Reversible Redox Regulation of Specificity of Arg-gingipain B in Porphyromonas gingivalis

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ABSTRACT

Arg-gingipain B (RgpB), a major virulence factor secreted by the periodontal pathogen Porphyromonas gingivalis is an Arg-specific cysteine proteinase. By monitoring proteolytic cleavage of a human salivary peptide histatin 5 using MALDI-TOF MS, RgpB purified from P. gingivalis HG66 was found to shift from a dominant Arg-X to dominant Lys-X activity, both in vitro and in vivo, upon reversible cysteine oxidation. Native PAGE analysis revealed the association of novel Lys-X activity with a reversible state change of the oxidized enzyme. The redox-regulated Lys-X activity of RgpB may provide a survival advantage to P. gingivalis against the oxidative host defence.

Keywords: Porphyromonas gingivalis, gingipain, specificity change, redox, histatin 5, MALDI-TOF MS

Abbreviations: FFRCMK, D-Phe-Phe-Arg-chloromethylketone; Bz-, benzoyl-; pNA, p-nitroanilide; Z-, benzyloxycarbonyl; L-BAPNA, Bz-L-Arg-pNA; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone;
PAGE, polyacrylamide gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GuHCl, guanidine hydrochloride.
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**Highlights**

- Arg gingipain B (RgpB) is an Arg-specific cysteine proteinase.
- Strain HG66 RgpB shifts to dominant Lys-X activity upon reversible Cys oxidation.
- Association of novel Lys-X activity with a reversible state change of RgpB.
- Novel Lys-X activity of RgpB was distinguishable from Kgp activity.
- The redox-regulated Lys-X activity of RgpB may provide a survival advantage for *P. gingivalis.*
Reversible Redox Regulation of Specificity of Arg-gingipain B in Porphyromonas gingivalis

1. Introduction

Porphyromonas gingivalis is an anaerobic, Gram-negative bacterium associated with chronic periodontitis, a destructive inflammatory disease of tooth supporting tissues. The pathogenicity of P. gingivalis has been attributed to a number of virulence factors, in particular the cell-associated and extracellular cysteine endoproteinases including Arg-gingipains (RgpA and RgpB) and Lys-gingipain (Kgp), which are known to be Arg-X specific and Lys-X specific, respectively. Gingipains contribute to around 85% of the proteolytic activity and 99% of the “trypsin-like” activity of the bacterium [1,2] and are capable of degrading a variety of host proteins and dysregulating host defenses [3]. The catalytic domains of the gingipains belong to the distinct cysteine proteinase family C25 [4]. Gingipains are encoded by separate genes. The rgpB gene encodes a calcium-stabilized enzyme that exists as a 70-90 kDa outer membrane-associated protein as well as a discrete 50 kDa C-terminally truncated protein released into the culture fluid [5-8]. An isogenic mutant of P. gingivalis W50 with rgpB insertionally inactivated was substantially less virulent than the wild type strain in murine lesion and periodontitis models [9,10]. The virulence and Arg-X activity of the mutant was restored by complementation with a plasmid carrying rgpB [9]. RgpB was first isolated from the culture fluid of P. gingivalis HG66 by Chen et al. [5,6]. The crystal structure (PDB ID: 1CVR) of the C-terminally truncated RgpB, from HG66, covalently bound with D-Phe-Phe-Arg-chloromethylketone (FFRCMK) inhibitor revealed a caspase-like catalytic domain [11]. All three gingipains require a reducing environment for full activity and are normally activated in vitro by pre-incubation with a thiol, in particular L-cysteine [5,7,12]. RgpB is classified as an Arg-X specific proteinase based on assay conditions that typically involve activation by 5-10 mM L-cysteine. During an autolysis study of purified RgpB from strain HG66 (unpublished), we discovered not only peptide fragments with a C-terminal Arg but also peptides with a C-terminal Lys.
Using a human salivary peptide histatin 5 with three Arg and four Lys residues as substrate, we demonstrate, for the first time, novel Lys-X activity from RgpB.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and growth conditions

*P. gingivalis* HG66 [13] was kindly provided by Prof Robert Pike (Monash University, Victoria, Australia). *P. gingivalis* YH522KAB, in which *rgpA*, *rgpB*, and *kgp* genes are disrupted with antibiotic resistance markers, and OPG37, a YH522KAB derivative bearing the shuttle plasmid pHY411, have been described previously [14]. *P. gingivalis* was maintained on blood agar and grown in batch culture anaerobically while *E. coli* was grown at 37 °C as described [14].

2.2. Cloning of *rgpB* and creation of *P. gingivalis* strain ECR129

A 2.7-kb PCR-derived fragment, encompassing the *rgpB* coding region and flanking DNA generated from *P. gingivalis* HG66 genomic DNA was ligated into pGEM®-TEasy (Promega, Alexandria, NSW, Australia) as described [9]. The *rgpB* gene was excised and inserted into the EcoRI site of pYH411 and fully sequenced. Purified recombinant plasmid DNA was electroporated into YH522KAB to create strain ECR129.

2.3. RgpB purification and characterization

Cell-free culture fluid prepared from a 2-day culture of *P. gingivalis* HG66 was concentrated and resolved using a two-column procedure essentially as described [15]. Arg-X and Lys-X activities of column fractions were measured using Bz-L-Arg-pNA (L-BAPNA; Sigma-Aldrich, St. Louis, MO) and Z-L-Lys-pNA (Novabiochem, Merck Millipore, Kilsyth, VIC, Australia), respectively. A sample of the protein fraction was first made up to 360 μL, pre-incubated with 10 mM L-cysteine (Sigma) for 10 min by adding 40 μL of 100 mM L-cysteine, pH 8.0, and then added with 2 mM substrate (400 μL) in 20 mM L-cysteine, pH 8.0, 400 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 30% (v/v) isopropanol. The initial rate of hydrolysis was measured at 10 s intervals over 3 min at 410 nm in a Hewlett Packard 8452A Diode Array
spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia). The results were expressed in units, where 1 U is equivalent to 1 μmol substrate hydrolyzed per min at 37 °C, pH 8.0. Protein concentrations were determined using Bradford assays (Bio-Rad, Gladesville, NSW, Australia) with BSA as standard.

Purified RgpB was subjected to SDS-PAGE, Edman sequencing and MALDI-TOF MS as described [15]. For native PAGE, RgpB samples (1 μg) before and after L-cysteine pre-incubation were diluted in 100 mM Tris-HCl, pH 8.8, 10% v/v glycerol, and 0.005% w/v bromophenol blue, immediately before electrophoresis in a Novex 4-20% w/v Tris-Glycine gel (Invitrogen, Life Technologies, Mulgrave, VIC, Australia) in 25 mM Tris, 192 mM glycine, pH 8.3 at 125 V at 4 °C. BenchMark Prestained Protein Ladder (Invitrogen) was used for gel to gel comparison. For free sulfhydryl group quantitation, purified RgpB was mixed with Ellman’s reagent [16] 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma), prepared in 0.1 M Tris-HCl, pH 8.0 without or with guanidine hydrochloride (GuHCl), and incubated for 15 min before 412 nm absorbance was recorded. DTNB and GuHCl was 240 µM and up to 6.8 M, respectively. L-cysteine (6-40 µM) was concomitantly assayed under identical conditions to generate standard curves.

2.4. In vitro and in vivo RgpB activity assays

For the in vitro assay, purified Rgp was pre-incubated with 1 mM L-cysteine at 1: 40 mol/mol at 37 °C. After 10, 20, and 60 min, an aliquot of the enzyme was mixed with 20 volumes of 10 μM histatin 5 (Sigma) in 10 mM NH₄HCO₃ at 1:320 or 1:8 mol/mol. An aliquot of the digest removed at various time points was acidified with formic acid (final 1% v/v), mixed 1:1 (v/v) with saturated α-cyano-4-hydroxycinnamic acid matrix solution in aqueous 33% (v/v) acetonitrile and 1% (v/v) formic acid and analyzed by linear MALDI-TOF MS using a Voyager-DE mass spectrometer (AB Sciex, Mt Waverley, VIC, Australia).

For the in vivo assay, bacterial whole cells, harvested from late-exponential-phase batch cultures of P. gingivalis strains ECR129 and OPG37 (negative control), were washed and maintained in TC150 buffer (20 mM Tris-HCl, pH 7.4, 5 mM CaCl₂ and 150 mM NaCl) at 4 °C. After pre-incubation with L-cysteine
at 37 °C or being stored in a capped tube in TC150 without reducing agents for 24 h at 4 °C, bacterial cells (1.25 x 10^7) were mixed with 20 volumes of 50 μM histatin 5. At various time points, an aliquot of the reaction mixture was clarified by centrifugation. The resulting supernatant fraction was acidified, mixed with the matrix on a ground steel target (Bruker Daltonics, Preston, VIC, Australia) and analyzed by reflectron MALDI-TOF MS using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics). The identified peptide fragments were sequenced using LIFT (MS/MS) in the same instrument when required using default settings.

3. Results and Discussion

3.1. Purification of soluble RgpB

Typically, cell-free culture fluid prepared from a 1-L culture of P. gingivalis HG66 exhibited 650-700 U Arg-X activity (L-BAPNA) containing 10-13 U/mg protein. More than 90% of the activity was found in the soluble fraction (supernatant) after ultracentrifugation (300,000 x g, 60 min, 4 °C). Cell-free culture fluid yielded 60-70% recovery and 2-fold purification of the Arg-X activity after 19-fold concentration. The concentrate was first resolved on a Q Sepharose HP column (Fig. 1A) that recovered at least 90% of both Arg-X and Lys-X (Z-L-Lys-pNA) activities. Six L-BAPNA-hydrolytic activity peaks were detected, including B1 (11% of the loaded activity), B2-B4 (66%), and B5-B6 (15%) while 99% of the loaded Z-L-Lys-pNA-hydrolytic activity was eluted in two peaks L1 and L2. Notably, L1 and L2 coincided with B5 and B6. Subsequent MS analysis of these peaks revealed the co-presence of Kgp and RgpA. Selected B2 peak fractions with no less than 70 U/mg were pooled and further resolved on Superose 12 (Fig. 1B). The pooled Superose 12 fractions eluted between 12-14 mL exhibited 79 U/mg, a single band on SDS-PAGE (Fig. 2A) and a measured mass of 48.2 kDa using MALDI-TOF MS (Fig. 2B). Edman sequencing revealed the N-terminal 50-amino acid sequence that matched 100% with the RgpB of the same strain [6]. No minor sequences, including Kgp, were detected.

3.2. Specificity change of purified RgpB
RgpB is typically assayed after pre-incubation (activation) with L-cysteine (5-10 mM), prior to incubation with L-BAPNA chromogenic substrate in L-cysteine-containing buffer. Although cysteine is the most abundant low molecular weight sulfhydryl compound in human plasma, its mean “total” concentration is only 0.26 mM [17-23]. Importantly, the reported “total” cysteine concentration in plasma includes oxidized forms of cysteine, such as cystine and protein-S-S-Cys. In fact, the predominant form of “cysteine” in human plasma is cystine (>40 μM) and the reduced Cys is in the range of 8–10 μM [24-26]. Therefore, in this study a lower concentration of L-cysteine (1 mM) was employed for “activation” and no additional L-cysteine was included in the substrate buffer to better reflect physiological conditions. In addition, histatin 5, a physiologically-relevant human host-derived salivary peptide, was used at low μM concentrations [27] as substrate. At a low enzyme:substrate molar ratio (1:320), incomplete digestion of histatin 5 was achieved to estimate total RgpB activity by MALDI-TOF MS. RgpB showed differential specificity after pre-incubation with 1 mM L-cysteine for different time periods. After 10-min pre-incubation, RgpB showed dominant Arg-X activity, with a minor Lys-X activity also noted in the 20 min digest (Fig. 3A). The Lys-X activity became stronger after 20-min (Fig. 3B) and 60 min (Fig. 3C) pre-incubation. As the Lys-X activity became dominant, the total activity dropped based on the increased digestion time required for reducing the full-length histatin 5 to a similar level (see peptide 7, Fig. 3).

To avoid additional oxidation during prolonged digestion, a higher enzyme:substrate molar ratio (1:8) and a short, fixed digestion time of 5 min were later employed. Under these conditions, an RgpB specificity change was again observed, from dominant Arg-X to dominant Lys-X (Fig. 4). To test whether the dominant Lys-X activity was derived from “oxidized” RgpB, fresh 1 mM L-cysteine was added to the same sample used in Fig. 4C, that instantly reverted the enzyme back to dominant Arg-X activity (Fig. 4D). Therefore, the novel specificity change was redox-regulated.

If completely cleaved at all three Arg residues (Arg6, Arg12, Arg22), only three histatin 5 fragments (peptides 1, 15 and 24) would be detected (Table 1). Consistent with being an Arg-X enzyme, these peptides were dominant in the digest with RgpB pre-incubated with 1 mM L-cysteine for 10 min or re-reduced with fresh L-cysteine (Fig. 4). Close examination revealed a minor Lys-X activity that produced
peptides 23 and 28, likely resulted from minor cleavage of peptide 24 at Lys17 (Fig. 4A; Table 1). In contrast, relatively oxidized RgpB, after 60-min pre-incubation with 1 mM L-cysteine, exhibited strong Lys-X activity cleaving all four Lys (Lys5, Lys11, Lys13, Lys17) and weak Arg-X activity cleaving Arg12 and Arg6, but not Arg22, leading to a more complex digestion profile with numerous MS peaks (Fig. 4C; Table 1). Interestingly, Arg12 and Arg6 are preceded by Lys while Arg22 is preceded by His. Importantly, oxidized RgpB cleaved Lys13 of histatin 5, that is preceded by Arg which is a non-cleavage site for Kgp [28,29]. Consistently, leupeptin at 100 µM, which has no effect on Kgp [28], abolished both dominant Arg-X and Lys-X activities of RgpB.

3.3. Association of a reversible state change of RgpB with novel Lys-X activity

Upon native PAGE, the RgpB form that exhibited mixed Arg-X and Lys-X activity showed four bands b1-b4 (Fig. 5, lane 1), with b3 and b2 being dominant. After 10-min incubation with 1 mM L-cysteine (1:40 mol/mol), the four bands were transformed into one, corresponding to b2 (lane 2). As the incubation time increased, a band corresponding to b4 appeared after 20 min (lane 3) and another band corresponding to b3 became visible after 60 min (lane 4). When fresh 1 mM L-cysteine was added to the same sample used for lane 4, only one band corresponding to b2 was observed (lane 5), indicating the association of multiple redox-regulated, reversible forms of RgpB with reversible specificity change. In comparison, when RgpB was incubated with 10 mM L-cysteine (1/400 mol/mol), only one band corresponding to b2 was detected after 10-min or 60-min incubation (lanes 6/7), consistent with unaltered dominant Arg-X activities. Further free thiol analysis revealed that RgpB with mixed Lys-X and Arg-X activity (Fig. 5, lane 1) contained 0.8 mol and 1.7 mol free thiols per mol enzyme in the absence and presence of 4.8 M GuHCl, respectively, at room temperature. The detectable free thiols increased significantly to 3.2 mol per mol enzyme when assayed at 37 °C with 6.8 M GuHCl. As RgpB has 7 Cys residues (Fig. 6), the above data support its oxidative susceptibility via cysteine residues, creating multiple reversible oxidative states responsible for reversible specificity change.

3.4. Reversible specificity change of cell-associated RgpB
*P. gingivalis* mutant strain ECR129 expressing recombinant RgpB derived from strain HG66 as the only gingipain was created in a triple gingipain mutant strain background YH522KAB. Freshly-prepared ECR129 cells exhibited dominant Arg-X activity even after 60-min pre-incubation with 1 mM L-cysteine (Fig. 7A). Under these conditions purified RgpB exhibited dominant Lys-X activity (Fig. 4C). This difference was attributed to prolonged air exposure of RgpB during purification as compared to whole cells freshly harvested from an anaerobic culture supplemented with 5 mg/mL L-cysteine (28 mM). To demonstrate the effect of oxidation on RgpB specificity in vivo, washed ECR129 cells were air-oxidized by incubation in Tris buffer without reducing agents for 24 h at 4 °C, resulting in mixed Arg-X and Lys-X activity (Fig. 7B). Pre-incubation of the oxidized cells with fresh L-cysteine reverted the specificity back to dominant Arg-X (Fig. 7C), consistent with the in vitro results.

4. Conclusion

Reversible redox-regulated specificity change of *P. gingivalis* HG66 RgpB occurs both in vitro and in vivo as a soluble or cell-associated enzyme, respectively. The novel Lys-X activity was distinct from Kgp activity as it was 1) demonstrated with purified RgpB and a *P. gingivalis* mutant expressing RgpB as the sole gingipain, 2) activated by reversible oxidation, 3) inhibited by leupeptin, and 4) active towards a non-cleavage site for Kgp (Arg-Lys-X). This redox regulated Lys-X activity of RgpB may provide a survival advantage against the oxidative host defence and may help explain why N\textsuperscript{\textprime}p-tosyl-L-lysine-chloromethyl ketone (TLCK) with a P1 lysine is a known inhibitor of the enzyme [1].

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References


Figure Legends

Fig. 1. RgpB purification. (A) Q Sepharose HP column chromatography of concentrated *P. gingivalis* HG66 cell-free culture fluid. (B) Superose 12 column chromatography of the B2 activity peak in panel A.

Fig. 2. Analysis of purified RgpB by SDS-PAGE (A) and MALDI-TOF MS (B).

Fig. 3. Effect of L-cysteine pre-incubation on RgpB specificity *in vitro*. Purified RgpB was pre-incubated with 1 mM L-cysteine for 10 min (A), 20 min (B) or 60 min (C) before being added to histatin 5 (1:320 mol/mol) and incubated for 20 min, 60 min and 100 min, respectively. The digests were analyzed by linear MALDI-TOF MS. The identified histatin 5 fragments are numbered according to Table 1; those marked with an asterisk indicate Lys-X cleavage. More than one MS peaks marked with the same number indicate the presence of both singly- and multiply-charged ions of the same peptide. A minor peak indicated with an asterisk without numbering was identified as histatin 5-(1-10).

Fig. 4. Reversible change of RgpB specificity *in vitro*. Purified RgpB was assayed as described (Fig. 3) except that histatin 5 was added at 1:8 mol/mol and digested for 5 min. An aliquot of the same RgpB enzyme used in panel C was re-reduced with fresh 1 mM L-cysteine immediately prior to addition of histatin 5 (D). The digests were analyzed as described (Fig. 3) and the results are summarized in Table 1. Peptide 8*, with the same m/z value as peptide 15, was also detected in panels B and C but not shown.

Fig. 5. Reversible change of RgpB redox states revealed by native PAGE. Purified RgpB was analyzed on a native Tris-glycine gel before (lane 1, Ori.) and after incubation with 1 mM L-cysteine for 10 min (lane 2), 20 min (lane 3), and 60 min (lane 4). An aliquot of the same sample used in lane 4 was re-reduced with fresh 1 mM L-cysteine immediately prior to gel analysis (lane 5). For comparison, purified RgpB was incubated with 10 mM L-cysteine for 10 min (lane 6) and 60 min (lane 7), both of which showed dominant Arg-X activity.
**Fig. 6.** Ribbon diagram of the crystal structure of RgpB reproduced from PDB ID: 1CVR [11] using PyMOL. Catalytic domain: subdomains A (blue) and B (green); immunoglobulin superfamily domain (red); $\gamma$ atoms of seven cysteine residues (yellow); Zn$^{2+}$ (dark grey); Ca$^{2+}$ (light grey); FFRCMK inhibitor (pink and blue sticks) covalently attached to the catalytic Cys244.

**Fig. 7.** Reversible change of RgpB specificity *in vivo*. Freshly-prepared strain ECR129 cells were pre-incubated with 1 mM L-cysteine for 60 min (A), oxidized for 24 h (B) or re-reduced after 24 h oxidation (C). Treated cells were added to histatin 5 for 5 min and the resulting supernatant fractions were analyzed using reflectron MALDI-TOF MS. The identified histatin 5 fragments were labelled as described in Fig.3.
Table 1
Predicted histatin 5 tryptic fragments with average MH⁺ greater than 600 and those found experimentally in RgpB digestion of histatin 5 as shown in Fig. 4. Numbered peptides marked with an asterisk indicate one or two Lys-X cleavages.

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