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学位授与の要件	医歯薬学総合研究科社会環境生命科学専攻 （学位規則第４条第１項該当）
学位論文の題目	*Porphyromonas gulae* proteases influence not only bacterial growth, coaggregation, and hemagglutination but also the maintenance of human protein （*Porphyromonas gulae* のタンパク分解酵素は細菌の増殖、共凝集、赤血球凝集だけでなく、ヒトタンパクの維持にも影響をおよぼす）
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学位論文内容の要旨

Porphyromonas gulae, previously known as the animal biotype of the human periodontal pathogen *P. gingivalis*, is Gram-negative, anaerobic, rod-shaped, asaccharolytic, black- pigmented, non-motile, non-spore-forming, and non-motile. *P. gulae* organisms have been isolated from the gingival sulcus of various animal species, including bear, brushtail, possum, dog, cat, coyote, kangaroo, monkey, ovine, wallaby, and wolf. Interestingly, it has also detected in human gingival tissues from healthy and diseased sites. Although recent studies have reported that *P. gulae* possesses a wide variety of virulence factors, such as including fimbriae, LPS, and proteases, the proteases have yet to be well clarified in details. The present study aimed to clarify the characters of the proteases from varieties of *P. gulae* strains available at this time. Based on biochemical and functional characters similar to the factors from *P. gingivalis*, enzyme roles, hemagglutination, and degradation of host proteins were clarified in this study.

***P. gulae* strains possess trypsin protease-like activity:** *P. gulae* exhibits several virulence characteristics similar to those of the human periodontal pathogen *P. gingivalis*. However, the proteolytic enzyme activities of *P. gulae* strains have not been fully elucidated. All of the examined *P. gulae* strains as well as the *P. gingivalis* ATCC 33277 strain consistently produced alkaline phosphatase and showed trypsin activity, while no other enzyme activities were detected in any of the strains tested. Moreover, protease activity was found in both cell extracts and supernatants, with negligible differences among the examined strains. Protease inhibitors, including antipain (cysteine protease inhibitor), phenylmethylsulfonyl fluoride (PMSF; serine proteinase inhibitor), tosyllysine chloromethyl ketone (TLCK; serine endopeptidase specific inhibitor) and leupeptin (serine protease inhibitor as bacterial metabolite, a cathepsin B inhibitor, a calpain inhibitor and a trypsin inhibitor), diminished *P. gulae* proteolytic activity up to 50%.

Hemagglutination activity: *P. gulae* and *P. gingivalis* reportedly possess protease-related and hemagglutinin genes. However, the hemagglutination ability of *P. gulae* has yet to be investigated. The present findings showed distinct hemagglutination activity in *P. gulae* ATCC 51700, and found that protease inhibitors, such as antipain, PMSF, TLCK, and leupeptin, failed to cause agglutination of

mouse erythrocytes. These results suggest that *P. gulae* proteases may contribute to the hemagglutination.

***P. gulae* growth:** Previous reports noted that *P. gingivalis* growth mediated by gingipains was reported to increase in chemically defined medium (CDM). After inoculation in CDM for the present assays, *P. gulae* ATCC 51700 was found to be in clearly in the stationary phase from 144 h. Furthermore, antipain, PMSF, TLCK, and leupeptin inhibited the growth of *P. gulae* ATCC 51700, suggesting that *P. gulae* proteases may be essential for bacterial growth.

Coaggregation reaction of *P. gulae* with *A. viscosus*: Coaggregation of *A. viscosus* with *P. gingivalis* has been previously reported. *P. gulae* ATCC 51700 was found to coaggregate with *A. viscosus* ATCC 15987, while inhibition of *P. gulae* proteases using protease inhibitors significantly abrogated that activity of the bacterium. These data suggest that coaggregation reactions between *P. gulae* ATCC 51700 and *A. viscosus* ATCC 15987 are regulated via the activity of *P. gulae* proteases.

Morphological changes and inhibition of proliferation of human cells by *P. gulae*: *P. gulae* ATCC 51700 caused rounding and detachment of human gingival carcinoma Ca9-22. The nature of morphological changes is reportedly linked to the proliferation rate of host cells infected with microorganisms. *P. gulae* ATCC 51700 inhibited proliferation of Ca9-22 cells in both multiplicity of infection (MOI) at 500. Pretreatments with antipain, PMSF, TLCK, and leupeptin prevented inhibition of Ca9-22 proliferation by *P. gulae* ATCC 51700, suggesting that *P. gulae* proteases cause morphological changes in Ca9-22 cells, leading to inhibition of their proliferation.

Degradation of human proteins by *P. gulae*: Previous studies have reported that focal contact and adherence junction components, including E-cadherin, β-catenin, focal adhesion kinase (FAK), and paxillin, were associated with epithelial morphology. Following *P. gulae* ATCC 51700 infection at an MOI of 500, E-cadherin, β-catenin, FAK and paxillin also shown to be cleaved at 6 hours. To further evaluate the role of *P. gulae* proteases, *P. gulae* ATCC 51700 was preincubated with several protease inhibitors prior to bacterial infection. Cleavage of focal contact and adherence proteins by *P. gulae* ATCC 51700 was diminished by antipain, PMSF, TLCK, and leupeptin. Furthermore, degradation of recombinant human proteins, γ-globulin and fibrinogen, was observed within 6 hours. Antipain, TLCK, and leupeptin were prevented the cleavage of γ-globulin and fibrinogen, while PMSF did not.

Conclusion: *P. gulae* proteases would be a crucial virulence factors factor for bacterial colonization, such as hemagglutination and coaggregation, and bacterial growth, as well as host defense and cell contact and adherence destruction.

論文審査結果の要旨

Porphyromonas gulae, a Gram-negative black-pigmented anaerobe, has been associated with periodontal disease in companion animals and human. Its virulence has been attributed to various factors, including LPS, fimbriae, and proteases. However, the characterization and function of *P. gulae* proteases have yet to be well clarified. In the present study, several proteases associated with *P. gulae* was characterized and clarified the functions of them. Based on biochemical and functional characters similar to the factors from *Porphyromonas gingivalis*, which is a periodontopathic bacteria in human and has been well characterized, enzyme roles, hemagglutination, and degradation of host proteins were clarified in this study.

Both alkaline phosphatase and trypsin activity were identified in all examined *P. gulae* strains, as well as *P. gingivalis* ATCC 33277. Moreover, proteolytic activity was observed for all *P. gulae* strains in either cell extract or culture supernatant samples. Protease inhibitors, including antipain (cysteine protease inhibitor), phenylmethylsulfonyl fluoride (PMSF; serine proteinase inhibitor), tosyl lysine chloromethyl ketone (TLCK; serine endopeptidase inhibitor), and leupeptin (serine protease inhibitor as bacterial metabolite, a cathepsin B inhibitor, a calpain inhibitor, and a trypsin inhibitor), diminished *P. gulae* proteolytic activity up to 50%. This inhibition resulted in the reductions of bacterial cell growth, hemagglutination, and coaggregation with *Actinomyces viscosus*. In addition, *P. gulae* inhibited gingival epithelial cells proliferation with small-rounded cell morphology at high multiplicity of infection (MOI 500) during 24-hour culture. Moreover, *P. gulae* caused decreases of cell adhesion-related proteins such as E-cadherin, β-catenin, FAK and paxillin at 6 hours. These inhibitions were relieved by adding the inhibitors mentioned above. Furthermore, recombinant human proteins such as γ-globulin and fibrinogen were degraded within 6 hours, but relieved by adding the inhibitors.

Together, these results demonstrated the characters and virulence of *P. gulae* proteases and the suggested the pathogenesis of this bacteria to the periodontal disease in either companion animals and human. This would contribute to understand the periodontal disease of companion animal but also the periodontal disease as a zoonotic disease.

This Dissertation Review Committee hereby accept this original article as a doctoral dissertation in dentistry.