Potential Value of a Rice Protein Extract, Containing Proteinaceous Inhibitors against Cysteine Proteinases from Porphyromonas gingivalis, for Managing Periodontal Diseases

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Arg-specific gingipain (Rgp) is a major pathogenic determinant of Porphyromonas gingivalis which is a major pathogen in periodontal disease. We prepared protein extracts with Rgp-inhibitory activity from polished rice (Oryza sativa) and evaluated the effects of these extracts on the growth and pathogenicity of P. gingivalis. The extracts inhibited the proteolytic degradation of human proteins by P. gingivalis proteases, and repressed the growth and homotypic biofilm formation of P. gingivalis. The disruption of adhesion of epithelial cells by P. gingivalis was also restricted by the rice protein extracts. Our results suggested that the rice protein extracts suppressed the pathogenicity and growth of P. gingivalis by inhibiting the bacterial protease activities, implying that the Rgp-inhibitory proteins prepared from rice may be potentially valuable as nutraceutical agents for preventing periodontal diseases.

Key words: Porphyromonas gingivalis; periodontal disease; Arg-specific gingipain; nutraceutical; rice protein

Periodontal diseases are highly prevalent chronic infections caused by microorganisms in the oral cavity, leading to inflammation of the gingiva and destruction of periodontal tissues. In addition to the serious consequences to oral health, emerging studies have reported a strong association between periodontal diseases and such systemic disorders as atherosclerosis and coronary heart diseases, diabetes, respiratory diseases, and preterm delivery with low birth weight. These findings suggest that controlling periodontal diseases would be important for maintaining good oral hygiene, and for preventing and managing these systemic disorders.

Porphyromonas gingivalis is a gram-negative black-pigmented anaerobic bacterium and is known to be one of the most important periodontal pathogens in the onset and progression of periodontitis. This bacterium produces various virulence factors, including outer membrane vesicles, adhesins, lipopolysaccharides, hemolysins and proteases. Among these, cysteine proteinases, which are called gingipains, are the main endopeptidases produced by P. gingivalis. Gingipains consist of two molecular species that show differing substrate specificity: Arg-specific (Rgp) and Lys-specific (Kgp) enzymes. These enzymes are essential for the growth and survival of the bacterium in vitro and in vivo, and play critical roles in the degradation of host proteins and cell invasion by the bacterium.

Gingipains are the major determinants in the pathogenicity of P. gingivalis, so that inhibitory agents towards these enzymes can be expected to be effective in preventing and improving P. gingivalis-associated diseases. Some inhibitors of gingipains have already been reported. Tetracycline analogues inhibited the amidolytic activity of Rgp and the Rgp-mediated enhancement of vascular permeability. FA-70C1, which is a potent Rgp inhibitor identified from the culture supernatant of Streptomyces species strain FA-70, suppressed disruption of the bacterial activity of polymorphonuclear leukocytes (PMNs) by P. gingivalis. FA-70C1 also protected human fibroblasts and umbilical vein endothelial cells from the cytotoxicity of P. gingivalis, in addition to inhibiting P. gingivalis growth. Kadowaki et al. have designed KYT-1 and KYT-36, which respectively are specific and potent inhibitors of Rgp and Kgp, based on the specificity and efficacy for the cleavage of histatins by these enzymes. These synthetic inhibitors strongly inhibited the bacterial growth and activities relevant to the pathogenesis of P. gingivalis such as the degradation of host proteins, disruption of the bactericidal activity of PMNs and enhancement of the vascular permeability. KYT-1 and KYT-36 could suppress the pathogenicity of P. gingivalis by a single use, and the effects of these inhibitors were also synergistic. These observations confirm the importance of Rgp and Kgp as molecular targets for the management of periodontal diseases.

“Nutraceuticals” or “functional foods” are defined as foods or dietary components that may provide a health benefit beyond the basic nutritional function of supplying nutrients. An aging population, increasing healthcare costs, and a rising interest in attaining wellness through the diet have prompted an increasing interest in nutraceuticals in recent years. These social and scientific trends have led to investigations into the use of bioactive food constituents in the management of various diseases, including periodontal diseases. A few polyphenols derived from plants such as green tea (Camellia

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Materials and Methods

Materials. Crushed rice, i.e., broken pieces of polished rice, was prepared from rice bran purchased from a local market by separating with an air classifier we had constructed, and then subsequently passed through a 2-mm sieve. Human plasma fibronectin, human plasma α2-macroglobulin (α2M) and human blood γ-globulin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), and human plasma holo-transferrin was obtained from Merck (Darmstadt, Germany).

Bacterial strains, culture conditions, and preparation of P. gingivalis enzymes. P. gingivalis ATCC 33277 and JC1 12257 were maintained at 37°C in enriched brain heart infusion broth (BHI), containing BHI (37 g/L, Oxoid, Hampshire, England) supplemented with yeast extract (5 g/L), hemin (1 mg/L), and cysteine (1 g/L), under anaerobic conditions using an anaerobic glove box (gas phase, 10% CO2/10% H2/80% N2; Model ANX-3; Hirasawa Co., Tokyo, Japan) or AnaeroPack system (Mitsubishi Gas Chemical Co., Tokyo, Japan). A proteinase fraction from the culture supernatant of P. gingivalis ATCC 33277 was prepared by the method of Kadowaki et al. Briefly, the bacterial culture was clarified by centrifugation (15,000 × g for 10 min), and ammonium sulfate was added to give 75% saturation. The precipitated proteins were dissolved in a 10 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer. After removing the insoluble materials by centrifugation, the clarified supernatant was stored at −80°C until needed. Rgp was purified from the culture supernatant of P. gingivalis ATCC 33277 as described previously. Partially purified Kgp was also prepared from the culture supernatant of this bacterial strain by size-exclusion chromatography (Sephadex G-100; GE Healthcare Bio-Science Corp., Piscataway, NJ, USA).

Cell culture. Sa3 (RBC0980), a human oral squamous carcinoma cell line, was obtained from Riken BioResource Center (Ibaraki, Japan). Gin-1, a human normal gingival fibroblast, was purchased from DS Pharma Biomedical Co. (Osaka, Japan). The cells were maintained at 37°C in a humidified 5% CO2 atmosphere, using Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/L of glucose, 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (pH 7.5) (D-MEM; Life Technologies) and 10% fetal bovine serum (FBS; ICN Biochemicals, Aurora, OH, USA).

Preparation of the rice protein solutions. The rice protein was extracted from crushed rice. Briefly, 2 kg of crushed rice was stirred with 20 L of a 30 mM citrate buffer (pH 6.0) at room temperature (RT) for 30 min and then passed through a 200-mesh nylon filter. The filtrate was heated by using a steam injection system equipped with a holding tube of 316L grade stainless steel sanitary pipe (35.7 mm internal diameter, 11 mm length; Osaka Sanitary Metal Industries Cooperative Union, Osaka, Japan) under the following conditions: direct steam injection at 130°C, flow rate of 661/L, and holding time of 10 min at 130°C. After the heat treatment, the extract was freeze-dried and stored under desiccated conditions. Ten grams of the powdered extract was dissolved in 200 mL of Milli-Q water (Millipore Corp., Bedford, MA, USA) and centrifuged at 38,900 × g for 30 min at 4°C. The resulting suspension was clarified by being sequentially passed through 3.0, 0.8, and 0.45-μm-pore membrane filters. Two kinds of rice protein solution were subsequently prepared: a water-soluble (W-S) solution whose clarified extract had been filtered through a 0.22-μm-pore membrane (Durapore; Millipore Corp.) and a dialyzed soluble (D-S) solution. The clarified extract of the latter was dialyzed against 20 mM HEPES (pH 7.5) by using a Spectra/Per 6 regenerated cellulose membrane with a molecular weight cut-off (MWCO) of 3,500 Da from Spectrum Laboratories, Laguna Hills, CA, USA) and then filtered through a 0.22-μm-pore membrane. The protein solutions were concentrated by freeze-drying and re-dissolution if necessary.

Gingipain inhibition assay. The Rgp- and Kgp-inhibitory activities were determined by using fluorogenic substrates purchased from Peptide Institute (Osaka, Japan): carbobenzoxyl (Z)-Phe-Arg-4-methylcoumaryl-7-amide (MCA) for Rgp and Z-His-Glu-Lys-MCA for Kgp. Purified Rgp and partially purified Kgp were used for the assay. Purified Rgp was diluted in the assay buffer (100 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM CaCl2, and 4 mM dithiothreitol (DTT)), including 0.05% 3-[3-cholamidopropyl]-dimethylammonio]-1-propane sulfonate (CHAPS), and incubated at 37°C for 15 min prior to use. The rice protein solution for each assay was pre-incubated for 5 min at 40°C with an appropriate amount of the enzyme preparation in an assay buffer with a total volume of 0.2 mL in a black polypropylene 96-well plate (Nunc-MicroWell plate; Thermo Fisher Scientific, Waltham, MA, USA). After the pre-incubation, a fluorogenic substrate was added to the reaction mixture to a final concentration of 50 μM for Rgp and 100 μM for Kgp. The release of 7-amino-4-methylcoumarin (AMC), which was associated with the enzyme reaction, was monitored at an excitation wavelength of 380 nm and an emission wavelength of 440 nm during incubation for 1–5 min at 40°C. The enzyme substrate was dispensed and AMC release monitored by using an Infinite M1000 microplate reader (Tecan, Männendorf, Switzerland). One unit of inhibition is defined as the reduction in the liberation of 1 μmol of AMC per minute.

Effect of a rice protein solution on proteolytic degradation of the host proteins by P. gingivalis proteases. For the human-derived proteins, 10 μg of fibronectin, transferrin, α2M, or γ-globulin was incubated at 37°C for 1 h with an appropriate amount of the proteinase fraction of P. gingivalis in 50 μL of the assay buffer used in the gingipain inhibition assay, in the presence or absence of the rice protein solution. The amounts of the proteinase fraction of P. gingivalis used in the reaction were 4 ng of protein for fibronectin and transferrin, and 200 ng of protein for α2M and γ-globulin. The rice protein solution was added at a final concentration of 40 μg/mL. The degradation products were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after the incubation.

Biofilm inhibition assay. A 48-h culture of P. gingivalis JC1 12257 in enriched BHI was diluted 100-fold in a fresh medium with or without a rice protein solution. The W-S rice protein solution was added to a final concentration of 50 or 100 μg/mL, and the D-S solution, to 100 or 150 μg/mL. Triplicates of 1-mL cultures were cultivated in 15-mL polybutadiene styrene centrifuge tubes (Thermo Fisher Scientific) for 24 h at 37°C in a humidified anaerobic atmosphere. The biofilm amount was measured by using crystal violet dye according to O’Toole et al. after cultivation. Briefly, the medium and unattached cells were removed, and the tubes were washed twice with 2 mL of distilled water. The biofilm was stained for 15 min with 1 mL of 0.1% crystal violet (Merck KGaA). The tube was then washed three times with 2.5 mL of distilled water to remove the unbound crystal violet dye and dried for 2 h at 37°C. After adding 0.5 mL of 95% (v/v) ethanol, the tube was shaken with a vortex mixer to release the stain from the biofilm, and the absorbance of the solution was
measured at 550 nm. The differences between two groups were statistically tested by using a two-tailed Student’s t test.

**Effect of a rice protein solution on the growth of *P. gingivalis*.** The growth inhibition assay was performed by using an α-ketoglutarate/bovine serum albumin (KGB) medium with slight modifications. The constituents were 10 mM NaH₂PO₄, 10 mM KCl, 2 mM citric acid, 1.25 mM MgCl₂, 50 μM MnCl₂, 10 μM CoCl₂, 25 μM ZnCl₂, 0.1 μM Na₂MoO₄, 5 μM CaCl₂, 5 μM H₃BO₃, 20 μM CaCl₂, 25 mM α-ketoglutaric acid, 50 mM HEPES, 2 mM cysteine, 3% bovine serum albumin (BSA), 1 mg/mL of transferrin, and 1 mg/L of vitamin K₁. The medium was adjusted to pH 7.3 prior to adding transferrin and vitamin K₁, and the medium was filter-sterilized by using a 0.22-μm-pore membrane filter.

*P. gingivalis* ICM 12257 was pre-cultured for 22 h in an enriched BHI medium containing 1 mg/mL of transferrin instead of hemin as an iron source and then diluted 10-fold with the modified KGB both supplemented with or without a rice protein solution. The W-S rice protein solution was added to the medium to a final concentration of 50 or 100 μg/mL, and the D-S solution, to 100 or 140 μg/mL. Triplicates of 1-mL cultures were cultivated in 5-mL polypropylene round-bottom tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at 37 °C in an anaerobic atmosphere. Aliquots of the cultures were collected at defined time intervals and stored at −80 °C. Bacterial growth was monitored by ATP-based luminescence quantification, using a BacTiter-Glo microbial cell viability assay (Promega Corporation, Madison, WI, USA). The differences between two groups were statistically tested by using a two-tailed Student’s t test. A fresh medium and culture supernatant at 93 h were diluted 50-fold with 100 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, and 0.05% CHAPS, before being loaded for SDS–PAGE to analyze the degradation of BSA as the sole carbon/energy source.

**Cell toxicity of a rice protein solution.** Sa3 cells were seeded into a 96-well culture plate (Nunc P96 MicroWell plates; Thermo Fisher Scientific) at a density of 1 × 10⁴ cells/well and cultivated for 2 d in D-MEM supplemented with 10% FBS. The culture medium was then replaced with serum-free D-MEM containing from 0 to 180 μg/mL of the D-S solution. The culture was incubated for 5 h, and the cell viability was then evaluated by using the WST-8 tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). A 10-μL aliquot of WST-8 was added to each well, and the culture was further incubated for 2 h at 37 °C in a CO₂ incubator. The production of water-soluble formazan was measured at 450 nm (650 nm reference) by using an Infinite M1000 microplate reader.

**Effect of a rice protein solution on the inhibition of adhesion of epithelial cells by *P. gingivalis* proteinases.** Sa3 cells were seeded into a 96-well culture plate at a density of 1 × 10⁴ cells/well, and then cultivated overnight in D-MEM supplemented with 10% FBS. The culture medium was then replaced with cultivation with serum-free D-MEM containing the proteinase fraction of *P. gingivalis* (10 μg of protein) and the D-S solution (0, 20, 40, 60, 80, 100, or 120 μg/mL). The culture was incubated for 4 h, and non-adhering cells were removed by aspiration. After washing the attached cells with PBS, the cells on the well surface were determined by using a CyQUANT NF cell proliferation assay kit (Life Technologies) based on the fluorescent staining of DNA, according to the manufacturer’s instructions. The fluorescence intensity was measured with an Infinite M1000 microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Assays were performed in triplicate, and the differences between two groups were statistically tested by using a two-tailed Student’s t test.

**Analyses.** The protein concentration was determined by using a Coomassie Plus assay kit (Thermo Fisher Scientific) based on the method of Bradford. SDS–PAGE was conducted using NuPAGE Novex 4–12% Bis-Tris Gel (Life Technologies), using a NuPAGE MOPS SDS running buffer (Life Technologies) according to the manufacturer’s protocol. The NOVEX Sharp unstained protein standard (Life Technologies) was used as an electrophoresis marker. The gel was stained after gel electrophoresis with the Oriole fluorescent gel stain (Bio-Rad Laboratories, Hercules, CA, USA).

**Results**

**Preparation of the rice protein solutions.** Rgp-inhibitory proteins were extracted from crushed rice, which was broken pieces of polished rice generated by a rice polishing process, on a pilot-scale, using a 30 mM citrate buffer (pH 6.0). Since the Rgp-inhibitory activity was highly thermostable, the extract was sterilized by heat treatment with a steam injection system at 130 °C for 10 min and then lyophilized. The rice protein extract included large amounts of citrate, and hence, two different rice protein solutions were prepared for a functional evaluation: the W-S solution, in which the powdered extract was dissolved in water; and the D-S solution, from which citrate had been removed from the W-S solution by dialysis. The respective pH values of the W-S and D-S solutions were 5.90 and 7.13, and the respective specific Rgp-inhibitory activities were 12.0 mU/mg and 7.9 mU/mg. These two rice protein solutions also had a low level of Kgp inhibitory activity.

**Effects of the rice protein solutions on protein degradation of the host proteins by *P. gingivalis* proteinases.**

The *P. gingivalis* proteinases that had been prepared from the culture supernatant degraded fibronectin, transferrin, γ-globulin, and α₂M, although the efficiency of the degradation differed according to the protein substrates (Fig. 1). Fibronectin and transferrin were efficiently degraded by 4 ng of the *P. gingivalis* proteinases, but the degradation of γ-globulin and α₂M was incomplete, even when 200 ng of the *P. gingivalis* proteinases was used. The *P. gingivalis* proteinases could degrade the heavy chain (approx. 55 kDa) of γ-globulin, producing a degradation product of approximately 31 kDa, but not the light chain (approx. 25 kDa). The W-S solution of the rice protein almost completely inhibited the degradation of fibronectin and transferrin.

![Fig. 1. Effect of the Rice Protein W-S Solution on the Degradation of Human Proteins by *P. gingivalis* Proteinases.](image-url)
Although inhibition of the degradation of α2M and γ-globulin heavy chain was partial, it was substantial. The D-S solution could also inhibit the degradation of human proteins by \textit{P. gingivalis} proteinases; the inhibition profile corresponded to the inhibition profile of the W-S solution (data not shown). These results indicate that the inhibition of protein degradation was attributable to the action of the Rgp-inhibitory proteins, but not to the disturbance of enzymatic activity due to a large amount of citrate.

**Effects of the rice protein solutions on biofilm formation by \textit{P. gingivalis}**

The colonization of \textit{P. gingivalis} on subgingival sites is an important step in the etiology of periodontal diseases, and this bacterium expresses cell surface structures that contribute to this step. Among these, fimbriae act as key factors facilitating the initial interaction in cell adhesion/colonization.\textsuperscript{36–38} Since Rgp was responsible for the processing of fimbriulin, a major component of fimbriae,\textsuperscript{38,39} we assessed the effects of the rice protein solutions on biofilm formation by \textit{P. gingivalis}.

The test strain was grown in an enriched BHI medium for the biofilm formation assays. This medium was nutrient rich and could support the growth of the gingipain-null mutant of \textit{P. gingivalis} as well as the wild-type strain.\textsuperscript{17} Biofilm formation was significantly inhibited by the W-S solution at concentrations of 50 μg/mL and 100 μg/mL (\(p < 0.05\); Fig. 2). Biofilm formation was also significantly inhibited by the D-S solution at concentrations of 100 μg/mL and 150 μg/mL (\(p < 0.05\)), although the level of inhibition was lower than that of W-S. The difference in inhibitory efficiency between the W-S and D-S solutions probably corresponded to the difference in Rgp inhibitory activity between these preparations.

**Inhibition of the bacterial growth of \textit{P. gingivalis}**

A growth inhibition assay was performed by using a modified KGB medium. This medium contained BSA as the sole energy/carbon source and human transferrin, which is a major iron-binding protein of gingival crevicular fluid, as an iron source. The wild-type strains of \textit{P. gingivalis} could grow in a KGB medium containing hemin as the iron source, although the Rgp/Kgp-null mutant could not grow in this medium, suggesting that the cysteine proteinases of \textit{P. gingivalis} were essential for growth as they would acquire peptides and amino acids via the degradation of exogenous proteins.\textsuperscript{17,34}

These results suggested that KGB would be a suitable medium to evaluate the effects of the rice protein solutions on the growth of \textit{P. gingivalis}.

Figure 3A shows that the growth of \textit{P. gingivalis} was significantly inhibited by the rice protein solutions. The W-S solution inhibited the growth in a concentration-dependent manner: after 93 h, the growth had decreased by about 50% and 80% at 50 μg/mL and 100 μg/mL, respectively. There was no difference in the inhibition level between 100 μg/mL and 140 μg/mL of the D-S solution, the growth after 93 h having decreased by about 87% and 91% at 100 μg/mL and 140 μg/mL, respectively. BSA was almost completely degraded by \textit{P. gingivalis} in the control culture, but the degradation...
of BSA was substantially inhibited in the presence of the rice protein solutions (Fig. 3B), corresponding to the growth inhibition of *P. gingivalis* by the rice protein extract.

**Effects of the rice protein solutions on the decrease in cell adhesion of epithelial cells by *P. gingivalis* proteases**

It has been reported that Rgp was responsible for the loss of adhesion of human gingival fibroblasts and umbilical vein endothelial cells, being induced by the culture supernatant proteins of *P. gingivalis*. We assessed the effects of the rice protein solutions on the deterioration of cell adhesion by the *P. gingivalis* proteases. Only the D-S solution was used in the subsequent cell-based assays, because the large amount of citrate present in the W-S solution acidified the medium.

Figure 4 shows the cell toxicity of the D-S solution to Sa3 cells. More than 80% of cell viability was maintained in the presence of up to 120 µg/mL of the D-S solution, the cell viability decreasing gradually with increasing concentration of the D-S solution. The concentration of the D-S solution was in the range of 20–120 µg/mL in further experiments to maintain the cell viability above 80%. Approx. 90% of the Sa3 cells had lost their adhesion ability after being treated with the proteinases of *P. gingivalis*, when compared with the cells without the proteinase treatment. The D-S solution prevented the deterioration of cell adhesion by the *P. gingivalis* proteinases in a concentration-dependent manner, cell adhesion being almost entirely protected at 120 µg/mL (Fig. 5).

**Discussion**

*P. gingivalis* is one of the most important periodontal pathogenic bacteria at the onset and progression of periodontitis. Rgp plays an essential role in the physiology and pathogenicity of this bacterium, leading to the notion that Rgp could be a promising therapeutic and preventive target for periodontal diseases. We had found in a previous study that the protein fraction from rice grains inhibited Rgp and that four proteins (i.e., a 26-kDa globulin, a plant lipid transfer/trypsin-α amylase inhibitor, an RA17 seed allergen, and an α-amylase/trypsin inhibitor) contributed to the majority of the inhibitory activity. Therefore, in this study, we produced the Rgp-inhibitory proteins on a pilot-scale for use as nutraceutical agents, and evaluated their actions on the growth and virulence of *P. gingivalis* in vitro. In addition to the W-S solution for the functional evaluation, a D-S solution was prepared by dialyzing the W-S solution to remove a large amount of citrate. A precipitates was generated in the D-S solution during the dialysis, suggesting a decrease in the solubility of the extracted Rgp-inhibitory proteins by the removal of citrate. The partial loss of the Rgp-inhibitory proteins in the D-S solution is thought to have reduced the Rgp-inhibitory activity and the effects on the biofilm formation and growth of *P. gingivalis* when compared to the W-S solution (Figs. 2 and 3). However, the findings obtained by using the D-S solution imply that the beneficial effect of the rice protein solution was attributable to the action of the Rgp-inhibitory proteins, but not to the large amount of citrate.

*P. gingivalis* is asaccharolytic, and its carbon and energy sources depend on small peptides and amino acids derived from the hydrolysis of host proteins. Kadowaki *et al.* have examined the effects of KYT-1, which is a synthetic inhibitor specific to Rgp, on the degradation of a panel of human proteins by a *P. gingivalis* culture supernatant. In this present study, we examined the effects of rice protein solutions on the degradation of host proteins according to the methods used in their study.

Figure 1 shows that the rice protein solutions inhibited the proteolysis of human proteins by the proteinases of *P. gingivalis*. Kadowaki *et al.* have reported that a single use of KYT-1 only slightly inhibited the degradation of human fibronectin, and that significant inhibition was only observed when KYT-1 was used in combination with KYT-36, a Kgp-specific synthetic inhibitor. However, the rice protein solutions inhibited the degradation of fibronectin almost completely at a concentration of 40 µg/mL. The weak but considerable Kgp inhibitory activity of the rice protein solutions may have contributed to this complete inhibition.

α2M is a major plasma proteinase inhibitor, which is known to contribute to the host defense mechanism, and
Kadowaki et al.\textsuperscript{25,26} and Bedi and Williams\textsuperscript{43} have indicated that Rgp was capable of degrading \textalpha2M. We confirmed the degradation of \textalpha2M by \textit{P. gingivalis} proteinases, and the rice protein solutions suppressed this \textalpha2M degradation (Fig. 1). These results corresponded to the actions of KYT-1 on the proteolysis of \textalpha2M by Rgp. On the other hand, Gron et al. have described that \textalpha2M inhibited Rgp.\textsuperscript{44} They determined the proteinase inhibitory capacity of \textalpha2M by a hide powder azure assay and did not directly measure the action of Rgp on \textalpha2M. These differences in the assay system may explain this apparent contradiction.

Transferrin is an iron-binding protein that is a major constituent of gingival crevicular fluid; \textit{P. gingivalis} acquires iron essential for its growth by degrading this protein.\textsuperscript{45} Inhibiting transferrin degradation should result in the growth inhibition of \textit{P. gingivalis}. Indeed, the rice protein solutions strongly inhibited the growth of \textit{P. gingivalis} in the modified KGB medium containing transferrin as an iron source at concentrations of 100 \(\mu\)g/mL and above (Fig. 3A). No growth inhibition by the rice protein solutions was apparent when hemin was used as the iron source (data not shown). Hemin can be used for the majority of anaerobic bacteria and has been widely employed in the growth media, since the acquisition of iron would not constrain bacterial growth in a hemin-containing medium. Similar results have been observed in the case of lactoferrin.\textsuperscript{45} Lactoferrin is an iron-binding protein which inhibits the growth of \textit{P. gingivalis} by disrupting the iron uptake system as it binds to the hemoglobin receptor. Consequently, a rice protein solution would suppress the growth of \textit{P. gingivalis} via its action on the iron acquisition process, similar to lactoferrin. Kadowaki et al. have also examined the effects of KYT-1 and KYT-36 on the growth of \textit{P. gingivalis} by using a KGB medium containing hemin as the iron source. In their study, almost complete inhibition of growth was obtained only by the combination of KYT-1 and KYT-36, inhibiting both Rgp and Kgp.\textsuperscript{26} The mechanism of action for growth inhibition could involve the inhibition of BSA degradation, which is used as a carbon/energy source, by the combined action of Rgp and Kgp. Since the majority of BSA was not degraded in the presence of the rice protein solution (Fig. 3B), the suppression of BSA degradation may have contributed to growth inhibition.

As well as bacterial growth, colonization of bacteria within a periodontal pocket is a prerequisite for the pathogenesis of \textit{P. gingivalis}. The rice protein solutions inhibited the homotypic biofilm formation by \textit{P. gingivalis} at 50 \(\mu\)g/mL for the W-S solution and 100 \(\mu\)g/mL for the D-S solution (Fig. 2). These concentrations correspond to those required for the inhibition of Rgp activity. Such agents as cranberry polyphenol\textsuperscript{28,29} and lactoferrin\textsuperscript{46} have also demonstrated inhibitory activities against both biofilm formation and Rgp/Kgp activity. However, in the case of cranberry polyphenol and lactoferrin, the concentration required for inhibiting biofilm formation was inconsistent with that required for inhibiting the proteinase activities. Furthermore, Kuboniuwa \textit{et al.}\textsuperscript{47} have examined the effects of gene disruption on homotypic biofilm formation by \textit{P. gingivalis} and reported that Rgp played a bifunctional role in coordinating the integrity of the biofilm by mediating microcolony formation and restraining biovolume. However, Kgp was demonstrated to be a negative regulator of microcolony formation. It is suggested from these findings that, in addition to proteinase inhibition, other mechanisms contributed to the prevention of biofilm formation by the rice protein solutions.

The rice protein solutions significantly restricted the loss of adhesion of Sa3 cells by the \textit{P. gingivalis} proteinases (Fig. 5). Similarly, other gingipain inhibitors as KYT-1 and leupeptin\textsuperscript{48} could protect cell adhesion, implying that the rice protein should protect cell-cell contact of oral epithelial cells/fibroblasts from \textit{P. gingivalis} infection by inhibiting the bacterial proteinase activities. The rice protein solution could markedly inhibit the degradation of human fibronectin, which is an extracellular matrix protein that plays important roles in the adhesion and migration of mammalian cells\textsuperscript{49} (Fig. 1), and the restriction of fibronectin digestion may be relevant to protecting the adhesion of Sa3 cells.

Our results demonstrate that the rice protein solutions should suppress the pathogenicity and growth of \textit{P. gingivalis} by inhibiting the bacterial proteinase activities, suggesting that the protein extract prepared from rice would be a promising candidate for a nutraceutical agent to prevent periodontal diseases. However, further studies are needed in order to verify the precise relationship between Rgp inhibition and the beneficial effects of the rice protein extract demonstrated in this study. We are now investigating this aspect by using purified preparations of the major Rgp-inhibitory proteins from rice.

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