	100	1
2. Ammonium sulphate (85%)	1186	98 090	0.012	95	95	1.2
3. Soluble fraction (S)	807	5 580	0.150	68	65	15
3.1 Affinity chromatography fraction S-B (bound) RI/BE	165	12.1	13 640	20	13	1364
3.2 DEAE-Sephacel chromatography of S-U (unbound) RIIA^S/BE	176	28.4	6 190	22	14	619
4. Pellet fraction (P)	161	1 364	0.120	14	13	12
4.1 Affinity chromatography fraction P-U (unbound)	148	1 064	0.140	92	12	14
4.2 DEAE-Sephacel chromatography of P-U RIIA^V/BE	28	15.6	1 800	17	2.3	180

Fig. 2. SDS-PAGE of Arg-specific proteases from *P. gingivalis* W50 and W50/BE1. Pure RI, RIA, RIB, RI/BE, RIIA^S/BE and RIIA^V/BE were treated with 1 mM leupeptin to inhibit protease activity and subjected to SDS-PAGE in 12.5% acrylamide gels. The molecular masses of marker proteins are indicated alongside the gel. Lanes: 1, RI; 2, RIA; 3, RIB; 4, RI/BE; 5, RIIA^S/BE; 6, RIIA^V/BE. Gels were stained for protein with Coomassie brilliant blue R250.

C-terminally truncated β -domain and the 30 kDa protein is derived from the γ -region of the PrpRI protein precursor (Table 2). This is shown in Fig. 3, in which the internal processing sites within the β - and γ -regions are indicated. Cleavage of the peptide bond between the pro- and α -regions, Arg₂₂₇-Tyr₂₂₈, and the bond between the α - and β -regions, Arg₇₁₉-Ser₇₂₀, generates the α -catalytic domain which is unaltered in the enzymes from W50 and W50/BE1. The putative processing site between the β - and γ -regions, Arg₁₂₆₂-Phe₁₂₆₃, would generate the $\sim$ 58 kDa β -adhesin domain seen in RI from the wild-type strain. However, the size of the β -domain seen in RI/BE suggests that processing of the

PrpRI precursor may occur at Arg₁₁₃₈-Ala₁₁₃₉, giving rise to the $\sim$ 36 kDa peptide (Ser₇₂₀-Arg₁₁₃₈). Hydrolysis of Arg₁₄₃₁-Ala₁₄₃₂ within the γ -region would release a $\sim$ 30 kDa C-terminal peptide. However, the intervening fragment, Ala₁₁₃₉-Arg₁₄₃₁, was not observed on SDS-PAGE of RI/BE. It is possible that the 'missing' Ala₁₁₃₉-Arg₁₄₃₁ peptide has been processed into smaller fragments which are not detected on either 12.5% SDS-PAGE or on SDS-gradient gels. Samples of RI/BE which were electroblotted onto PVDF membranes after SDS-PAGE for N-terminal amino acid sequencing also showed the presence of two minor bands (<2% intensity of all Coomassie-stained bands) of $\sim$ 78 kDa and $\sim$ 57 kDa. N-terminal amino acid sequence analysis revealed the same N-terminus as the 36 kDa truncated β -region (SGQAE...; see Table 2). However, the size of these fragments indicates that they probably encompass all of the β -region and part of the γ -region of PrpRI (Aduse-Opoku *et al.*, 1995), i.e. Ser₇₂₀-Arg₁₄₃₁. These large fragments have never been observed in preparations of RI from *P. gingivalis* W50. In all previous work (Rangarajan *et al.*, 1997b; Pavloff *et al.*, 1995), the largest peptide from the β - and γ -regions of PrpRI has been the β -adhesin domain of RI from *P. gingivalis* W50. It is possible that the $\sim$ 78 kDa and $\sim$ 57 kDa domains co-purify with RI/BE during affinity chromatography on Arg-agarose columns.

RIA/BE gave two protein bands on SDS-PAGE corresponding to $\sim$ 54 kDa and $\sim$ 26 kDa, which differs from the parent RIA, which gives only a single band of $\sim$ 54 kDa. N-terminal amino acid sequencing (Table 2) revealed that the $\sim$ 54 kDa peptide was derived from *prR2* since it contained the characteristic Glu residue at position 8 and Pro residue at position 17 (versus Gln₈ and Ala₁₇ in *prR1*-derived enzymes). Furthermore, the N-terminal amino acid sequence of the $\sim$ 26 kDa peptide (GRNPQ...) is located in the pro-region of *prR2* [Rangarajan *et al.*, 1997b; accession no. AF007124

Table 2. N-terminal amino acid sequence of *P. gingivalis* W50/BE1 Arg-specific enzymes

Enzyme	Molecular mass (kDa)	N-terminal amino acid sequence*
RI/BE	54	YTPVEEK ...
	36	SGQAE ...
	30	ANEA ...
RIIA ^S /BE	54	YTPVEEKENGRMIVIVPKKY ...
	26	GRNPQVRLLSAE ...
RIIA ^V /BE	54	YTPVEEKENGRMIVIVPKKY ...
	26	GRNPQVRL ...

* Amino acid residues which differ from those present in corresponding enzymes from *P. gingivalis* W50 are indicated in bold.

Fig. 3. Generation of the three forms of Arg-specific proteases in *P. gingivalis* W50/BE1. The organization of PrpRI and PrpRII are as in Fig. 1. (a) Arrowheads above PrpRI indicate the proteolytic processing sites required to generate the heterodimer of RI. Arrowheads below PrpRI indicate putative processing sites required to generate the multimer form of RI/BE. Open arrowheads indicate the additional processing sites proposed by Pavloff *et al.* (1995) which generate a multimeric, non-stoichiometric complex of polypeptides which in association with the α-domain generates HMG. (b) Arrowheads above PrpRII indicate the protease processing site required to generate RIIA and RIIB. Arrowheads below PrpRII indicate processing sites required to generate RIIA^S/BE and RIIA^V/BE.

(N. Slakeski and others, complete cds)] and would be generated by processing at Arg₂₉–Gly₃₀ and Arg₂₃₀–Tyr₂₃₁, which would release a peptide fragment of 201 residues (Fig. 4).

In the case of the membrane/vesicle-bound enzyme, we did not observe a highly modified form equivalent to RIB from the parent strain. Instead, RIB/BE gave two bands of ~ 54 kDa and ~ 26 kDa on SDS-PAGE as we had observed for RIA/BE. The N-terminal amino acid sequences of the ~ 54 and ~ 26 kDa bands from RIB/BE were identical to the N-terminal sequences obtained for RIA/BE, indicating that RIB/BE was also derived from *prR2*. Throughout the remainder of the manuscript we will therefore refer to these *prR2*-derived enzymes in W50/BE1 as RIIA^S/BE (soluble) and RIIA^V/BE (vesicle-bound) to indicate that they are derived from *prR2* (Fig. 3) and are probably the same isoform with different extracellular locations.

Antibody reactivity of purified proteins

Western blotting with antibodies raised either to *P. gingivalis* W50 whole cells, the β-region of *P. gingivalis* W50 or *P. gingivalis* W50 LPS was carried out as a means of comparing W50/BE1 enzymes with those from W50.

The anti-whole-cell antibody recognized all major components of the protein samples apart from the ~ 30 kDa fragment of RI/BE and the ~ 26 kDa pro-peptide domain of RIIA^S/BE and RIIA^V/BE (Fig. 5a). Antibody 1A1, a monoclonal recognizing the β-region of RI, gave a positive reaction with the ~ 78, ~ 57, ~ 54 and ~ 36 kDa bands (Ser₇₂₀–Arg₁₁₃₈) of RI/BE, confirming that these polypeptides contain Gly₉₀₇–Thr₉₃₁ recognized by the antibody (Curtis *et al.*, 1996) (Fig. 5b). Antibody 1B5, a monoclonal which recognizes *P. gingivalis* W50 LPS (unpublished observation) and which reacts with both RIA and RIB, failed to recognize

PtrR2	1	MKKNFSRIVSIVAFSSLLGGMAFAQPAERGNPQVRL. SAEQSMKSVQF	49
PrpRI	1	.MKNLNKFSVIALCSSLLGGMAFAQQTTELGRNPVRLLESTQQSVTKVQF	49
PtrR2	50	RMDNLQFTGVQTSKGVAVPTFTTEGVNISEKGTPIPLILSRSLAVSETRA	99
PrpRI	50	RMDNLKFTEVQTPKGMQVPTYTEGVNLSEKGMPTLPILSRSLAVSDTRE	99
PtrR2	100	MKVEVVSSKFIEKKDVLIAPSKGVISRAENPDQIPYVYGQSYNEDKFFPG	149
PrpRI	100	MKVEVVSSKFIEKKNVLIAPSKGMIMRNEDPKIPYVYGKSYQNKFFPG	149
PtrR2	150	EIATLSDFILRDVRGQVNVFAPLQYNPVTKTLRIYTEIVVAVSETAEAG	199
PrpRI	150	EIATLDDPFILRDVRGQVNVFAPLQYNPVTKTLRIYTEITVAVSETSEQG	199
PtrR2	200	QNTISLVKNSTFTGFEDIYKSVFMNYEATRYTPVEEKNGRMIVIVPKKY	249
PrpRI	200	KNILN. .KKGTFAGFEDTYKRMFMNYEPGRYTPVEEKQNGRMIVIVAKKY	247

Fig. 4. Alignment of N-terminal amino acid sequences of PtrR2 and PrpRI. The ~26 kDa peptide of RIIA^S/BE and RIIA^V/BE is generated by cleavage of the bonds at Arg₂₉-Gly₃₀ and Arg₂₃₀-Tyr₂₃₁. The fragment is derived from the *prR2* locus (analogous to *ptrR2*), indicated by the characteristic Glu₈ and Pro₁₇ (versus Gln₈ and Ala₁₇ in *prpR1*-derived enzymes).

Fig. 5. Western blot analysis of Arg-specific proteases of *P. gingivalis* W50/BE1. Pure protease samples were immunoblotted onto nitrocellulose membranes and probed with a polyclonal antibody raised to *P. gingivalis* W50 whole cells (α -wc), and mAbs raised to *P. gingivalis* W50 RI β -chain (1A1) and *P. gingivalis* W50 LPS (1B5). Antibody binding was detected using horseradish-peroxidase-conjugated anti-species immunoglobulins and diaminobenzidine. The molecular masses of marker proteins are indicated alongside the blots. (a) Lanes 1–3, antibody α -wc against RI/BE, RIIA^S/BE and RIIA^V/BE, respectively. (b) Lanes 1–3, antibody 1A1 against RI/BE, RIIA^S/BE and RIIA^V/BE, respectively. (c) Lanes 1–7, antibody 1B5 against RI, RIA, RIB, RI/BE, RIIA^S/BE, RIIA^V/BE and *P. gingivalis* W50 LPS, respectively.

RIIA^S/BE or RIIA^V/BE, indicating that the proteases from W50/BE1 lack glycan modifications (Fig. 5c).

Enzymology

Enzyme kinetic studies were carried out to establish the similarity of the proteases from the mutant to the proteases from the wild-type (Table 3). Optimum enzyme activity was obtained when cysteine (10 mM) was used in the assay buffer. The pH optimum for both the mutant and wild-type enzymes was 7.9–8.1, measured in the presence of 10 mM DTT. However, RI/BE appeared to have a broader pH optimum for L-BRpNA activity. K_m and V_{max} were measured at pH 8.1

in the presence of 10 mM L-cysteine as the reducing agent: the K_m of the mutant enzymes (9–13 μ M using the chromogenic substrate L-BRpNA) was found to be of the same order of magnitude as the K_m for the wild-type enzymes whereas the V_{max} of RIIA^S/BE and RIIA^V/BE was approximately three- to fivefold lower than that of the corresponding enzymes from W50 and W501 (Table 3). Active-site titration of the enzymes indicated that the percentage of active enzyme in the purified preparations from W50/BE1 was only 28% for RIIA^S/BE and 20% for RIIA^V/BE, which could account for the low V_{max} compared to enzymes from *P. gingivalis* W50. This inactivation could be a result of the number of steps needed to isolate the pure enzymes. Protease RI/BE,

Table 3. Kinetic constants for Arg-specific protease activity from *P. gingivalis* W50 and W50/BE1

Strain	Enzyme	pH optimum	K_m (μ M; $\pm$ SE)*	V_{max} (U mg ⁻¹ ; $\pm$ SE)*
W50	RI	7.9–8.1	6 $\pm$ 0.90	11 $\pm$ 0.05
	RIA	7.9–8.1	10 $\pm$ 1.30	17 $\pm$ 0.13
	RIB	7.9–8.1	14 $\pm$ 1.25	17 $\pm$ 0.08
W501 (<i>prpR1</i> isogenic mutant)	RIIA	7.9–8.1	4 $\pm$ 1.10	17 $\pm$ 0.09
	RIIB	7.9–8.1	5 $\pm$ 1.40	12 $\pm$ 0.06
W50/BE1	RI/BE	8.0–8.6	9 $\pm$ 1.25	13 $\pm$ 0.13
	RIIA ^s /BE	7.9–8.1	13 $\pm$ 1.57	6 $\pm$ 0.16
	RIIA ^v /BE	7.9–8.1	13 $\pm$ 1.13	3 $\pm$ 0.07

* K_m and V_{max} were measured at 30 °C in 0.1 M Tris/HCl/10 mM Ca²⁺/10 mM cysteine, pH 8.1, using L-BRPN as the chromogenic substrate.

which required fewer purification steps, contained 73 % of active enzyme and the V_{max} value was comparable to that of RI. This suggests that the presence of extra polypeptides in RI/BE does not affect protease activity.

DISCUSSION

The Arg-specific proteases of *P. gingivalis* are thought to be important virulence determinants of the organism. This has been supported by studies using spontaneously occurring and transposon-induced protease-deficient mutants which demonstrate reduced virulence (Kesavalu *et al.*, 1996; Fletcher *et al.*, 1995). The present work confirms previous reports of reduced Arg-specific activity in the avirulent *P. gingivalis* W50/BE1 mutant. Recent progress in determining the genetic origin of the Arg-specific proteases from *P. gingivalis* W50 has allowed us to investigate whether reduced activity in W50/BE1 reflects reduced levels of all the isoforms or specific reduction of individual components.

Purification of the Arg-specific activity yielded three enzymes, RI/BE, RIIA^s/BE and RIIA^v/BE, which differ in subunit organization and N-terminal amino acid sequence from the equivalent enzymes from the parent strain. There was a decrease in extracellular proteases derived from the *prpR1* locus – RI/BE accounted for 13 % of the total Arg-specific enzyme activity (compared with 20 % in the parent strain W50) and RIA and RIB were absent. Activity was partially compensated by expression of *prpR2* products RIIA^s/BE and RIIA^v/BE. Therefore, there is an overall reduction in activity in the mutant as well as different synthesis/distribution of *prpR1* and *prpR2* products.

In addition to the altered distribution of the *prpR1* and *prpR2* products in W50/BE1, it appears that the proteases may be differently processed relative to the parent strain. RI/BE is composed of three peptide chains of approximately 54, 36 and 30 kDa in contrast to the $\alpha\beta$ -heterodimer structure of RI from W50. RI/BE is similar to a multimeric high-molecular-mass complex termed high-molecular-mass gingipain (HMG), which is derived from a homologous gene, *rgp-1*, in *P. gingivalis*

strain HG66 (Potempa *et al.*, 1997; Pavloff *et al.*, 1995). *rgp-1* encodes a polypeptide of 1704 amino acids which is processed to give peptides of 54, 44, 27, 17 and 15 kDa. The 54 kDa peptide is analogous to the 54 kDa α -catalytic domain of RI/BE, the 44 kDa peptide has the same N-terminal amino acid sequence as the 36 kDa β -adhesin domain of RI/BE, and the 27 kDa fragment is derived from the γ -region and is analogous to the 30 kDa fragment from RI/BE. However, the 15 and 17 kDa peptides, generated by cleavage between Arg₁₁₃₈–Ala₁₁₃₉, Lys₁₂₇₃–Pro₁₂₇₄ and Arg₁₄₃₁–Ala₁₄₃₂, respectively, are not seen on SDS-PAGE gels of RI/BE. This region contains at least five Arg residues, and it is possible that the peptides undergo autolytic degradation into smaller peptide fragments which cannot be detected on SDS-PAGE gels.

There may be a number of explanations for the observed differences in the subunit organization of RI purified from *P. gingivalis* W50 versus W50/BE1. It has already been established that there are a range of phenotypic differences between W50 and W50/BE1, including cell surface properties, proteolytic enzyme production and haemagglutination (McKee *et al.*, 1988). It is plausible that these differences may also include alterations to the processing/export machinery of the RI protease which leads to the generation of a different subunit organization of this enzyme from W50/BE1. Alternatively, the altered subunit organization of W50/BE1 RI may simply reflect differences in the proteolytic degradation of the enzyme in stationary-phase culture compared to the parent strain, since the overall proteolytic activity in these culture supernatants is markedly different. Some support for this proposal comes from the studies of Smalley & Birss (1991), which demonstrated that high-molecular-mass forms of extracellular Arg-specific enzyme are present in exponential-phase cultures of W50/BE1 and these are reduced in cultures older than 24 h. The purifications in the present report were deliberately performed on late-stationary-phase cultures to maximize the levels of enzyme in the culture supernatant and it is possible that this has accentuated a differential effect on extracellular degradation. A similar

explanation may account for the inconsistencies in the literature on the subunit organization of this protease isolated from different *P. gingivalis* strains (Pavloff *et al.*, 1995; Rangarajan *et al.*, 1997a). Conversely, however, we have failed to observe any changes to this enzyme purified from isogenic *prR2* mutants of *P. gingivalis* W50 in which the total extracellular Arg-specific protease levels are significantly lower than the parent strain (Aduse-Opoku *et al.*, 1998). Hence, while the reduction in the overall proteolytic activity of the culture supernatant of W50/BE1 may have some influence on the resultant subunit organization of RI, it may not represent the only factor.

There is some evidence that the RI heterodimer may have higher virulence potential than the other isoforms. HMG is a more potent activator of blood coagulation through activation of factor X than the 50 kDa gingipain R (analogous to RIA) by five- to sixfold, probably due to the adhesin domain increasing affinity to factor X and promoting cleavage of the substrate (Imamura *et al.*, 1997). Activation of factor X by HMG increases in the presence of phospholipids and calcium ions, while activation by the 50 kDa form is unaffected, perhaps due to calcium-ion-mediated binding of the adhesin domain to phospholipids. Furthermore, the 50 kDa gingipain R lacks the adhesin domain and does not bind to fibronectin and other matrix proteins which coat extracellular biosurfaces *in vivo*. In contrast, HMG binds and degrades fibrinogen with high affinity and fibronectin and laminin with lower affinity, suggesting a role for this isoform in adhesion to host cell surfaces and interference with host clotting mechanisms (Pike *et al.*, 1996). Low expression of RI/BE by the mutant may consequently contribute to the avirulence of W50/BE1.

Differences were also observed between RIIA^S/BE and RIIA^V/BE and their counterparts from the wild-type W50. N-terminal amino acid sequencing revealed that these two enzymes contained a ~ 54 kDa α -catalytic chain and a ~ 26 kDa peptide chain derived from *prR2* (Rangarajan *et al.*, 1997b) (Fig. 3). The N-terminal amino acid sequence of the ~ 26 kDa peptide indicated that it was derived from the pro-region of the polypeptide. Many proteases are synthesized as inactive precursors which are dependent upon proteolytic removal of the associated pro-region for activation. These propeptides are thought to act as mediators of protein folding and intramolecular chaperones involved in stabilization and cellular localization of their associated proteases. The propeptide of cathepsin B, a cysteine protease, acts as a potent inhibitor until it is removed by autoproteolysis (Fox *et al.*, 1992). The propeptide of subtilisin E, a serine protease, can also act as an inhibitor for its enzyme, and is essential for conversion of inactive pro-subtilisin E to the active form via mediation of correct folding of the subtilisin molecule (Ohta & Inouye, 1990; Zhu *et al.*, 1989). The propeptide of PrRII remains associated with RIIA^S/BE and RIIA^V/BE, even after rigorous purification, and these pure preparations are only 28 and 20% active, respectively. It is therefore possible that cleavage and dissociation of the propeptide

are required for correct folding to give the mature active protease. The retention of the propeptide in these forms may reflect either modification to the processing/export of the enzyme in W50/BE1 or alteration to their extracellular processing as described above for RI/BE from this strain.

The α -chain present in RIIA^V/BE did not contain large glycan modifications and this was confirmed by lack of reactivity with anti-LPS antibodies. However, RIIA^V/BE was still associated with the membrane/vesicle fraction and required the presence of 0.05% Zwittergent to solubilize it for purification purposes. This suggests that targeting of RIIA^V/BE to the vesicle fraction is independent of glycan modification of the α -catalytic chain. This is an important observation but it is not yet apparent how RIIA^S/BE and RIIA^V/BE become distributed between the soluble and membrane/vesicle fractions. Indeed, increase in soluble extracellular Arg-specific protease activity at the expense of vesicle-associated activity in W50/BE1 compared to the parent strain has been previously reported (Smalley *et al.*, 1989); the authors suggested that the enzyme from W50/BE1 may be unable to bind to or be retained on cellular and vesicular membranes. This study drew a comparison between *P. gingivalis* W50/BE1 and a 'rough' strain of *Salmonella typhimurium*, where extracellular accumulation of alkaline phosphatase (normally located in the periplasm) is a result of defective LPS in the outer membrane causing a change in binding affinity of the enzyme for the membrane (Lindsay *et al.*, 1973). Recent studies on the nature of the outer membrane of *P. gingivalis* have demonstrated the loss of key antigenic determinants from the LPS of W50/BE1 compared to the parent strain (Cutler *et al.*, 1996). It is therefore possible that membrane composition and post-translational modification of enzyme both contribute towards the final localization of the enzymes in the parent and mutant strains.

Whilst the differences between the Arg-specific enzymes of the W50/BE1 mutant and the parent strain W50 do not significantly affect the kinetics of the enzymes measured against the small chromogenic synthetic substrate BRpNA, the structural differences may have an effect *in vivo* by modulating enzyme activity on physiological substrates. This is currently under further investigation. Furthermore, recent work has established a role for the Arg-specific proteases in fimbriation of the *P. gingivalis* outer membrane (Nakayama *et al.*, 1996), and therefore disruption of these proteases may lead to disruption of fimbriae and other protease-dependent virulence determinants.

Consequently, the reduced virulence potential of W50/BE1 may reflect an overall reduction in Arg-specific activity, altered distribution of *prpR1* and *prR2* products, and differently processed isoforms. The nature of the mutation(s) in W50/BE1 is currently under investigation by determination of mRNA levels and complementation studies using the parent strain *prpR1*. There may be multiple defects in gene expression at

many loci, or alternatively a single mutation in a regulator which affects not only protease gene expression but also accessory genes involved in the processing pathway. Given the numerous phenotypic differences observed in W50/BE1 compared to the parent strain W50, and the apparent stability of the mutant, the latter hypothesis seems more likely. Therefore, further study of *P. gingivalis* W50/BE1 may provide important insights into the maturation processes of *prpR1*- and *prR2*-derived enzymes and the regulatory influences which control their expression.

ACKNOWLEDGEMENTS

This work was supported by Medical Research Council grant no. PG9318173 and by an MRC PhD studentship to L.M.C.

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Received 21 January 1998; revised 5 May 1998; accepted 12 May 1998.