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Comparative studies on the formation of pathogenicity related enzymes and protein among four strains of *Porphyromonas gingivalis*

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Abbreviations : prolyl tripeptidyl peptidase ; PTP, dipeptidylpeptidase ; DPP ; *p*–nitroanilide ; pNA

Porphyromonas gingivalis has been implicated as an etiological agent of chronic periodontitis[1-4]. RGP (arginine–gingipain) hydrolyses the peptide bonds of arginine–X amino acid, and KGP (lysine–gingipain) splits those of lysine–Y amino acid. These enzymes are major proteinases of *P. gingivalis*, and have been regarded as important pathogenic factors[5–7]. In addition, peptidases are also significant enzymes for survival of anaerobic bacteria, since these organisms utilize peptides as energy sources[8]. *P. gingivalis* lacks a siderophore system[9,10] which allows it to acquire iron in bacteria. Therefore, hemoglobin binding activity due to hemoglobin binding protein may contribute to the uptake of iron[11]. Based on this background information, it may be worthwhile to assess and compare the formation of proteinases, peptidases and binding activity to hemoproteins among several strains of *P. gingivalis*.

Strains of *P. gingivalis* employed in this work were ATCC33277, 381, W50 and W83, which were cultured at 37°C for 3 days anaerobically in a glove box filled with a mixture of gases containing N_2 : H_2 : CO_2 = 85 : 10 : 5. The culture medium consisted of Trypticase peptone (17 g), yeast extract (3 g), K_2HPO_4 (2.5 g), NaCl (5 g), hemin (5 mg) and menadione (0.5 mg) per liter.

Preparations of particle free culture fluid, crude extract and envelope were carried out according to methodology outline in previous reports[12,13]. Briefly, cells were collected by centrifugation at 15,000 xg for 20 min. The supernatants were further centrifuged at 100,000 xg for 60 min to obtain the particle free culture fluids. Cells washed twice by centrifugation in saline were disrupted by ultrasonic treatment at 150 W for 20 min, followed by centrifugation at 6,000 xg for 15 min to remove unbroken cells. Then this supernatant was centrifuged at 100,000 xg for 60 min and the centrifugal precipitates and the supernatants were referred to as the envelope and crude extracts, respectively. The envelope was solubilized by a zwitterionic detergent, (3–[(3–cholamidopropyl)– dimethylammonio]–1–propanesulfonate (CHAPS).

Activities of RGP and KGP were determined using Bz–Arg–pNA and Tosyl–Gly–Pro–Lys–pNA

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as substrates, respectively[14,15]. Azocoll hydrolysis was estimated as follows. The reaction mixtures contained 4 mg of azocoll powder, 100 μl of enzyme source and 900 μl of 50 mM Tris–maleate buffer, pH 7.2 were incubated at 37°C for 30 min. After incubation, reaction mixtures were cooled immediately in ice–water bath to stop reaction, followed by centrifugation at 15,000 xg for 3 min at 4°C. Azodye released from the substrate by the function of enzymes in the supernatant was measured by absorbance at 520 nm (A_{520}). The activity was defined as the increase of the A_{520} by 1.0 per min. PTP, DPP–I, DPP–II and DPP–IV were assayed using H–Ala–Ala–Pro–pNA, H–Gly–Phe–pNA, H–Lys–Ala–pNA and H–Gly–Pro–pNA as substrates, respectively[16,17].

The binding of hemoproteins (hemoglobin and myoglobin) at pH 5.0, 7.0 and 8.5 to the intact cell was evaluated by the methods described earlier[18–20] ; mixtures containing 800 μl of hemoprotein solutions (300 μg/ml in water), 50 μl of bacterial suspensions in saline (480 mg/ml) and 150 μl of 1.0 M buffers (acetate buffer, pH 5.0 ; phosphate buffer, pH 7.0 or Tris–HCl buffer, pH 8.5) were incubated at 37°C for 30 min. After incubation, unbound hemoproteins in the centrifugal supernatants were measured by absorbance at 410 nm. The amounts of bound hemoproteins were determined by subtraction.

The cellular locations of RGP, KGP and azocoll proteinase were investigated first. As shown in Table 1, rates of distribution of RGP and KGP of strain ATCC 33277, 381 and W 50 were quite similar. Both proteinases located mainly in the culture fluids. However, in the case of strain W 83, they localized largely in the culture fluids ; almost all the activities were found in these fractions. Azocoll proteinase activities were detected also mainly in the culture fluids. Azocoll proteinase activities were detected also mainly in the culture fluids.

Interestingly, significant amounts of peptidase were found in the crude extracts and the envelops but not in the culture fluids.

In the location profiles of proteinases, they were detected mainly in the culture fluids ; however, no peptidase activity was detected in the culture fluids, while most activities were found in the crude extracts. This difference suggests that no mechanism to transport the enzymes extracellularly is provided in the peptidases. Peptidases in the envelopes accounted for approximately only 2 to 11 % of those in crude extracts (Table 2 & 3).

Table 1 : Comparison of locations of proteolytic enzymes among 4 strains of *P. gingivalis*.

Strain	Fraction	RGP (U)	KGP (U)	Azocoll (U)
ATCC33277	culture fluid	12.0 (55)	7.6 (62)	0.7 (78)
	crude extract	6.6 (30)	4.1 (33)	0.2 (22)
	envelope	3.3 (15)	0.6 (5)	0.0 (0)
381	culture fluid	18.2 (66)	7.7 (64)	1.4 (82)
	crude extract	8.0 (29)	4.1 (34)	0.3 (18)
	envelope	1.2 (4)	0.3 (2)	0.0 (0)
W50	culture fluid	10.2 (58)	16.4 (69)	1.0 (77)
	crude extract	4.9 (28)	6.3 (27)	0.3 (23)
	envelope	2.5 (14)	0.9 (4)	0.0 (0)
W83	culture fluid	24.9 (94)	32.4 (94)	1.6 (89)
	crude extract	1.5 (6)	1.8 (5)	0.1 (6)
	envelope	0.2 (1)	0.4 (1)	0.1 (6)

cell harvested ; 2.5 g

Numbers in parentheses are percentages to the total units

Table 2 : Comparisons of peptidase activities in the crude extracts among 4 strains of *P. gingivalis*.

	Total Units*			
Strain	PTP	DPP–I	DPP–II	DPP–IV
ATCC33277	27.33	0.88	2.46	5.83
381	10.84	0.65	1.99	3.32
W50	23.02	0.85	1.79	4.06
W83	15.67	1.19	2.08	2.85

* ; Units in the crude extracts prepared from 2.5 g cell

Table 3 : Comparisons of peptidase activities in the solubilized envelope fractions among 4 strains of *P. gingivalis*.

	Total Units*			
Strain	PTP	DPP–I	DPP–II	DPP–IV
ATCC33277	1.32	0.09	0.14	0.21
381	1.15	0.02	0.03	0.13
W50	0.79	0.09	0.05	0.10
W83	0.90	0.10	0.09	0.16

* ; Units in the solubilized envelope fractions prepared from 2.5 g cell

Table 4 : Comparison of binding activities to hemoglobin and myoglobin.

	Binding Rate (%)					
	Hemoglobin			Myoglobin		
Strain	pH 5.0	pH 7.0	pH 8.5	pH 5.0	pH 7.0	pH 8.5
ATCC33277	92.5	7.2	0	86.4	6.9	2.1
381	87.8	10.0	1.1	78.9	8.5	6.1
W50	86.3	9.8	1.3	75.9	6.7	0.6
W83	89.0	13.1	3.2	81.8	9.9	11.2

Binding activities to hemoglobin and myoglobin are shown in Table 4. Binding mode of the four strains to hemoproteins appears entirely to be extremely pH dependent ; high binding rates were observed in a low pH buffer and low binding were in neutral and high pH buffers. Binding of these strains to other hemoproteins such as cytochrome *c* and catalase occurred as the similar pH dependent manner. However, the binding rates were significantly lower than those to hemoglobin and myoglobin (data not shown).

In this report, we discussed the formation and cellular locations of proteinases and peptidases, as well as evaluation of binding activities to hemoproteins of *P. gingivalis*, which are related, if not directly, to the pathogenicity of this species. To conclude, these enzymes were found to be elaborated commonly in all strains. A similar tendency appeared for the protein responsible for the hemoprotein binding function.

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抄録：***Porphyromonas gingivalis*** 4株における病原性関連酵素とタンパクの比較研究

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Porphyromonas gingivalis の研究上使われる代表的な4株（ATCC33277，381，W50，W83）を用いて，病原因子と深い関係のある，RGP，KGP を含むプロテイナーゼ，ペプチダーゼおよびヘモグロビンとミオグロビン結合タンパクを通しての結合活性について比較検討した．

プロテイナーゼに関して，4株ともにその産生が見られ，それぞれの株で3画分での分布状況を調べたところ，RGP，KGP，アゾコル分解酵素ともに，培養上清，粗抽出液，エンベロープの順に多かった．RGP，KGP に関しては，ATCC33277，381，W50で培養上清にほぼ60%ほど含まれていたが，W83では94%を含んでいた．これに反して，ペプチダーゼ（PTP，DPP–I，DPP–II，DPP–IV）は4株ともに培養上清には検出されず，そのほとんどが粗抽出液に認められ，数%のみがエンベロープに分布しており，ペプチダーゼには細胞外への分泌機構が作用しないものと思われる．いずれの株でも PTP 産生単位数が DPP のどれよりも高かった．

菌体細胞のヘムタンパクへの結合もいずれの株でも見られたが，ともに pH 依存性が見られ，酸性で強く，中性，アルカリでは非常に弱かった．結合の度合いはヘモグロビンとミオグロビンでは違いはほとんど認められず，これはミオグロビンがヘモグロビン分子の四分の一から成っていることから当然と言えよう．他のヘムタンパク（チトクローム C，カタラーゼ）への結合も弱いながら観察された．