Periodontal diseases are infectious in nature and induce an inflammatory response in the gingival and underlying supportive periodontal tissues in response to bacterial accumulation in the subgingival crevice. *Porphyromonas gingivalis*, a gram-negative black-pigmented anaerobe, has been strongly implicated as an etiologic agent of periodontitis in adults (16). The organism has been shown to produce a variety of virulence factors in both cell-associated and cell-free forms. Among them, fimbriae, fine fibrous proteinaceous appendages emerging from the cell surface, are thought to be critical factors in the adherence of the organism to the gingival margin (4). Purified fimbriae have been reported to bind to epithelial cells, fibroblasts, human salivary components, collagen, and fibronectin (1, 4, 11–13). We previously purified an arginine-specific cysteine protease (RC-protease) from *P. gingivalis* 381. The purified enzyme was found to enhance fimbrial binding to fibroblasts or extracellular matrix (ECM) proteins. The protease exposed a cryptic receptor on the host matrix proteins, (X)_n–Arg-COOH, and this site increased fimbrial binding to the host proteins. We have simultaneously found that addition of L-Arg, or oligopeptides containing Arg residues at the C terminus, clearly inhibit fimbrial binding to cryptic receptors (11, 12). In this regard, it should be noted that RC-protease appears to be very similar to Arg-gingipain, encoded by rggB, as reported by Nakayama et al. (15), in terms of molecular size, amino-terminal sequence, and some enzymatic properties. Thus, these findings suggest that RC-protease of *P. gingivalis* plays an important role in adherence of the organism to host tissue.

The rationale of this study was to seek natural peptides rich in the Arg residue to obtain good inhibition of *P. gingivalis* RC-protease. Should such agents be found and used on a wide scale, elimination or reduction in the numbers of *P. gingivalis* would eventually be possible.

Protamines are found in fish spermatozoan nuclei in large quantities, where their presence appears to compact DNA. Protamines are highly basic peptides (pI > 10) due to a high arginine content (21 arginines out of 32 amino acids) (2). In our survey of RC-protease inhibitors from natural resources, we found that protamines exhibited a marked inhibitory effect on protease activity. Therefore, we examined the inhibitory mechanisms of protamines for the proteolytic activity of RC-protease and the interaction of fimbriae with immobilized fibronectin treated with RC-protease.

**Inhibitory effect of protamines on the protease activity of *P. gingivalis*.** RC-protease was extracted from cell paste by sonication in phosphate buffer containing 0.2% Triton X-100 and was purified by column chromatography, as described previously (12). The proteolytic activity of the purified RC-protease (7 U/ml; specific activity, 54 U/mg) was determined with benzoyl-L-arginine 4-methyl-coumaryl-7-amide, as described previously (12). Inhibitory effects of various reagents on proteolytic activity were measured by using a 300 μM concentration of a synthetic chromogenic substrate, benzoyl-L-arginine-p-nitroanilide (Bz-L-Arg-pNA) (Peptide Inst., Minoo-Osaka, Japan), in 12.5 mM sodium phosphate buffer (pH 7.5) containing 375 mM CaCl₂ and 560 mM cysteine (1-m final volume). After an appropriate incubation period at 37°C, the amount of released p-nitroanilide (pNA) was colorimetrically read at 405 nm. Among various protamines, salmon (composed of 32 amino acid residues: PRRRRSSSVPRVRRRRVRVRRRRRVRGRRR) from salmon and clupeine (31 residues: ARR RRRSSPRRRRRRVRTRRRAAGRRR) from herring were obtained from Sigma Chemical Co. (St. Louis, Mo.). They were dissolved in distilled water and used immediately after filtration (pore size, 0.2 μm). Chymostatin, a chymotrypsin inhibitor, and pepstatin, an aspartic-protease inhibitor, were purchased from Peptide Inst. and used as control enzyme inhibitors. As shown in Fig. 1, protamines were found to markedly inhibit the proteolytic activity of RC-protease, and a 50% inhibitory effect was observed at a 60 μM concentration of each protamine. The inhibitory levels were significantly higher than those of pepstatin when ≥30 μM concentrations of protamines or pepstatin were used.

**Inhibitory effects of the protamines against RC-protease activity were also demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** Proteolytic activities on human proteins such as plasma fibronectin (Sigma) were examined. These native proteins (100 μg) dissolved in 1 ml of 20 mM sodium phosphate buffer (pH 7.5) containing 1.0 mM CaCl₂ and 1.0 mM cysteine with or without protamines (100 μg/ml [salmine or clupeine]) were mixed with RC-protease (1 mU) and incubated at 37°C for 1 h. The reac-
tion mixtures were then analyzed by SDS-PAGE, and the proteins were stained with Coomassie brilliant blue (Sigma). Degraded peptides from native proteins were visualized as bands with smaller molecular sizes than those of the intact molecules (Fig. 2). When fibronectin or collagen was treated with R. protease in the presence of salmine or clupeine (100 \( \mu g/mL \)), these proteins remained intact.

**Fimbrial binding to immobilized protamines or fibronectin.** Fimbriae were prepared from *P. gingivalis* 381 cells, as described previously (11). The purified fimbriae (10 mg) were biotinylated by incubation with 1.2 mg of biotin-N-hydroxysuccinimide (Calbiochem, La Jolla, Calif.) in 11 ml of 100 mM NaH2CO3 (pH 8.0) at 25°C for 2 h, followed by dialysis against phosphate-buffered saline, pH 7.4 (PBS). Various concentrations of protamines (100 \( \mu l \) of 0 to 100 \( \mu g/mL \) in PBS) were added to microtiter wells (Maxisorp; Nalge Nunc International) and left overnight at 4°C for immobilization. After

![FIG. 1. Inhibitory effect of protamines (salmine or clupeine) against *P. gingivalis* RC-protease. Proteolytic activity was measured by using the synthetic chromogenic substrate Bz-Arg-pNA (300 \( \mu M \)) in 12.5 mM sodium phosphate buffer (pH 7.5) containing 375 \( \mu M \) CaCl2, 560 \( \mu M \) cysteine, and protamines (0 to 1,000 \( \mu g/mL \)). After an appropriate incubation period at 37°C, the amount of released pNA was read colorimetrically at 405 nm. Values are presented as means ± standard deviations of triplicate assays performed on three separate occasions.](image1)

![FIG. 2. SDS-PAGE profile of collagen and fibronectin with or without pretreatment of *P. gingivalis* RC-protease. Protein specimens were incubated at 37°C for 1 h in the presence or absence of RC-protease, and the protein profile of each reaction mixture was determined by SDS-PAGE followed by staining with Coomassie brilliant blue. Lanes A, reaction mixture in the absence of RC-protease and protamines; lanes B, reaction mixture in the presence of RC-protease (1 mU/ml) and in the absence of protamines; lanes C, reaction mixture in the presence of RC-protease (1 mU/ml) and salmine (100 \( \mu g/mL \)); lanes D, reaction mixture in the presence of RC-protease (1 mU/ml) and clupeine (100 \( \mu g/mL \)).](image2)

![FIG. 3. Binding of biotinylated fimbriae to immobilized protamines. After immobilization of salmine or clupeine to wells, biotinylated fimbriae (0.7 \( \mu g \)) were added to the wells and incubated. The amount of fimbrial bound was colorimetrically determined by using streptavidin-alkaline phosphatase. Values are means ± standard deviations (SD) of triplicate assays performed on three separate occasions. SD were within ±5% of the means.](image3)

![FIG. 4. Inhibitory effect of salmine and clupeine on binding of fimbriae to immobilized fibronectin with treatment of *P. gingivalis* RC-protease. After immobilization of fibronectin to wells, biotinylated fimbriae (0.7 \( \mu g \)) were added with the protease (7 mU/ml) and salmine or clupeine (0 to 300 \( \mu g/mL \) in PBS) to the wells. The amount of bound fimbriae was colorimetrically determined by using streptavidin-alkaline phosphatase. Results are expressed relative to the fimbrial binding of the control (without RC-protease treatment or protamines). Values are means ± standard deviations of triplicate assays performed on three separate occasions.](image4)
being washed with PBS three times, biotinylated fimbriae (0.7 µg/100 µl) were added to the wells and incubated for 0.5 h. The amount of bound fimbriae was colorimetrically determined after washing of the wells with PBS. As shown in Fig. 3, fimbriae were found to clearly bind to the immobilized salmine and clupeine. The amount of fimbrial binding increased as the concentrations of protamines used for immobilization increased. Fimbrial binding to bovine serum albumin was negligible.

We then examined the effect of protamines (0 to 300 µg/ml) on the binding of biotinylated fimbriae to immobilized fibronectin that had been treated with RC-protease. Aliquots (100 µl) of human fibronectin solution (20 µg/ml) (Koken Co.) were added to microtiter wells and left overnight at 4°C for immobilization of the protein. After the wells were washed extensively with PBS, biotinylated fimbriae (0.7 µg/100 µl) and increasing concentrations of protamines were added to the wells with or without RC-protease (7 mU/ml) in PBS containing 1 mM CaCl₂ and 1 mM cysteine, followed by incubation for 1 h at 37°C. The amount of bound fimbriae was determined as described above. RC-protease treatment markedly enhanced the binding of fimbriae to the immobilized fibronectin (Fig. 4). Both salmine and clupeine were found to inhibit the enhanced fimbrial binding to fibronectin treated with RC-protease was obtained at approximately a 15 µM concentration of protamines, and the maximum inhibition reached about 60% at a protamine concentration of 150 µM.

Kinetic analyses of the inhibitory effect of protamines on RC-protease. Kinetic analyses were then carried out to demonstrate the mechanism of inhibition of protamines against RC-protease. Briefly, Bz-L-Arg-pNA was added at final concentrations of 2, 4, and 8 µM to the assay mixture composed of 12.5 mM sodium phosphate buffer (pH 7.5), 375 µM CaCl₂ and 560 µM cysteine, followed by incubation at 37°C for 5 min, the reaction was started by the addition of RC-protease (7 mU/ml final concentration). The amount of released pNA was read by the change in absorbance at 405 nm. All assays were done in triplicate, and mean values were used for determination of $K_m$ and $K_i$ values.
mined from Lineweaver-Burk plots to be 0.2 to 0.4 μM (Fig. 5B and D). These results showed that protamines were competitive inhibitors to the substrate, Bz-L-Arg-pNA.

Protamines present in fish spermatozoan nuclei are a group of highly basic peptides with an isoelectric point of 10 to 13 because of a high Arg content (1). Various protamines have been reported to exhibit antibacterial activity (5, 8, 9), which may be ascribed to their polycationic nature. Protamines also modify cellular functions in the periplasmic spaces of gram-positive and gram-negative bacteria; however, their effects on gram-negative bacteria appear to be less significant (7, 21).

This may be due to the effective barrier function of the outer membrane (14). At the same time, protamines are suggested to penetrate gram-negative bacteria and increase the permeability of the outer membrane (17, 18). The antibacterial effects of these polycationic peptides are due to their ability to form channels in the cytoplasmic membrane. The action of these peptides may result in uncoupling of electron transport, reduction of the electromotive force, or lowering of the pH gradient across the cell envelope (10).

In this study, protamines, i.e., salmine and clupeine, were found to effectively inhibit the proteolytic activity of RC-protease. It was also shown that these protamines exhibit a clear inhibitory effect on fimbrial binding to host matrix proteins treated with RC-protease. RC-protease is thought to be an important virulence factor of this organism and to be involved in degradation of ECM proteins such as collagen and fibronectin (3). We have previously reported that RC-protease exposes a cryptic receptor in the host matrix proteins, which should play a significant role in the initial attachment of the organism to host tissues through the fimbriae (11, 12). The participation in this step of C-terminal Arg residues of ECM proteins in the binding of P. gingivalis fimbriae has been demonstrated.

In conclusion, this study shows that fimbriae having a strong affinity for the Arg residue may participate in cell-to-surface interactions of P. gingivalis and that protamines have multiple functions, i.e., interaction with fimbriae, inhibition of RC-protease, and fimbrial binding. Polyamines found in gingival fluid, which have been reported to play a local role in modulating the fibronectin-binding proteins. Infect. Immun. 64:756–762.


