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Basic peptide protamine exerts antimicrobial activity against periodontopathic bacteria

——Growth inhibition of periodontopathic bacteria by protamine

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ABSTRACT

Protamine was investigated for its antibacterial activity against the periodontal pathogens, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*. We determined the minimum inhibitory concentrations of protamine and its hydrolysate and their bactericidal activity. Protamine inhibited the growth of all periodontopathic bacteria tested on agar plates. Protamine, which MIC was 6.3×10^{-7} g L^{-1}, was most effective against *P. gingivalis*. The antibacterial effect of native protamine was higher than that of its hydrolysate. An ATP bioluminescence assay revealed that protamine showed bactericidal activity against *P. gingivalis* in a time-dependent manner. These results indicate that protamine could be candidate peptide for prevention of *P. gingivalis* infection.

Keywords: Antimicrobial Peptide; Periodontopathic Bacteria; Implantitis; Protamine

1. INTRODUCTION

Bacterial infection arising from an accumulation of microbial plaque around dental implants is a major cause of periodontal disease including peri-implantitis. Five species of periodontal pathogen detected in cases where a titanium implant was used [1]. This indicates the importance of maintaining biofilm-free surfaces on both the sub-gingival and supra-gingival portions of dental implants in preventing peri-implantitis. *Porphyromonas gingivalis* [2] and *Aggregatibacter actinomycetemcomitans* [3] are believed to be major etiologic bacteria in many cases of human periodontitis. *Prevotella intermedia* [4] has also been associated with human periodontal disease. Research at our laboratory has focused on developing a system of defense against infection on dental implant surfaces [5]. Reducing plaque has been emphasized in the prevention of periodontal diseases, including peri-implantitis [6]. It is possible that using antimicrobial materials can help reduce oral bacteria.

The loading of antimicrobial peptides onto the surface of a dental implant is an important candidate for achieving antimicrobial activity. Antimicrobial peptides, a promising new type of antimicrobial agent, offer the advantage of not easily acting as antigens against the host [7]. One of the aims of our ongoing study is to create a defense system against peri-implantitis. We are currently exploring the antimicrobial potential of peptides affixed to the surfaces of dental materials [8].

We investigated the anti-periodontopathic activity of the antimicrobial peptide, protamine and its hydrolysate. Protamine, a basic peptide [9], discovered from salmon testicles by F. Miescher in 1869 was later found to be involved in the folding of nucleic acids in salmon sperm [10]. Protamine has been isolated from more than 50 kinds of fish, and is used as a natural food preservative. Protamine has several characteristics, including high stability under heat and a preservative effect in neutral or alkaline food. It does not influence the texture, smell, or taste of food to which it is added. It can also be eaten as a raw material, and has been used as a food additive for many years. An acute toxicity test of protamine in mouse [11] and a sub-long-term toxicity test in rat [12] demonstrated its safety and confirmed that it was an excellent antibacterial agent in milt. The antibacterial activity of protamine is strongest against Gram-positive bacteria such as the Bacillus species [13] and lactobacilli [14], whereas its activity against fungi and yeast [15] is weak. Our previous study revealed that hydrolyzed protamine,

was most effective against the biofilm formation of *C. albicans* [16]. In this study, we investigated the inhibitory effect of protamine and its hydrolysate on growth of periodontopathic bacteria.

2. MATERIALS AND METHODS

2.1. Bacteria and Culture Condition

For liquid culture, *P. gingivalis* ATCC 33277, ATCC53977, W50 (ATCC, American Type Culture Collection) and *P. intermedia* ATCC 25611 were cultured in trypticase soy broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with hemin (5 g L^{-1}; Sigma Chemical Co., St Louis, MO) and menadione (0.5 g L^{-1}; Wako Pure Chemical Industries, Osaka, Japan). *A. actimomycetemcomitans* 310a and Y4 were cultured in Todd Hewitt Broth (Becton Dickinson and Company) supplemented with Yeast Extract (10 g L^{-1}; Becton Dickinson and Company). For plate culture, the bacteria were grown on blood agar plates consisting of Tryptic soy agar (Becton Dickinson and Company) supplemented with 10% defibrinated horse blood, hemin (5 g L^{-1}), and menadione (0.5 g L^{-1}). Cultures were performed at 37°C in an anaerobic chamber filled with an atmosphere of 80% N_2, 10% H_2 and 10% CO_2.

2.2. Preparation of Protamine and its Hydrolysates

Protamine (designated as Prot) was obtained from Maruha Nichiro foods, Inc., Tokyo, Japan. This product is prepared from the milt of salmon (*Oncorhynchus keta*), living in the northern part of Japan. This milt has been reported to contain four different molecular species [17] rich in arginine, the primary structures of which were reported to be as follows:

PRRRRRSSSRPIRRRRRPRASRRRRRGGRRRR,
PRRRRSSRRPVRRRRRPRVSRRRRRRGGRRRR,
PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR,
PRRRRASRRIRRRRRPRVSRRRRRGGRRRR.

The Prot consisted of 30 or 32 amino acids in this study, and the arginine residues constituted 60-70% of the amino acid sequences. Protamine hydrolysate (designated as Brom) obtained by digestion with the enzyme bromelain [18], a cysteine protease, was kindly donated by Maruha Nichiro foods, Inc.

2.3. Evaluation of Minimum Inhibitory Concentration.

Agar plates containing Prot or Brom were used to determine minimum inhibitory concentration (MIC). Peptide concentration was adjusted by stepwise dilution. Using a loop, each bacterial strain was streaked onto an agar plate. The agar plates were then incubated for 3-7 days in the anaerobic chamber at 37°C. Minimum inhibitory concentration was defined as the lowest concentration of peptides that would inhibit the visible growth of the microorganism after incubation. The values were expressed as the mean ± SD of four experiments.

2.4. Antibacterial Activity of Protamine against *P. gingivalis* ATCC 33277

P. gingivalis ATCC 33277 was anaerobically grown at 37°C to the early-stationary phase in the broth described above. The harvested cells were washed once in autoclaved water and then resuspended in freshly autoclaved water containing adequate concentrations of protamine. Cell suspensions were incubated at 37°C, and every 30 minutes the samples were examined for cell viability. Cell viability was determined by ATP-bioluminescent assay using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega, Madison, USA). Briefly, a volume of BacTiter-Glo reagent equal to the volume of each suspension was added and briefly mixed. The luminescence of the solution was then recorded by using the AUTO-LUMICOUNTER Model 1422EX (Microtec Co., LTD, Funabashi, Japan). The value obtained was expressed as the ratio to that at the start of incubation. The results were expressed as the mean ± SD of three experiments.

2.5. Statistical Analysis

Data were analyzed for statistical significance using a two-way analysis of variance (ANOVA) followed by the Scheffe test for multiple comparisons.

3. RESULTS AND DISCUSSION

As shown in **Figure 1**, protamine and its derivative showed an inihibitory effect on growth of all the periodontopathic bacteria tested, with inhibitory effect greatest on growth of the *P. gingivalis* strains. The inhibitory effect of native protamine was greater than that of its hydrolysate ($p < 0.05$). The MIC values for *P. gingivalis* ranged from 6.3×10^{-7} to 7.5×10^{-7} g L^{-1}, while those for *A. actinomycetemcomitans* and *P. intermedia* required a higher concentration, of almost double or more. Recently, we reported that protamine absorbed onto PMMA or PMMA treated with oxygen (O_2) plasma caused a marginal decrease in initial attachment of *C. albicans* [16]. In the case of *C. albicans*, the initial amount of fungal attachment to Brom- or O_2 plasma-treated PMMA diminished slightly in comparison to that treated with native peptide. These findings suggest that protamine exhibits selective inhibitory action against growth of oral microorganisms.

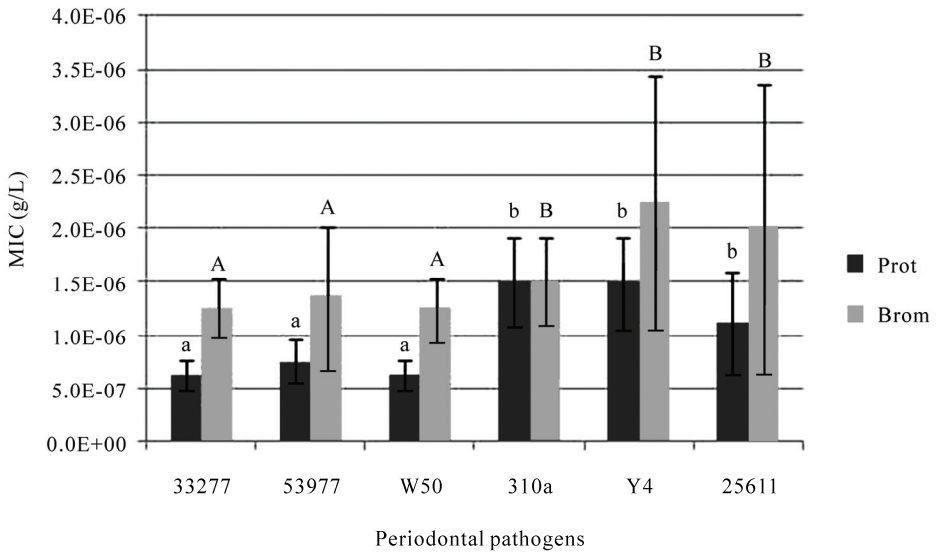

Figure 1. Minimum Inhibitory Concentration of Prot. and Brom. against periodontopathic pathogens. Identical letters indicate no significant difference ($p > 0.05$). 33277, 53977 and W50: *P. gingivalis*; 310a, Y4: *A. actinomycetemcomitans*; 25611: *P. intermedia.*

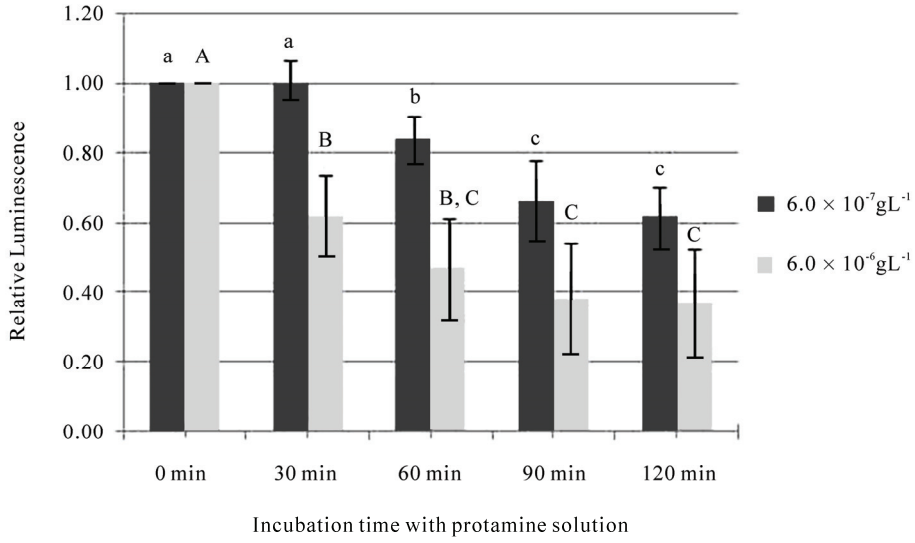

Figure 2. Influence of protamine on cell viability of *P. gingivalis* ATCC33277. Identical letters indicate no significant difference ($p > 0.05$).

To further investigate possible mechanisms of inhibition of *P. gingivalis* growth, the bactericidal activity of protamine was assessed. As shown in **Figure 2**, protamine and its derivative possessed bactericidal activity in a dose- and time-dependent manner against *P. gingivalis*. At 6.0×10^{-6} g L^{-1}, which is approximately ten-fold the value of the MIC (6.0×10^{-7} g L^{-1}), the number of *P. gingivalis* cells after two hours incubation was less than 40% of that at 30 min incubation. A higher concentration of protamine induced further inhibition of growth of *P. gingivalis*. A significant difference was observed in inhibitory action between the two concentrations used ($p < 0.01$). This indicates that this peptide has an inhibitory effect on the growth of oral bacteria. In this study, we found that protamine had an inhibitory effect on periodontal pathogens, as well as fungi. Further investigation is necessary to elucidate the properties of this peptide, for example, using immobilization methods established at our laboratory. We believe that the application of protamine to dental implants would offer ad-

vantages in the prevention of periodontal diseases such as peri-implantitis and oral care.

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