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# Comparisons of the Isozymes of Arginine-Specific Proteinases in the Culture Supernatant of *Porphyromonas gingivalis*

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## Summary

By anion-exchange chromatography of the concentrated culture fluids of *P. gingivalis* W 50, two proteolytic enzymes, referred to as RGP-I and RGP-II were, eluted from column at the different NaCl concentrations in the eluant buffer. The two enzymes were purified until the preparations displayed single bands with sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) by gel filtration and isoelectric focusing. Comparative studies between the two enzymes revealed that the same molecular masses (44 kDa) of RGP-I and II were obtained both with gel filtration and SDS-PAGE. However, the isoelectric points of RGP-I and II were 5.0 and 5.7, respectively. Both enzymes were thiol proteinases and were inhibited by sulfhydryl group blocking reagents, tosyl-L-lysine chloromethylketone, EDTA, leupeptin, L-trans-epoxy-succinylleucylamido-(4-guanidino) butane, and antipain. Both enzymes were activated by glycylglycine. RGP-I and RGP-II hydrolyzed synthetic substrates containing arginine in the P-1 positions, but those containing lysine in the same position were not cleaved.

## Introduction

The putative etiological agent of adult periodontitis is *Porphyromonas gingivalis* which is an oral indigenous resident, anaerobic gram negative rod, and able to characteristically form jet black colonies on blood agar. This species elaborates many biologically active proteinaceous substances such as proteinases[1,2,3,4,5], hemolysin[6,7], hemagglutinin[8,9,10], DNase[11], hemin-binding protein[12,13,14], and hemoglobin-binding protein[15]. Of these proteinases have been most noticed and studied in the last decade by many investigators because of their possible function as pathogenic factors of this species. In the proteolytic system of *P. gingivalis*, arginine specific proteinase and lysine specific proteinase, designated Arg-gingipain (RGP) and Lys-gingipain (KGP), respectively[16] are regarded as the key enzymes. The former proteinase, also called "trypsinlike enzyme" is a cysteine protease, is inhibited strongly by leupeptin or tosyl-L-lysine chloromethyl ketone (TLCK) and catalyzes peptidyl bonds of arginine in the P-1 positions. The latter enzyme, which hydrolyzes those of lysine in the P-1 positions, is also a cysteine protease and is characteristically activated by EDTA[17]. We found the existence of two kinds of RGPs sepa-

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rated clearly by ion-exchange chromatography in the culture supernatant of *P. gingivalis* W 50, and a comparative characterization of these proteinases was undertaken.

## Materials and Methods

*P. gingivalis* strain W 50 was grown anaerobically in the medium described by Sawyer et al.[18], but containing no glucose or sodium bicarbonate. Subsequent incubation and cultivation were carried out in an atmosphere of nitrogen, hydrogen, and carbon dioxide (85 : 10 : 5) at 37℃ for 3 days.

The culture supernatant was obtained by centrifugation of the whole culture at 20,000 xg for 20 min. To prepare the vesicle fraction, ammonium sulfate was added to this culture supernatant at 30% saturation and collected by centrifugation at 20,000 xg for 20 min. Suspension of the vesicles was dialyzed against 50 mM Tris-HCl buffer (pH 8.2). An envelope was prepared from the harvested cells by ultrasonic treatment and 100,000 xg centrifugation[19]. The supernatant was kept as the cell extracts. The vesicle and envelope were solubilized by 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) at 1.0% in a 50 mM Tris-HCl buffer (pH 8.2)[15]. Ammonium sulfate was added to the supernatant at 75% saturation and the precipitate was dissolved in a 50 mM Tris-HCl buffer (pH 8.2) and thoroughly dialyzed against the same buffer. After the dialysis procedure, the insoluble materials were removed by centrifugation at 40,000 xg for 15 min. The RGP activity was determined routinely using benzoyl-L-arginyl *p*-nitroanilide (Bz-Arg-pNA, Sigma Chemical Co.) as a substrate[20]. One unit (U) of activity was defined as the liberation of 1 μ mole of *p*-nitroaniline per min. The KGP activity was assayed by hydrolysis of toluenesulfonyl-glycyl-L-prolyl-L-lysine *p*-nitroanilide (Tosyl-Gly-Pro-Lys-pNA, Sigma)[17]. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide) was carried out according to the methods of Laemmli[21]. The sample was heated in boiling water for 2 min in the presence of 0.75 M mercaptoethanol and 3 % SDS before application of gel electrophoresis. Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydlase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa) were employed as the molecular mass markers.

## Results and Discussion

The cellular distribution of RGP of a 1,800 ml culture of *P. gingivalis* W 50 in the cell fractions of the culture supernatant, cell extracts, envelope, and vesicle from the 1,800 ml culture was 70 U, 5 U, 13 U, and 5 U, respectively. For reference, the corresponding distribution values of KGP were 25 U, 2 U, 1 U, and 0.5 U, respectively.

Purification of RGPs (RGP-I and RGP-II) was initiated from the concentrated culture supernatant by ammonium sulfate. The sample was reduced by addition of 2-mercaptoethanol at 10 mM and applied to a Q-Sepharose (1.5 by 70 cm) (Pharmacia Biotech AB), equilibrated with 50 mM Tris-HCl buffer (pH 8.2) containing 10 mM 2-mercaptoethanol (TM, pH 8.2), followed by washing the column with the same solution. Then the column was eluted with a linear concentration gradient of NaCl, which was generated by mixing 150 ml of TM containing 0.5 M NaCl into an equal volume of TM. Two peaks of RGP activity emerged from the column at NaCl concentrations of about 0.20 M and 0.35 M (Fig. 1). RGPs eluted at these NaCl concentrations were referred to as RGP-I and RGP-II, respectively. The active fractions of RGP-I and RGP-II, as indicated in Fig. 1, were combined, concentrated using a rotary vacuum evaporator, and dialyzed against TM containing 0.2 M NaCl (TMS). The concentrated active fractions from the Q-Sepharose column were applied separately to a Sephacryl S-300 (Pharmacia Biotech AB) column (2.6 by 70 cm), previously equilibrated with TMS and eluted with

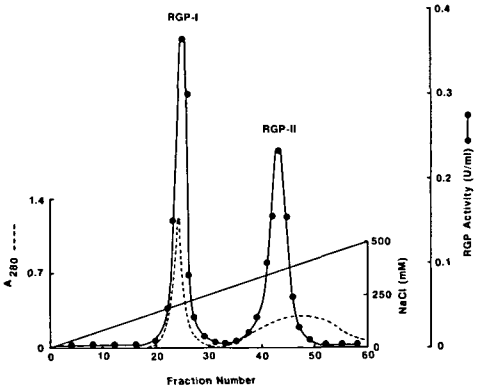


**Fig. 1** : Q-Sepharose column chromatography of the concentrated culture supernatant of P. gingivalis W 50. Experimental conditions are described in text.

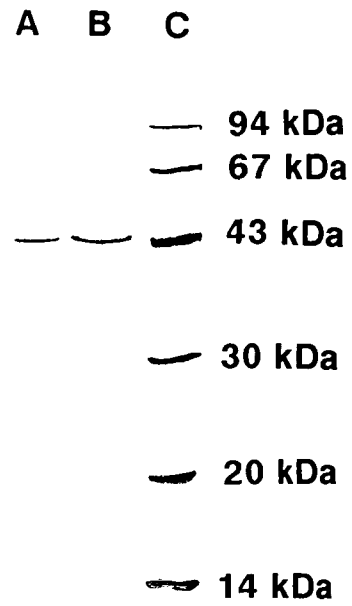


**Fig. 2** : SDS-PAGE of the purified RGP-I and RGP-II.
lane A : RGP-I, lane B : RGP-II, lane C : marker proteins. Gel was stained with Coomassie brilliant blue R-250.

this buffer saline. In both cases of gel filtration of RGP-I and GP-II, a single peak of the enzyme activity was found at the same elution volume, which is in the vicinity to that of ovalbumin. To purify RGP-I and RGP-II further, each active fraction was subjected to isoelectric focusing after dialysis against 1% glycine solution containing 10 mM of 2-mercaptoethanol. The electrophoresis was performed at 400 V for 20 h (final current was 2.3 mA) in a 110 ml column using pH 3-10 ampholine under the presence of 10 mM mercaptoethanol. The column was cooled with tap water (about 6℃) during the electrophoresis.

The peak of activity of RGP-I was focused at pH 5.0 and RGP-II was at pH 5.7. Examinations by SDS-PAGE of both purified samples exhibited single stained protein bands at the same migration position (Fig. 2). The molecular masses of RGP-I and RGP-II were calculated as 44 kDa from the electrophoretograms. The subsequent comparisons of the enzymatic properties between RGP-I and RGP-II were carried out using these purified samples. The effects of various group specific reagents, specific inhibitors of proteinases, and a peptide are summarized in Table 1. No substantial difference in activation or inhibition by various reagents was seen for the two enzymes. Both enzymes were significantly activated by thiol reagents (2-ME, cysteine, DTT), whereas different types of SH-blocking reagents (PCMB, NEM) inhibited the enzyme activities. Similar to arginine specific proteases isolated from the other strains of *P. gingivalis*, both enzymes were almost completely inhibited by low concentrations of TLCK, a lysine alkylating reagent. RGP-I and RGP-II were also inhibited by EDTA, indicating these proteinases harbor divalent metal ions within the molecule. However, the inhibitory effect of EGTA was quite low. Inhibitors of thiol proteinases of microorganism origin (leupeptin, antipain) and synthetic chemical [L-trans-epoxy-succinylleucylamido-(4-guanidino) butane = E 64] inactivated exhaustively both enzymes. The same degree of activation was seen by the presence of glycylglycine as was reported by Chen et al.[22].

Substrate specificity of the RGP-I and RGP-II was examined using various p-nitroanilide derivatives of amino acids or peptides (Table 2). Both enzymes split X-Y-Arg-pNA but compounds of X-

**Table 1**：Effects of various reagents on RGP-I and RGP-II of *P. gingivalis* W 50

| Reagents | concentration | Relative activities (%) RGP-I | RGP-II |
|---|---|---|---|
| Control | − | 100 | 100 |
| 2-ME | 10 mM | 130 | 141 |
| | 30 mM | 165 | 179 |
| DTT | 10 mM | 139 | 151 |
| | 30 mM | 208 | 227 |
| Cysteine | 10 mM | 120 | 143 |
| | 30 mM | 187 | 221 |
| PCMB | 0.02mM | 5 | 8 |
| | 0.2 mM | 2 | 2 |
| NEM | 0.1 mM | 13 | 18 |
| | 1.0 mM | 7 | 6 |
| TLCK | 0.05mM | 0 | 1 |
| EDTA | 2.0 mM | 4 | 2 |
| EGTA | 2.0 mM | 61 | 70 |
| Leupeptin | 0.02mM | 0 | 0 |
| E 64 | 0.5 mM | 1 | 1 |
| | 2.5 mM | 0 | 1 |
| Antipain | 0.1 mM | 0 | 0 |
| Gly-Gly | 200 mM | 188 | 189 |

abbreviations ; 2-ME : 2-mercaptoethanol, DTT : dithiothreitol, PCMB : *p*-chloromercuribenzoate, NEM : *N*-ethyl maleimide, TLCK : tosyl L-lysine chloromethylketone, EGTA : ethylene glycol-bis (β-aminoethyl ether) -*N*, *N*, *N'*, *N'*-tetraacetic acid, E 64 : L-trans-epoxy-succinylleucylamido-(4-guanidino) butane, Gly : glycine.

**Table 2**：Substrate specificity of RGP-I and RGP-II of *P. gingivalis* W 50

| Substrates | Relative activities (%) RGP-I exp. 1 | RGP-I exp. 2 | RGP-II exp. 1 | RGP-II exp. 2 |
|---|---|---|---|---|
| Bz-Arg-pNA | 100 | 100 | 100 | 100 |
| Tosyl-Gly-Pro-Arg-pNA | 206 | 127 | 162 | 136 |
| Val-Leu-Arg-pNA | 231 | 137 | 203 | 145 |
| Bz-Phe-Val-Arg-pNA | 328 | 194 | 346 | 171 |
| Bz-Val-Gly-Arg-pNA | 311 | 184 | 231 | 152 |
| Bz-Pro-Phe-Arg-pNA | 253 | 139 | 206 | 159 |
| Boc-Val-Leu-Gly-Arg-pNA | 295 | 170 | 225 | 157 |
| Boc-Leu-Gly-Arg-pNA | 384 | 192 | 283 | 173 |
| Boc-O-benzyl-Ser-Gly-Arg-pNA | 409 | 145 | 324 | 140 |
| Tosyl-Gly-Pro-Lys-pNA | 4 | ND | 2 | ND |
| Val-Leu-Lys-pNA | 8 | ND | 3 | ND |
| Bz-Lys-pNA | 4 | ND | 0 | ND |
| Suc-Ala-Ala-Ala-Pro-Phe-pNA | 1 | ND | 4 | ND |
| Glt-Phe-pNA | 0 | ND | 2 | ND |
| Bz-Tyr-pNA | 5 | ND | 6 | ND |

Duplicate experiments (exp. 1, exp. 2) for the determination of substrate specificity were carried out using the same enzyme samples.

abbreviations ; Bz : benzoyl, Tosyl : toluenesulfonyl, Boc : butyloxycarbonyl, Suc : succinyl, Glt : glutaryl, ND : not determined.

Y-Lys-pNA and other substrates were not hydrolyzed. These results suggest that the hydrolytic activities of RGP-I and RGP-II of *P. gingivalis* W 50 were limited to the compounds with arginine in the P-1 positions.

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## 抄録：*Porphyromonas gingivalis*培養上清中のアルギニン特異的プロテアーゼのアイソザイムの比較

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*P. gingivalis* W 50の培養上清の陰イオン交換クロマトグラフィーにおいて，2種類のアルギニン特異的プロテアーゼ（RGP-ⅠとRGP-Ⅱ）の存在が認められ，それぞれをゲル濾過と等電点電気泳動で精製した．RGP-ⅠとⅡを比較すると，ともに分子量は44 kDaで等電点はそれぞれ5.0と5.7であった．酵素学的には両者の間に大きな違いはなく，ともにチオール酵素でTLCK，EDTA，ロイペプチン，E 64，アンチパインで阻害を受け，グリシルグリシンで活性化される．P-1の位置にアルギニンをもつ合成基質のみを加水分解し，リジンも含めて他の基質には作用しなかった．このアイソザイムの生成機序については未だ不明である．