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. 
termedia ATCC 25611 were cultured in trypticase soy 
broth (Becton Dickinson and Company, Sparks, MD, 
USA) supplemented with hemin (5 g L\(^{-1}\); Sigma Chemi-
cal Co., St Louis, MO) and menadione (0.5 g L\(^{-1}\); Wako 
Pure Chemical Industries, Osaka, Japan). A. actinomy-
cetemcomitans 310a and Y4 were cultured in Todd Hew-
itt Broth (Becton Dickinson and Company) supple-
mented 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 Ma-
ruha 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:

\[ \text{Prot}: \text{PRRRRSSSPRIRRRRPRASRRRRGGRRRR,} \]
\[ \text{Protamine hydrolysate}: \text{PRRRRSSRPPRIRRRPRASRRRRGGRRRR,} \]
\[ \text{Protamine hydrolysate}: \text{PRRRRSSRPPRIRRRPRASRRRRGGRRRR,} \]
\[ \text{Protamine hydrolysate}: \text{PRRRRSSRPPRIRRRPRASRRRRGGRRRR,} \]

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 (des-
ignated 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 de-
termine minimum inhibitory concentration (MIC). Pept-
ide 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 in-
hibitory concentration was defined as the lowest concen-
tration of peptides that would inhibit the visible growth 
of the microorganism after incubation. The values were 
expressed as the mean \( \pm \) 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 auto-
claved 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 lumines-
cence of the solution was then recorded by using the 
AUTO-LUMICOUNTER Model 1422EX (Microtec Co., 
Ltd, Funabashi, Japan). The value obtained was ex-
pressed as the ratio to that at the start of incubation. 
The results were expressed as the mean \( \pm \) 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 inhibitory effect on growth of all the perio-
dontopathic bacteria tested, with inhibitory effect great-
est 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 \( 6.3 \times 10^{-7} \) to \( 7.5 \times 10^{-7} \) 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 mar-
ginal 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 di-
minished slightly in comparison to that treated with na-
tive peptide. These findings suggest that protamine ex-
hibits 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*.

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 $6.0 \times 10^{-6}$ g L$^{-1}$, which is approximately ten-fold the value of the MIC ($6.0 \times 10^{-7}$ 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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