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Characterization of protease from animal periodontal pathogen *Porphyromonas gulae*

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Abstract

***Porphyromonas gulae* is an animal-derived oral microorganisms known also to be associated with periodontal disease in humans. We previously reported that *P. gulae* proteases are possible one of virulence factors related to adhesion and invasion of gingival epithelial cells, and host cell destruction. Here we attempted to characterize bacterial proteases associated with *P. gulae*. Both alkaline phosphatase and trypsin activity were identified in all examined *P. gulae* strains and *P. gingivalis* ATCC33277. Each of the *P. gulae* strains also showed proteolytic activity in cell extract and/or culture supernatant samples. In addition, there was a significant increase in protease activity level seen in living bacterial cells, dependent on cell number, while there were no significant differences regarding proteolytic activity among the *P. gulae* strains. The present results indicate that antipain and PMSF are effective inhibitors of *P. gulae* proteases as well as *P. gingivalis*. In addition, TLCK and leupeptin significantly inhibited proteolytic activity in a dose-dependent manner. On the other hand, AEBSF, ALLN, aprotinin, bestatin, chymostatin, E64, EDTA, pepstatin, and phosphoramidon showed no inhibitory effects, while those of KYT-1 and KYT-36, *P. gingivalis* gingipain-specific inhibitors, were negligible. These results suggest that both *P. gulae* and *P. gingivalis* produce and secrete trypsin-like serine proteases, while the structure of those proteases differ between the two bacteria.**

Key Words: API-ZYM, bacterial protease, *P. gulae*, protease inhibitor, proteolytic activity

Introduction

Porphyromonas gulae has been isolated from the gingival sulcus of a lot of animal species, including bear, canine, cat, coyote, marsupial, monkey, ovine, and wolf [2,12,34)]. *P. gulae* organisms are a black-pigmented, Gram-negative coccobacillus, asaccharolytic, anaerobic, non-

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motile, and non-spore-forming characteristics[12,42], and has been observed in significant numbers of canines with periodontal diseases as compared to those with healthy gingiva[42]. *P. gulae* as well as human periodontal pathogen *P. gingivalis* have abilities to adhere to and invade human gingival epithelial cells, dependent on fimbria characteristics[19].

Periodontal diseases, including gingivitis and chronic periodontitis, are common infection in human and dogs, left untreated, can lead to tooth loss[17,35,49]. The prevalence of periodontal disease in dogs reach up to 85% in individuals over the age of 4 years[28]. In addition, previous reports noted that *P. gulae* was detected in human gingival tissues from subjects with and without periodontitis[49].

Bacterial protease, one of virulence factors, has been reported a large group of enzymes that catalyze the hydrolysis of peptide bonds and are classified into seven catalytic types; aspartyl, cysteine, serine, metallo, glutamic, threonine peptidase, and asparagine peptide lyase[7,40]. The roles of protease in bacterial pathogens have been reported to be degradation of host tissue components for growth and proliferation, and avoidance of the host immune defense system[40]. Moreover, secreted protease has been implicated in bacterial pathogenesis and shown to have several immunomodulating activities, including release of pro-inflammatory cytokines, extracellular matrix degradation, IgG cleavage, and degradation of other immunoglobulins[40]. Bacterial protease has been reported to cause a large number of infectious diseases, such as cholera, salmonellosis, Legionnaires' disease, bronchiectasis, cystic fibrosis, botulism, tetanus, and anthrax[40].

Serine and cysteine proteases are important pathogenesis factors of the periodontal pathogens, such as *P. gingivalis, Treponema denticola, Tannerella forsythia*, and *Fusobacterium nucleatum*[7,16]. Protease produced by periodontal pathogens has been reported capable of degrading periodontal tissues and shown to inactivate host defense effectors, which promote pathological alterations associated with development and progression of periodontal disease[10,15]. *P. gulae* protease in particular has been demonstrated to have arginine- and lysine-specific proteolytic activity[30], and involved in initial attachment of that organism to human gingival epithelial cells[19]. Moreover, *P. gulae* protease play an important role in bacterial biology, such as hemagglutination activity, coaggregation and bacterial growth, as well as degradation of human proteins[48].

Here we report that all examined strains of *P. gulae* mainly possess trypsin-like activity, and proteolytic activity was observed in both cell extracts and culture supernatants, as well as *P. gingivalis*. In addition, proteolytic activity of *P. gulae* was reduced by serine and cysteine protease inhibitors, such as antipain, phenylmethylsulfonyl fluoride (PMSF), Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and leupeptin. Our findings provide proteolytic characterization of *P. gulae* organisms using protease inhibitors.

Materials and Methods

Bacterial culture and API-ZYM test

P. gingivalis ATCC33277 and *P. gulae* ATCC51700, D040, D044, D049, D066, D077 were selected from our culture collections[19,23]. Bacterial cells were grown in Trypticase soy broth supplemented with yeast extract (1 mg/ml), menadione (1 μg/ml), and hemin (5 μg/ml), as described previously[19]. The phenotypic enzyme profiles of all strains and their supernatants were estimated using an API-ZYM test (API biomèrieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

Cell culture

SAS cells, an oral squamous cell carcinoma cell line, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan), and cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2.

Zymography

SAS cells were incubated with *P. gulae* strains ATCC 51700, D040, D044, D049, D066, and D077, and *P. gingivalis* ATCC 33277 at an MOI of 1 for 24 h. Culture supernatant samples (proMMP9) from SAS cells were collected and analyzed for proMMP9 activation using gelatin zymography, as previously described[20]. Briefly, samples (3 μg/lane) were mixed with SDS sample buffer without reducing reagents, then separated on 10% SDS-polyacrylamide gels containing 0.1% gelatin. The gels were incubated at 37°C with 2.5% Triton X-100 for 1 h, followed by 48 h with reaction buffer [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, 0.02% NaN_3]. After staining with 5% Coomassie brilliant blue R-250, gelatinolytic activities were visualized as clear bands against a blue background.

Chemicals

Eleven inhibitors were used in this study (Table 3) as follows; antipain, cysteine and serine protease inhibitor, TLCK, serine protease inhibitor; PMSF, serine protease inhibitor, were purchased from Sigma-Aldrich (St. Louis, MO). Leupeptin, cysteine and serine protease inhibitor, KYT-1, an arginine-gingipain inhibitor, and KYT-36, a lysine-gingipain inhibitor, were purchased from Peptide Institute (Osaka, Japan). A protease inhibitor set containing AEBSF, ALLN, antipain, aprotinin, bestatin, chymostatin, E-64, EDTA-Na2, leupeptin, pepstatin, phosphoramidon, and PMSF was purchased from G-Biosciences (St. Louis, MO).

Protease activity assay

The protease activity of *P. gulae* cells and supernatants was determined using a Pierce Protease assay kit (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. Briefly, samples were mixed with a casein solution and incubation buffer, and then incubated at 37ºC, after which trinitrobenzene sulfonic acid (TNBSA) was added and incubation was continued at 37ºC. TNSBA reacts with exposed primary amines to produce an orange-yellow product, which was measured on SH-1000 Lab microplate reader (Corona Electric, Ibaraki, Japan) at 450 nm. Samples were preincubated with various concentrations of each inhibitor at 37°C for 2 h before addition of the substrates. All inhibitors (1-100 μM) were dissolved in dimethyl sulfoxide.

Statistical analyses

Data were analyzed by two-way analysis of variance (ANOVA) with Tukey multiple-comparison test and are presented as means standard deviations (SDs). Statistical significance was considered at $P < 0.01$ and $P < 0.05$. The data presented are representative of at least three biological replicates.

Results

P. gulae exhibits several virulence characteristics similar to those of the human periodontal pathogen *P. gingivalis*[19,30,48,49]. *Porphyromonas* species, isolated from companion animals, have been shown to have arginine- and lysine-specific proteolytic enzyme activities[30]. However, the proteolytic enzyme activities of *P. gulae* strains are not fully understood. Having previously tested the API-ZYM system, we first examined that the enzyme activities of *P. gulae* strains were assayed using bacterial cells and culture supernatants. As shown in Tables 1 and 2, all tested *P. gulae* strains as well as the *P. gingivalis* ATCC33277 consistently demonstrated alkaline phosphatase and trypsin activities, while no other enzyme activities were detected in any of the tested strains. In addition, protease activity increased in cell number dependent manner (. 1A). Protease activity has been reported to differ considerably among all strains of *P. gingivalis* with type II fimbriae[18]. *P. gulae* protease was found to activate in both cell extracts and supernatants, with negligible differences among the strains (Fig. 1B and C).

Several protease inhibitors, including

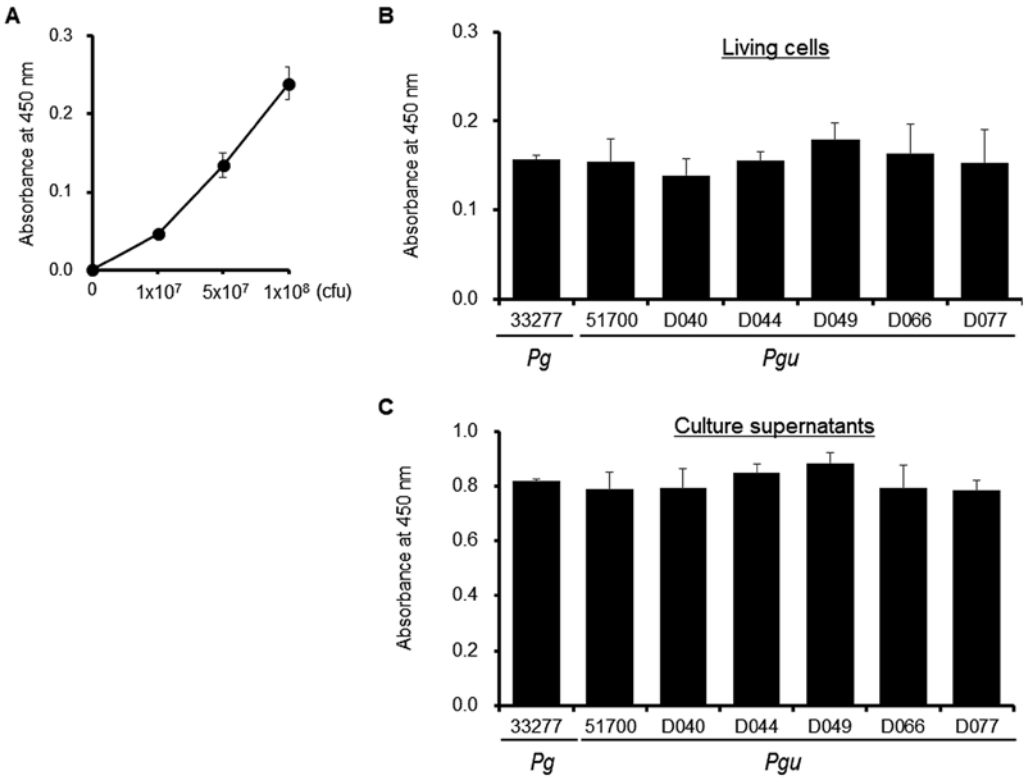


Fig. 1. Proteolytic activity of *P. gulae* strains.
(A) Quantification of protease activities of *P. gulae* ATCC 51700 ($1x10^7$, $5x10^7$, $1x10^8$ cfu), (B) ATCC 51700, D040, D044, D049, D066, and D077 ($5x10^7$ cfu), and (C) supernatants (10 μg; the amount of protein contained in the supernatant), as compared with *P. gingivalis* ATCC33277. Protease activity was determined using a Pierce Protease assay kit, as described in Materials and Methods. Enzyme activities were recorded with a microplate reader and are expressed as arbitrary units. Data are shown as the mean ± SD of three independent experiments. * $P < 0.01$ by ANOVA with Tukey multiple-comparison test.

serine and/or cysteine protease inhibitors, have been reported to inhibit the proteolytic activity of bacteria[21,37]. To clarify the characteristics of *P. gulae* protease, protease activity was determined using protease inhibitors, including antipain, PMSF, EBSF, ALLN, aprotinin, bestatin, chymostatin, E64, EDTA, pepstatin, phosphoramidon (Table 3). Antipain decreased *P. gulae* protease activation by 17.54 ± 3.83%, and PMSF decreased *P. gulae* protease activation by 38.27 ± 3.32%, whereas other inhibitors showed negligible effects. A time course analysis revealed that *P. gulae* as well as *P. gingivalis* protease activity was reduced by antipain and PMSF. In addition, both inhibitors diminished *P. gulae* protease as well as *P. gingivalis* in a concentration-dependent manner (Fig. 2). These findings also suggest that *P. gulae* protease may possess serine protease and/or cysteine protease. Previous studies have noted that TLCK and leupeptin are inhibitors of trypsin-like and cysteine proteases[14,45]. In addition, TLCK and leupeptin reportedly diminishes *P. gingivalis* proteas[21,37]. Therefore, we examined the effects of TLCK and leupeptin on *P. gulae* protease activity using *P. gingivalis* cells as control. Both inhibitors separately and in combination significantly decreased the activities of *P. gulae* protease as well as that of *P. gingivalis* protease in time- and dose-dependent manner (Fig. 3). Together, these results suggest that *P. gulae* may produce and secrete some kinds of proteases. KYT-1 and KYT-36, specific inhibitors of *P. gingivalis* protease gingipains, have also showed significant inhibitory

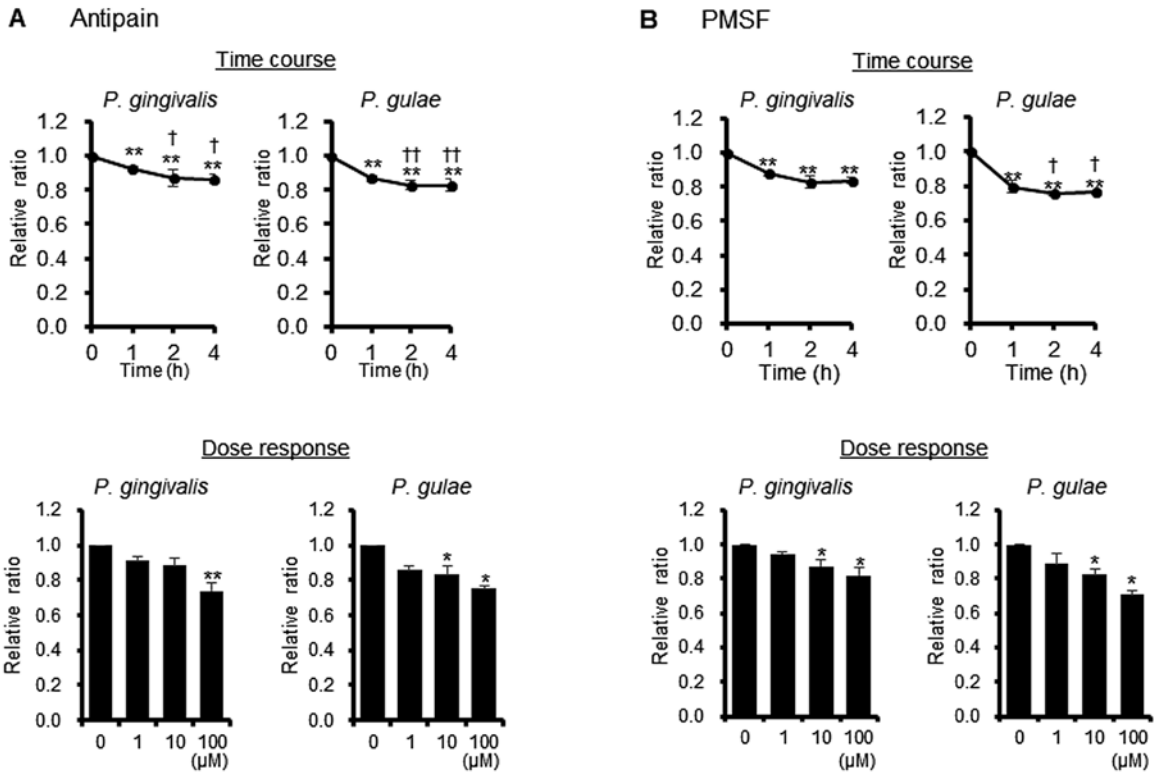


Fig. 2. Effects of antipain and PMSF inhibitors on *P. gulae* proteolytic activity.
Quantification of protease activity of *P. gulae* ATCC 51700 ($5x10^7$ cfu) and *P. gingivalis* ATCC 33277 ($5x10^7$ cfu) with antipain or PMSF. A: Antipain. B: PMSF. Time course studies indicated for 0-4h, and dose response evaluations indicated at 100 μM. Enzyme activities were recorded with a microplate reader and are expressed as arbitrary units. Data are shown as the mean ± SD of three independent experiments. ** $P < 0.01$ and * $P < 0.05$ by ANOVA with Tukey multiple-comparison test (compared with 0 h). †† $P < 0.01$ and † $P < 0.05$ by ANOVA with Tukey multiple-comparison test (compared with 1 h).

effects, as has been reported[22], whereas no inhibition of *P. gulae* protease activity was noted (Fig. 4). *P. gingivalis* protease has been reported to process the proenzyme to the active form of MMP9[20]. Therefore, we examined the effects of selected *P. gulae* strains on proMMP9 activation. Zymography findings revealed that activation of proMMP9 was shown to be induced in vitro by all *P. gulae* strains as well as by *P. gingivalis* ATCC33277 (Fig. 5).

Discussion

The present findings showed that all of the examined *P. gulae* strains produce serine protease with trypsin-like activity. Although details regarding the classification and activities of *P. gulae* protease have yet to be reported, because of the enzyme characteristics similar to *P. gingivalis*, some conclusions can be drawn.

Bacterial protease activities were previously shown to have negligible differences among *P. gingivalis* strains in cell extracts and culture supernatants[18], consistent with the present findings of protease activities of *P. gulae* strains (Fig. 1, Table 2 and 3). On the other hand, *P. gingivalis* strain H66 was found to have negligible gingipain protease activities in cell extract samples, while those were remarkably increased in culture supernatant samples, in contrast to *P. gingivalis* strains ATCC33277 and W50, which showed protease activities in cell extracts[38]. API ZYM systems has been

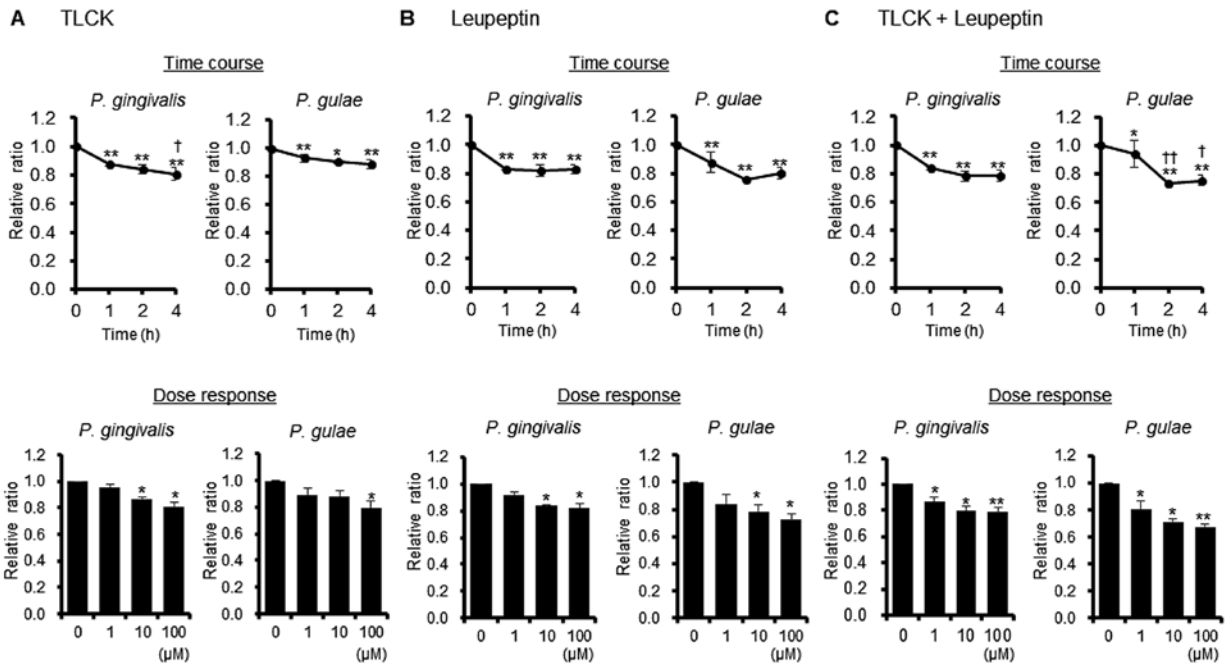


Fig. 3. Effects of TLCK and leupeptin on *P. gulae* proteolytic activity.
Quantification of protease activity of *P. gulae* ATCC 51700 ($5x10^7$ cfu) and *P. gingivalis* ATCC 33277 ($5x10^7$ cfu) with TLCK and/or leupeptin. A: TLCK. B: leupeptin. C: TLCK + leupeptin. Time course studies indicated for 0-4h, and dose response evaluations indicated at 100 μM. Enzyme activities were recorded with a microplate reader and are expressed as arbitrary units. Data are shown as the mean ± SD of three independent experiments. ** $P < 0.01$ and * $P < 0.05$ by ANOVA with Tukey multiple-comparison test (compared with 0 h). †† $P < 0.01$ and † $P < 0.05$ by ANOVA with Tukey multiple-comparison test (compared with 1 h).

reportedly used to charently recognized in regard to taxonomic treatments[1,24,41]. Among them, three speciacterize the enzymatic profiles of the genus *Porphyromonas*. These bacteria are listed the 18 enzymes that curres, *P. gingivalis, P. macacae,* and *P. loveana*, have been reported to possess both alkaline phosphatase and trypsin activities[8,43,46]. On the other hand, other *Porphyromonas* species, such as *P. asaccharolytica, P. bennonis sp, P. canoris, P. cangingivalis, P. crevioricanis, P. endodontalis, P. leveii, P. somerae,* and *P. uenonis*, have alkaline phosphatase but not trypsin activities[5,6,8,9,46,47]. In this study, all tested *P. gulae* strains produced both alkaline phosphatase and trypsin activities (Table 1 and 2), suggesting that only four *Porphyromonas* species are phenotypically positive for trypsin and alkaline phosphatase activity. In addition, this is consistent with reports showing that phylogenetic analysis findings presented indicating that *P. gingivalis, P. gulae* and *P. loveana* are relatively closely related[1], though this issue requires additional study.

Protease inhibitors are known to be useful tools for identification and classification of several different bacterial proteases, including *Escherichia coli* OmpT, *Vibrio parahaemolyticus* protease A, *Yersinia pestis* YopT, and *Bacillus halodurans* keratinase[26,29,39,44]. Our results showed that *P. gulae* protease activity was inhibited by serine/cysteine protease inhibitors, such as antipain, PMSF, TLCK, and leupeptin, while the cysteine protease inhibitor E64 did not cause inhibition (Fig. 2 and 3, and Table 3), nor did metalloprotease, an aspartic protease inhibitor, or calpain (Table 3). Thus, those findings indicate

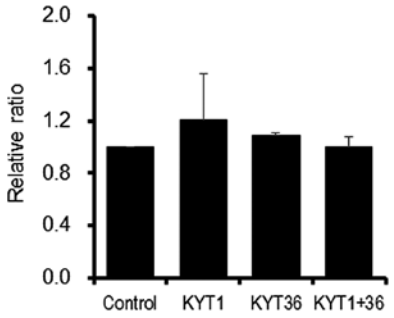


Fig. 4. Effects of gingipains inhibitors on *P. gulae* proteolytic activity.
Quantification of protease activity of *P. gulae* ATCC 51700 ($5x10^7$ cfu) with KYT-1 and KYT-36 gingipain inhibitors (100 μM). Enzyme activities were recorded with a microplate reader and are expressed as arbitrary units. Data are shown as the mean ± SD of three independent experiments and were analyzed with ANOVA with Tukey multiple-comparison test.

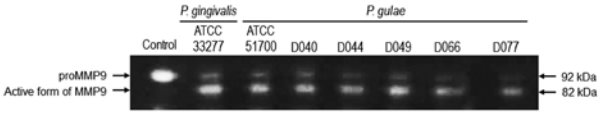


Fig. 5. Effects of protease inhibitors on *P. gulae* proteolytic activity.
Following growth of SAS cells, *P. gulae* and *P. gingivalis* strains (MOI=1) were added to the culture supernatant and incubated for 24 h. proMMP9 activation was examined using gelatin zymography. No infection medium was used as a control.

the possibility that *P. gulae* expresses trypsin-like serine proteases based on their inhibition profile, while serine proteases, such as AEBSF and aprotinin, did not work. The inhibitory effects of antipain and PMSF on proteolysis by bacteria indicate the presence of both trypsin-like serine and papain-like cysteine proteinases[3,32], while AEBSF and aprotinin only inhibit the serine protease[4,13]. Thus, those findings suggest the possibility that *P. gulae* expresses both trypsin-like serine and papain-like cysteine proteinases based on their inhibition profile. Together, these findings suggest that both inhibitors may be unusual regarding inhibition of *P. gulae* proteolytic activity.

P. gulae ATCC51700 reportedly possesses *P. gingivalis* gingipain-related genes, including *rgpA, rgpB,* and *kgp*[30]. The matrix metallopeptidase 9 (MMP9) proenzyme (92KD) is activated by cleavage on the C-terminal side of arginine and lysine by trypsin, and a *P. gingivalis* gingipain, resulting in generation of active mature MMP 9 (82KD)[11,20]. In the present study, *P. gulae* protease exhibited arginine and lysine proteolytic activities in zymography findings, the same as *P. gingivalis* (Fig. 5). These results suggest that *P. gulae* protease cleave different positions in arginine and lysine residues. Additionally, there is a possibility that Rgp and/or Kgp-like proteases of *P. gulae* are released into the extracellular environment. Those proteases have not yet been purified and their structures not observed, thus these issues require additional study. Previous study has noted that KYT-1 and KYT-36, *P. gingivalis* gingipain-specific inhibitors, were designed and synthesized using a series of small peptide analogs containing arginine at the P1 position and lysine at the P2 position[22]. *P. gulae* protease activity was not inhibited by KYT-1 or KYT-36, suggesting the protein structures of protease differ between *P. gulae* and *P. gingivalis*.

In conclusion, all tested *P. gulae* strains as well as *P. gingivalis* have trypsin-like protease activities. However, our results suggest that the structure of *P. gulae* protease may be different from those of *P. gingivalis* among genus Porphyromonas.

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Table 1. Enzymatic characterization of living cells of *P. gulae* strains using API ZYM® system

	P. gingivalis ATCC 33277	*P. gulae* ATCC 51700	*P. gulae* D040	*P. gulae* D044	*P. gulae* D049	*P. gulae* D066	*P. gulae* D077
Alkaline phosphatase	+	+	+	+	+	+	+
Esterase (C4)	−	−	−	−	−	−	−
Esterase-lipase (C8)	−	−	−	−	−	−	−
Lipase (C14)	−	−	−	−	−	−	−
Leucine arylamidase	−	−	−	−	−	−	−
Valine arylamidase	−	−	−	−	−	−	−
Cystine arylamidase	−	−	−	−	−	−	−
Trypsin	+	+	+	+	+	+	+
α-chymotrypsin	−	−	−	−	−	−	−
Acid phosphatase	−	−	−	−	−	−	−
Naphthol-AS-BI-phosphohydrolase	−	−	−	−	−	−	−
α-galactosidase	−	−	−	−	−	−	−
β-galactosidase	−	−	−	−	−	−	−
β-glucuronidase	−	−	−	−	−	−	−
α-glucosidase	−	−	−	−	−	−	−
β-glucosidase	−	−	−	−	−	−	−
N-acetyl-β-D-glucosaminidase	−	−	−	−	−	−	−
α-mannosidase	−	−	−	−	−	−	−
α-fucosidase	−	−	−	−	−	−	−
Negative control	−	−	−	−	−	−	−

Table 2. Enzymatic characterization of culture supernatants of *P. gulae* strains using API ZYM® system

	P. gingivalis ATCC 33277	*P. gulae* ATCC 51700	*P. gulae* D040	*P. gulae* D044	*P. gulae* D049	*P. gulae* D066	*P. gulae* D077
Alkaline phosphatase	+	+	+	+	+	+	+
Esterase (C4)	−	−	−	−	−	−	−
Esterase-lipase (C8)	−	−	−	−	−	−	−
Lipase (C14)	−	−	−	−	−	−	−
Leucine arylamidase	−	−	−	−	−	−	−
Valine arylamidase	−	−	−	−	−	−	−
Cystine arylamidase	−	−	−	−	−	−	−
Trypsin	+	+	+	+	+	+	+
α-chymotrypsin	−	−	−	−	−	−	−
Acid phosphatase	−	−	−	−	−	−	−
Naphthol-AS-BI-phosphohydrolase	−	−	−	−	−	−	−
α-galactosidase	−	−	−	−	−	−	−
β-galactosidase	−	−	−	−	−	−	−
β-glucuronidase	−	−	−	−	−	−	−
α-glucosidase	−	−	−	−	−	−	−
β-glucosidase	−	−	−	−	−	−	−
N-acetyl-β-D-glucosaminidase	−	−	−	−	−	−	−
α-mannosidase	−	−	−	−	−	−	−
α-fucosidase	−	−	−	−	−	−	−
Negative control	−	−	−	−	−	−	−

Table 3. Potency of inhibitors against *P. gulae* protease activity

Inhibitors	Target		Inhibition of Protease activity (%)	References
	Groups	Specificity		
AEBSF	Serine protease	Chymotrypsin, kallikrein, plasmin, thrombin, and trypsin	2.62 ± 6.71	4)
ALLN	Cysteine protease	Calpain	1.61 ± 2.74	37)
Antipain	Serine protease	Trypsin	17.54 ± 3.83	32)
	Cysteine protease	Papain, cathepsin A and B	1.75 ± 4.04	
Aprotinin	Serine protease	Chymotrypsin, kallikrein, plasmin, thrombin, and trypsin	0.37 ± 6.97	13)
Bestatin	Metalloprotease	Aminopeptidase N	1.53 ± 1.08	31)
Chymostatin	Serine protease	α, β, γ, δ Chymotrypsin	1.52 ± 1.04	25)
E64	Cysteine protease	Papain, cathepsin A and L	0.68 ± 3.75	3)
EDTA	Metalloprotease	Gelatinase	1.61 ± 2.74	33)
Pepstatin	Aspartic protease	Cathepsin D, chymosin, pepsin, and rennin	3.99 ± 5.10	3)
Phosphoramidon	Metalloprotease	Thermolysin, collagenase, and endthelin coverting enzyme	3.09 ± 3.56	27)
PMSF	Serine protease	Chymotrypsin, thrombin, and trypsin	38.27 ±3.32	3)
	Cysteine protease	Papain		

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