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Cysteine Protease of *Porphyromonas gingivalis* 381 Enhances Binding of Fimbriae to Cultured Human Fibroblasts and Matrix Proteins

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It has been shown that *Porphyromonas gingivalis* 381, a suspected periodontopathogen, possesses fimbriae on its cell surface. The organism is also known to produce proteases which can degrade the host cell surface matrix proteins. In this study, we investigated the effect of protease on the binding of the purified *P. gingivalis* fimbriae to cultured fibroblasts or matrix proteins. A protease that can hydrolyze benzoyl-L-arginine *p*-nitroanilide was obtained from *P. gingivalis* 381 cells by sonication in phosphate-buffered 0.2% Triton X-100 and was purified by column chromatography. The molecular size of the protease was estimated to be 55 kDa by gel filtration or 47 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The enzyme activity was markedly inhibited by sulfhydryl reagents, antipain, and leupeptin. The protease degraded various host proteins, including collagen and fibronectin, and cleaved the COOH terminus of the arginine residue in peptides such as benzoyl-L-arginine *p*-nitroanilide. However, *P. gingivalis* fimbriae were not degraded by this protease activity. The enzyme activity was enhanced in the presence of reducing agents or $CaCl_2$. When cultured fibroblasts were partially treated with the protease, the binding of the purified *P. gingivalis* fimbriae to the fibroblast monolayer was increased significantly. However, this enhancing effect was suppressed upon the addition of antipain and leupeptin. Similarly, binding of the fimbriae to the collagen or fibronectin immobilized on the microtiter wells was also enhanced. Addition of these host matrix proteins efficiently inhibited the binding of fimbriae to the fibroblast monolayer. The binding assay of fimbriae using dipeptidyl ligand affinity column chromatography demonstrated a clear interaction between fimbriae and the arginine residue. Taken together, these results indicate that the *P. gingivalis* protease at least partially degrades the host matrix proteins, which, in turn, may lead to an increased exposure of the cryptic ligands that can result in enhanced fimbria-mediated binding of this organism to periodontal tissues.

Porphyromonas gingivalis, a black-pigmented gram-negative anaerobe, has been recognized as an important pathogenic organism in adult periodontitis, a chronic inflammatory disease (23). *P. gingivalis* possesses fine fibrillar proteinaceous appendages, fimbriae, which are arranged peritrichously, emerging from the cell surface (14). Bacterial adherence to the host tissue is an essential step in the initiation of infectious diseases, and an adhesive function held by many pathogenic bacteria is now well recognized and documented (29). It has been suggested that *P. gingivalis* fimbriae are involved in adherence to host cells (16, 26, 30).

P. gingivalis produces various enzymes such as protease, collagenase, gelatinase, peptidase, and hyaluronidase, which are now recognized as virulence factors of this organism. Among these enzymes, proteolytic enzymes are thought to be most important, since they degrade host tissue proteins (27), plasma proteinase inhibitors such as α-1-antitrypsin and α_2-macroglobulin (4), immunoglobulins (12, 24, 35), complement components (43), and key factors of the plasma proteinase cascade systems (28), which may perturb the host defense mechanism. In addition, proteases from *P. gingivalis* also degrade basement membrane components such as collagens (3, 34, 40) and fibronectin (36, 41). Furthermore, proteases from *P. gingivalis* may initiate inflammatory reactions in gingival fibroblasts through activation of proteinase and collagenase derived from host cells (38, 41). These findings suggest that proteases from *P. gingivalis* may play an important role in the progression of periodontitis.

That there is a positive correlation between the protease activity and the adherence of *P. gingivalis* is a unique concept that may have been suggested by some experimental findings wherein pretreatment of epithelial cells with trypsin, papain, chymotrypsin, or neuraminidase significantly enhanced the adherence of *P. gingivalis* cells (6, 8, 9, 25). *P. gingivalis* protease may also contribute to coaggregation with *Actinomyces viscosus* cells, since the protease-defective mutant of *P. gingivalis* did not demonstrate a coaggregating reaction (22). Furthermore, it was found that strains of *P. gingivalis* exhibiting high levels of trypsin-like protease activity adhered more efficiently to human erythrocytes and epithelial cells than strains with lower levels of activity (10).

In this study, we have purified and characterized a cell-associated protease from *P. gingivalis* and examined its effect on the molecular binding of *P. gingivalis* fimbriae.

MATERIALS AND METHODS

Bacteria. *P. gingivalis* 381 was grown in GAM broth (Nissui, Tokyo, Japan) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) in an anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2) at 37°C for 3 days.

Purification and characterization of protease. Unless stated otherwise, all steps in the purification of *P. gingivalis* protease were carried out at 4°C, and the enzyme activity was quantitated by measuring benzoyl-L-arginine *p*-nitroanilide (Bz-L-Arg-*p*NA; Peptide Institute, Osaka, Japan) cleaving activity. Organisms were harvested from 12-liter batch cultures by centrifugation at 9,000 × *g* for 20

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min, washed with phosphate-buffered saline (PBS; pH 7.4), and resuspended in 400 ml of PBS containing 0.2% Triton X-100 and 1 mM $CaCl_2$. The bacterial suspension was sonicated at 180 W for 5 min in ice with a model W-375 ultrasonicator (Heat Systems-Ultrasonics Inc., New York, N.Y.). After cell debris was removed by centrifugation, the supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.0). The dialyzed sample was applied on an Arginine Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) column (2.8 by 25 cm) which had been equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The column was washed extensively with the same buffer and was eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer to release bound proteins. Protease fractions hydrolyzing Bz-L-Arg-*p*NA were collected, combined, and dialyzed against 10 mM sodium phosphate buffer (pH 6.0).

The dialyzed protease sample was then applied on a DEAE Sepharose CL-6B (Pharmacia LKB Biotechnology) column (2.8 by 30 cm) that had been equilibrated with 10 mM sodium phosphate buffer (pH 6.0). After the column was washed with the same buffer (500 ml), protease was released by a linear gradient elution of 0 to 0.5 M NaCl in the buffer. Fractions exhibiting Bz-L-Arg-*p*NA hydrolyzing activity were combined (total volume, 120 ml), and aliquots of the protease were stored at −80°C until further purification.

The protease sample (1 ml) was applied to a HiLoad 16/60 Sephacryl S-200HR column (Pharmacia LKB Biotechnology) equilibrated with PBS. Glutamate dehydrogenase (290 kDa), lactate dehydrogenase (146 kDa), ovotransferrin (77 kDa), enolase (67 kDa), adenylate kinase (32 kDa), carbonic anhydrase (30 kDa), myoglobin (17.2 kDa), cytochrome *c* (12.4 kDa), and phenol red were used as molecular mass markers. The total volume (V_t) of the column was calibrated by using phenol red as a marker, and the relative elution volume (V_e/V_t) was utilized for molecular mass estimation. Fractions hydrolyzing Bz-L-Arg-*p*NA were applied on a HiTrap Q (1 ml) column (Pharmacia LKB Biotechnology) equilibrated with 10 mM sodium phosphate buffer (pH 6.0) and washed with 20 column volumes of the buffer. The bound proteins were released with a linear gradient elution of 0 to 1.0 M NaCl in the buffer. Gel filtration and HiTrap Q ion exchange chromatography was then carried out, and the active fractions were used as the purified protease preparation.

Amino-terminal sequence analysis of the purified protease was performed with a protein sequencer model PSQ-1 (Shimadzu, Kyoto, Japan), using the program provided by the manufacturer.

Enzyme assays. The proteolytic activity of each chromatographic fraction was measured by using the synthetic chromogenic substrate Bz-L-Arg-*p*NA (300 μM) in 12.5 mM sodium phosphate buffer (pH 7.5) containing 375 μM $CaCl_2$ and 560 μM cysteine. After an appropriate incubation period at 37°C, the amount of released *p*NA was read colorimetrically at 405 nm. The enzyme activity unit was determined by using benzoyl-L-arginine 4-methyl-coumaryl-7-amide (Bz-L-Arg-MCA; Peptide Institute) as a substrate. One unit of protease activity was defined as the amount of enzyme releasing 1 μmol of 7-amino-4-methyl-coumarin (AMC) per min, which was measured at 460 nm by excitation at 355 nm with a Titertek Fluoroskan II (Labsystems, Helsinki, Finland). The substrate specificity was also determined by using various AMC-conjugated peptides (Peptide Institute) at a concentration of 10 μM.

Hydrolysis of matrix proteins by the protease. The proteolytic activity against native proteins, such as purified fimbriae from *P. gingivalis*, bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), plasma fibronectin (Chemicon International Inc., Temecula, Calif.), and type I collagen from placenta (Becton Dickinson Labware, Bedford, Mass.), was determined; these native proteins (10 μg) dissolved in 100 μl of 75 mM sodium phosphate buffer (pH 7.5) containing 1.0 mM $CaCl_2$ and 1.5 mM cysteine were mixed with the protease and incubated at 37°C for 30 min. Each reaction mixture was then examined for its protein profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the description of Laemmli (19). The protein bands developed by SDS-PAGE were stained with Coomassie brilliant blue (Sigma).

Binding of fimbriae to fibroblasts or various immobilized proteins. Fimbriae were detached from *P. gingivalis* 381 cells and purified chromatographically as described previously (14, 15, 45). The purified fimbriae (10 mg) were incubated with 1.2 mg of biotin-*N*-hydroxysuccinimide (Calbiochem, La Jolla, Calif.) in 11 ml of 100 mM NaH_2CO_3 (pH 8.0) at 25°C for 2 h; this was followed by dialysis against PBS. The product was used as biotinylated fimbriae for colorimetric determination of the binding of fimbriae to the following proteins: collagen from calf skin (Sigma), fibronectin from bovine plasma (Sigma), laminin from basement membrane of Engelbreth-Holm-Swarm Mouse sarcoma (Sigma), and β-lactoglobulin from bovine milk (Sigma). These proteins (0 to 1,000 μg/ml of PBS) were added to microtiter wells and left overnight at 4°C for immobilization of proteins. After incubation, the wells were washed extensively with PBS. Biotinylated fimbriae (0.7 μg) were then added to each well and incubated for 30 min at 25°C. The amount of bound fimbriae was determined colorimetrically with streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Ala.).

Normal human dermal fibroblasts (Kurabou, Osaka, Japan) were grown to confluent culture in serum-free F-GM medium (Kurabou). Trypsin-EDTA-treated fibroblast cells were seeded in flat-bottomed culture plate wells at a concentration of 5.0×10^3 cells per well. After incubation at 37°C in a 5% CO_2 atmosphere for 2 days, the monolayer of fibroblasts was washed twice with PBS, and biotinylated fimbriae (0.7 μg) were added to the wells in the presence of matrix proteins, e.g., fibronectin, collagen, and laminin (25 μg), in 100 μl of PBS. The biotinylated fimbria solution containing bovine serum albumin was used as a control solution. Bound fimbriae were quantitated as described above.

The effect of *P. gingivalis* protease on the binding of biotinylated fimbriae to the fibroblast monolayer or immobilized matrix proteins was then determined. The monolayer of cultured fibroblasts was washed twice with PBS and treated with purified *P. gingivalis* protease (0.001 U/ml) in PBS containing 1 mM $CaCl_2$–1 mM cysteine in the presence or absence of various protease inhibitors for 30 min at 37°C. After being washed three times with PBS, biotinylated fimbriae (0.7 μg) were added to each well and incubated for 30 min at 25°C. Similar experiments were carried out with the collagen- or fibronectin-coated wells instead of the monolayer of cultured fibroblasts. To determine the amount of fimbriae bound to the fibroblasts, we prepared ^{125}I-labeled fimbriae. Briefly, fimbriae (10 μg) and Bolton-Hunter reagent (100 μCi; CAL-RAD Co., Santa Ana, Calif.) were dissolved in 20 mM sodium phosphate buffer (pH 8.0) to a total volume of 30 μl. After 30 min of incubation, 0.5 M glycine (50 μl) was added. The ^{125}I-labeled fimbriae were purified by gel filtration on a NICK column prepacked with Sephadex G-50 (Pharmacia LKB Biotechnology).

Binding of fimbriae to various dipeptides. Binding of fimbriae to dipeptidyl ligands was also determined by using HiTrap affinity chromatography. Glycyl-glycine, glycyl-arginine, glycyl-lysine, glycyl-histidine, glycyl-glutamate, glycyl-glutamine, glycyl-alanine, glycyl-leucine, glycyl-phenylalanine, and glycyl-serine (Kokusan Chemical Works, Tokyo, Japan) were coupled to biotin-*N*-hydroxy-succinimide-activated HiTrap columns (1.0 ml; Pharmacia) according to the manufacturer's instructions. These columns were equilibrated with 10 mM Tris-HCl buffer (pH 7.8). Fimbriae were loaded (25 μg each) on these columns and washed with the buffer; this was followed by linear gradient elution of 0 to 0.5 M NaCl in the same buffer. The effluents were monitored for protein by measuring A_{280}.

Statistical analysis. All data presented are means ± standard deviations (SD). Statistical significance was assessed with the use of analysis of variance and is shown where appropriate.

TABLE 1. Purification of the protease from *P. gingivalis* cell extracts

Purification step	Total amt (mg) of protein	Total activity (U)[a]	Sp act (U/mg)	Purification (fold)	Yield (%)
Arg Sepharose	123	140	1.1	1	100
DEAE Sepharose	23	107	4.7	4.3	76
Sephacryl S-200	0.70	32	46	42	23
HiTrap Q	0.48	26	54	49	19

[a] The enzyme units were determined as described in Materials and Methods by using Bz-L-Arg-MCA as the substrate. One unit is defined as the amount of enzyme releasing 1 μmol of AMC in 1 min at 37°C.

RESULTS

Biochemical characterization of *P. gingivalis* protease. The sonic extract of *P. gingivalis* 381 cells was found to exhibit strong Bz-L-Arg-*p*NA hydrolyzing activity. The proteases demonstrating this activity were purified by column chromatography with Arginine Sepharose 4B, DEAE Sepharose CL-6B, Sephacryl S-200HR, and HiTrap Q (Table 1). A gel filtration profile of the purified protease indicated that the Bz-L-Arg-*p*NA hydrolyzing activity product was eluted with an apparent molecular mass of 55 kDa. On the other hand, SDS-PAGE analysis of the same enzyme preparation gave a single band, indicating a molecular mass of 47 kDa. Amino-terminal analysis of the protease gave a sequence of Y-T-P-V-E-E-K-E-N-G-R-M-I-V-I-V-A-K-K-Y.

The enzyme activity was enhanced severalfold by the addition of cysteine and/or divalent cations such as Ca^{2+}, Mg^{2+}, or Mn^{2+}, and the following enzyme assays were done in the presence of cysteine and Ca^{2+}. The enzyme exhibited maximum activity in the pH range of 7.3 to 7.7 and in the temperature range of 30 to 50°C. Its activity was not affected significantly in the presence of 0.1% SDS or 8 M urea, whereas the activity was inhibited upon the addition of Cu^{2+}. Among a series of class-specific inhibitors, sulfhydryl-reactive reagents were moderately effective (Table 2), indicating that this proteolytic enzyme was classified as cysteine protease. Furthermore, the pro-

TABLE 2. Effect of various inhibitors on the protease[a]

Inhibitor (class inhibited)	IC_{50}[b]
Class-specific	
p-Chloromercuribenzoic acid (cysteine)	110 μM
N-Ethylmaleimide (cysteine)	310 μM
Iodoacetamide (cysteine)	315 μM
EDTA (metallo)	460 μM
Phenylmethanesulfonyl fluoride (serine)	1 mM
Diisopropyl fluorophosphate (serine)	11 mM
Peptide and protein	
Leupeptin	2 nM
Antipain	9 nM
Nα-tosyl-L-lysyl chloromethyl ketone	20 nM
Chymostatin	500 nM
N-tosyl-L-phenylalanyl chloromethyl ketone	920 nM
α-1-Antitrypsin	35 μM
Elastatinal	44 μM
Bowman-Birk inhibitor (BBI)	78 μM
trans-Epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64)	105 μM
Aprotinin	480 μM
Ac-Leu-Leu-Nle[c]-al	2 mM
Ac-Leu-Leu-Met-al	>2 mM
Pepstatin	>2 mM

[a] Inhibition of MCA release from Bz-L-Arg-MCA by highly purified *P. gingivalis* protease in the presence of inhibitor was determined fluorometrically.
[b] IC_{50}, the concentration of inhibitor that inhibits 50% of the protease activity under the assay condition as described in Materials and Methods.
[c] Nle, norleucine.

tease was strongly inhibited by antipain, leupeptin, and Nα-tosyl-L-lysyl chloromethyl ketone (TLCK). Chymostatin and *N*-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK) moderately inhibited the activity. However, other inhibitors, including α-1-antitrypsin, were less effective against the enzyme (Table 2). These results indicated that arginine- or lysine-containing peptides would be very effective in inhibiting the protease activity. Various synthetic substrates were examined for hydrolysis by *P. gingivalis* protease. It was found that specific cleavage occurred at the COOH-terminal side in the arginine residue of these peptides, except with Z-Arg-Arg-MCA and Arg-MCA (Table 3).

The hydrolyzing activity of the enzyme was assessed by degradation of native proteins (Fig. 1). The degraded peptides were visualized as bands with smaller molecular sizes by SDS-PAGE. As shown in Fig. 1, the protease was found to degrade bovine serum albumin, fibronectin, and type I collagen into small, fragmentary peptides. It was also noted that *P. gingivalis* fimbriae were not affected by the proteolytic activity of the enzyme under these conditions.

Effect of the protease on the binding of fimbriae to fibroblasts or matrix proteins. Our experiments indicated that ^{125}I-labeled fimbriae bound to cultured fibroblasts at 0.24 pg per cell. We then determined colorimetrically if biotinylated fimbriae would bind to matrix proteins immobilized on the microtiter wells. Collagen, fibronectin, and laminin were found to bind biotinylated fimbriae, while β-lactoglobulin was not (Fig. 2). The fimbrial binding to the monolayer of fibroblasts was effectively inhibited by the addition of collagen, fibronectin, or laminin in the assay mixture (Fig. 3), suggesting that fimbrial binding to the fibroblasts occurred through cell surface matrix proteins such as collagen, fibronectin, and laminin. The effect of the purified *P. gingivalis* protease on the adherence of fimbriae to fibroblasts was then examined. The pretreatment of the fibroblast cultures with the protease markedly enhanced the binding of biotinylated fimbriae to the fibroblast monolayer (Fig. 4A), although no significant changes in the number of cells, cell morphology, or viability were observed (data not shown). This effect was abrogated by heat inactivation of the protease or addition of protease inhibitors such as antipain, leupeptin, and TLCK (Fig. 4A). Similar enhanced bindings of the biotinylated fimbriae to the collagen or fibronectin immobilized on microtiter wells were obtained; Fig. 4B shows the results obtained when collagen was used. In addition, among 11 amino acids, L-Arg was found to inhibit significantly the binding of fimbriae with or without pretreatment of protease. The inhibitions occurred dose dependently (Table 4).

Binding of fimbriae to peptide ligands containing arginine residue. Since the surface matrix proteins of the fibroblasts were degraded by the arginine-specific protease, it could be possible that COOH-terminal arginine would be exposed from the matrix protein. To determine the validity of this hypothesis, various dipeptides ligated with Sepharose beads were examined for their ability to bind to the fimbriae. The fimbriae did not bind to glycyl-glycine, glycyl-lysine, glycyl-histidine, glycyl-glutamate, glycyl-glutamine, glycyl-alanine, glycyl-leucine, glycyl-phenylalanine, and glycyl-serine; only glycyl-arginine ligand demonstrated an affinity for fimbriae. Figure 5 shows the typical elution profiles, indicating that only the affinity column with the glycyl-arginine ligand retained the fimbriae and that fimbriae were eluted from the column by increasing the molarity of NaCl in the elution buffer to 0.2 M.

DISCUSSION

Ample evidence has indicated that strains of *P. gingivalis* produce three types of protease activities: trypsin-like activity (11, 17), collagenase activity (2, 21, 37), and peptidase activity

TABLE 3. Hydrolysis of various synthetic peptidyl aminomethyl coumarins by the *P. gingivalis* protease

Substrate[a]	Relative hydrolyzing activity
Bz-Arg-MCA	100[b]
Boc-Asp(OBzl)-Pro-Arg-MCA	30
Pro-Phe-Arg-MCA	164
Z-Phe-Arg-MCA	112
Z-Arg-Arg-MCA	12
Boc-Gln-Gly-Arg-MCA	119
Boc-Phe-Ser-Arg-MCA	166
Boc-Ile-Glu-Gly-Arg-MCA	70
Boc-Leu-Ser-Thr-Arg-MCA	131
Boc-Gln-Ala-Arg-MCA	116
Glt-Gly-Arg-MCA	93
Bz-Lys-MCA	0
Boc-Val-Leu-Lys-MCA	2
Boc-Glu-Lys-Lys-MCA	0
Gly-Pro-MCA	2
Suc(OMe)-Ala-Ala-Pro-Val-MCA	0
Suc-Ala-Pro-Ala-MCA	0
Suc-Ala-Ala-Ala-MCA	0
Suc-Ala-Ala-Pro-Phe-MCA	0
Suc-Leu-Leu-Val-Tyr-MCA	0
Arg-MCA	2
Lys-MCA	0
Ala-MCA	0
Leu-MCA	0

[a] Some substituent groups in the substrates are abbreviated as follows: Bz, benzoyl; Bzl, benzyl; Boc, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; Suc, succinyl (3-carboxypropionyl); MCA, 4-methyl-coumaryl-7-amine.
[b] The protease activity releasing AMC from Bz-Arg-MCA was defined as 100.

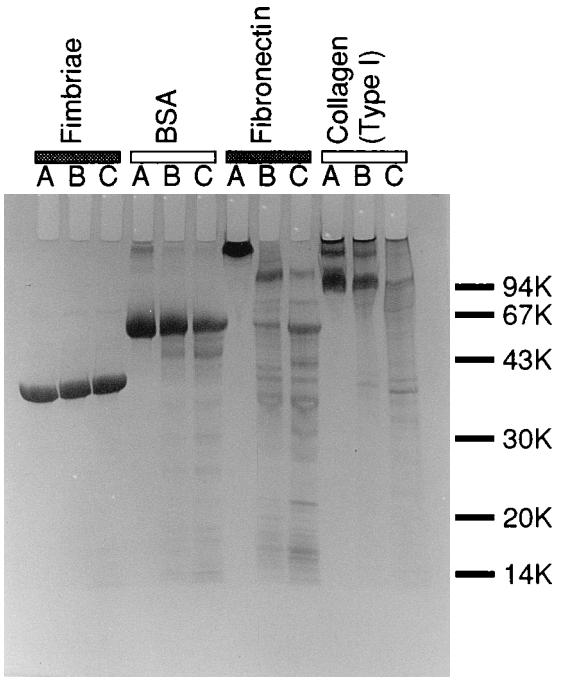

FIG. 1. SDS-PAGE profile of *P. gingivalis* fimbriae and various proteins with or without pretreatment of *P. gingivalis* protease. Protein specimens were incubated at 37°C for 30 min in the presence or absence of *P. gingivalis* protease, and the protein profile of each reaction mixture was determined by SDS-PAGE. The protein bands were stained with Coomassie brilliant blue. Lanes A, reaction mixture in the absence of the protease; lanes B, reaction mixture in the presence of 0.027 U of protease per ml; lanes C, reaction mixture in the presence of 0.08 U of protease per ml.

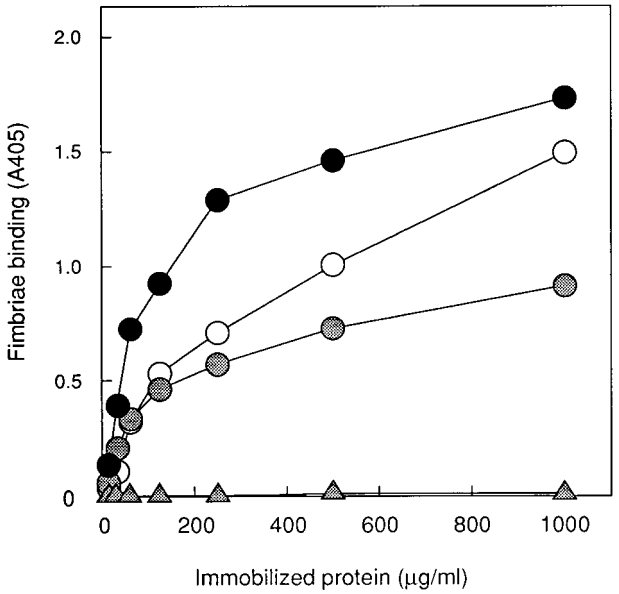

FIG. 2. The binding of the biotinylated fimbriae to various proteins immobilized on microtiter wells. Proteins (0 to 1,000 μg/ml) were added to the wells and left overnight at 4°C. After wells were washed, biotinylated fimbriae (0.7 μg) were added to the wells and incubated. The amount of the bound fimbriae was determined colorimetrically. Immobilized proteins used collagen (○), fibronectin (▨), laminin (●), and β-lactoglobulin (△). Values are the means ± SD of five determinations per experiment. Three separate experiments showed similar results, and a representative result is shown here. The SD values were within ±5% of the mean value.

(1, 13, 39). These proteolytic activities of this gram-negative anaerobic species have been recognized as important attributes in the pathogenesis of periodontal diseases. These proteases may play significant roles in the development of periodontal disease by degrading the connective tissues (3, 34, 40), inactivating components involved with the host defense mechanisms (4, 12, 24, 35), activating the complement systems (43), activating the kallikrein cascade (28), or disrupting the functions of the polymorphonuclear leukocytes (44). However, the precise role of these proteases is still not clearly understood.

In this study, we have identified a strong proteolytic activity in the sonic extract of organisms of *P. gingivalis*. This cell-associated protease was found to be an arginine-specific cysteine protease. Our sequence analysis of the amino-terminal portion of the enzyme strongly suggested that the enzyme is very similar to Arg-gingipain, which has been reported recently by other investigators (5, 31–33). Our cell-associated protease reported here and Arg-gingipain were identical except for 1 residue among the first 20 amino acid sequences, i.e., the eighth amino acid residue from the N-terminal side of these enzymes, glutamic acid and glutamine, respectively. It is known that the Glu-to-Gln conversion may occur by one point mutation of the genetic codon. In this regard, Fujiwara et al. (7) reported that there are considerable differences among strains of *P. gingivalis* in the nucleotide sequence of the *fimA* gene that encodes fimbrillin. It is likely that the difference in the amino-terminal sequence between our cell-associated cysteine protease and Arg-gingipain can be ascribed to a genetic polymorphism among strains, as has been seen with other cellular proteins of this species (14). It has been suggested that Arg-gingipain is synthesized as a polyprotein that contains adhesion molecules at the C-terminal side (31), and multiple forms of Arg-gingipain are found in the culture supernatant (i.e., 95- and 50-kDa proteins) and are cell-associated forms (i.e., 110- and 70- to 90-kDa forms) (33). It was found that our protease was very similar to the 50-kDa form of Arg-gingipain that has been found predominantly in culture supernatant (33). Furthermore, our enzyme and Arg-gingipain shared a number of similar properties, i.e., optimum pH, molecular weight, sub-

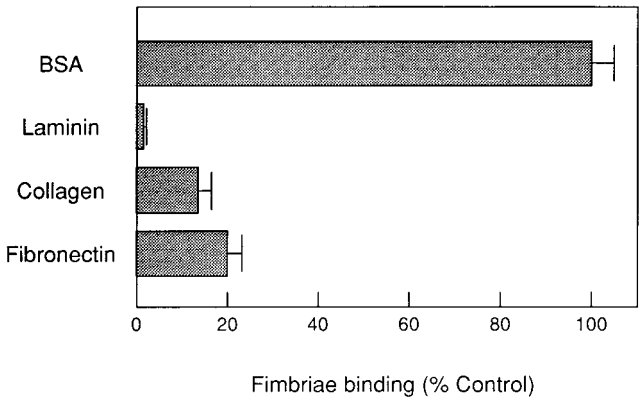

FIG. 3. Effect of the matrix proteins on the binding of fimbriae to the monolayer of the cultured fibroblasts. The monolayered fibroblasts in the microtiter wells were washed twice with PBS, and biotinylated fimbriae (0.7 μg) were added to the wells in the presence or absence of matrix proteins (25 μg) in 100 μl of PBS and incubated for 30 min at 25°C. The amount of bound fimbriae was determined colorimetrically. The binding of the biotinylated fimbriae in the presence of bovine serum albumin (BSA) was used as a control (100%). Values are the means ± SD of five determinations per experiment. A representative result from three separate experiments is shown.

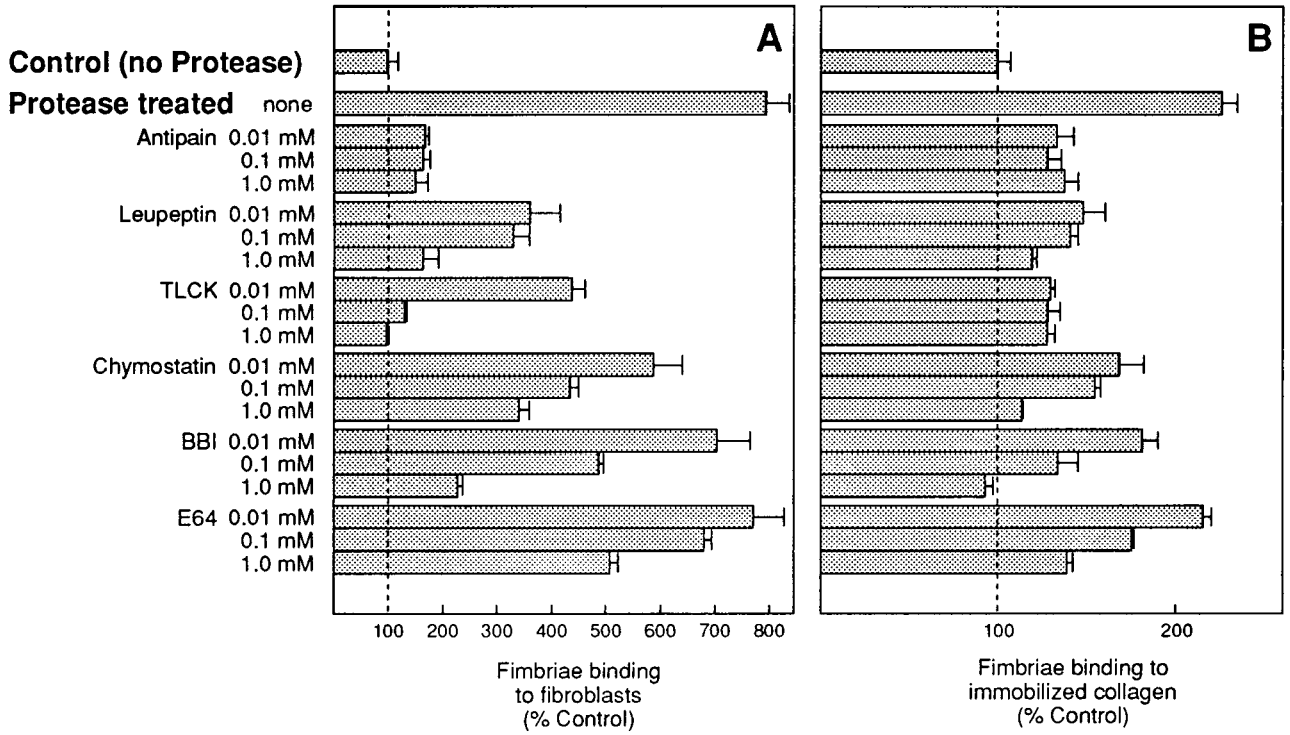

FIG. 4. Effect of *P. gingivalis* protease on the binding of fimbriae to the monolayer of the fibroblasts (A) or collagen (B). The monolayer of the fibroblasts and the immobilized collagen were pretreated with the protease, and 0.7 μg of biotin-conjugated fimbriae was added to the wells. The amount of bound fimbriae was determined colorimetrically. Results are expressed relative to the fimbrial binding of the control (without protease pretreatment), which is 100%. Values are the means ± SD of triplicate assays per experiment. A representative result from three separate experiments is shown.

strate specificities, and the susceptibilities to selected protease inhibitors.

More recently, an arginine-specific cysteine proteinase (arg-ingipain) and a collagen-degrading protease from the culture supernatant of *P. gingivalis* 381 have been reported (2, 18). These enzymes also shared properties with our protease, such as effectively degrading type I and IV collagens. Chen et al. (5) did not mention the collagenolytic activity of their Arg-gingipain from *P. gingivalis* H66. It was also reported that *P. gingivalis* W12 possessed a 150-kDa cell surface protein that could bind and degrade fibronectin (20).

Of more interest is that the protease did not degrade fimbriae but enhanced the binding of purified *P. gingivalis* fimbriae to the cell surface of the monolayered fibroblasts and the matrix proteins immobilized on the microtiter wells. This enhanced binding of fimbriae was clearly abrogated by heat inactivation of the protease or by using protease inhibitors such as antipain, leupeptin, and TLCK, suggesting that the enhanced binding of fimbriae may be dependent on the function of the protease. In addition, experiments investigating the binding of fimbriae to various dipeptide ligands revealed that a strong affinity existed between fimbriae and glycyl-L-arginine. As the protease degrades matrix proteins on the surface layer of fibroblasts, arginine residues, to which *P. gingivalis* fimbriae could bind effectively, would be exposed. In this regard, we showed that L-Arg inhibited the binding of fimbriae to the immobilized collagen especially when the collagen coat was pretreated with *P. gingivalis* protease. Thus, it is possible to speculate that the cell-associated protease of *P. gingivalis* plays an important role in the initial attachment of the organism to the host tissue through its fimbriae. It has also been claimed that fimbriae bind to collagen- or saliva-coated hydroxyapatite through their hydrophobic interaction (26). *P. gingivalis* protease may also modify the binding sites of the oral mucosal surfaces by exposing the arginine residues, so that bacterial adherence and colonization in the subgingival area would be enhanced through fimbria-arginine interaction, an interesting host-parasite interaction.

A unique mechanism explaining adherence has been proposed, introducing the concept of cryptitope, a hidden segment of cell adhesion molecules, that could be exposed after enzymatic degradation of host matrix proteins (8, 9). One of the cryptitopes in the infection of *P. gingivalis* might be the arginine residue exposed by its own arginine-specific cysteine protease. Similar observations were noted in the attachment of *Treponema denticola* to fibroblasts (42).

TABLE 4. Effect of L-Arg on the binding of fimbriae to immobilized collagen[a]

Concn of L-Arg added (mM)	Bound fimbriae expressed as A_{405} (% of control)[b]	
	No protease	Protease treated
0.0	0.245 ± 0.005 (100.0)	0.539 ± 0.040 (100.0)
0.1	0.214 ± 0.012* (87.3)	0.505 ± 0.045 (93.7)
1.0	0.194 ± 0.020** (79.1)	0.430 ± 0.032** (79.8)
10	0.094 ± 0.015** (38.5)	0.136 ± 0.011** (25.2)

[a] Biotinylated fimbriae (7 μg/ml) were added in the presence or absence of L-Arg to the immobilized collagen with or without pretreatment of protease and incubated for 30 min at room temperature. The amount of bound fimbriae was determined colorimetrically and expressed as A_{405}.

[b] Values are means ± SD (n = 5). Statistical intragroup differences among control group without L-Arg and test groups with L-Arg were determined by analysis of variance; *, $P < 0.02$; **, $P < 0.0001$. The amount of bound fimbriae without L-Arg was defined as 100.

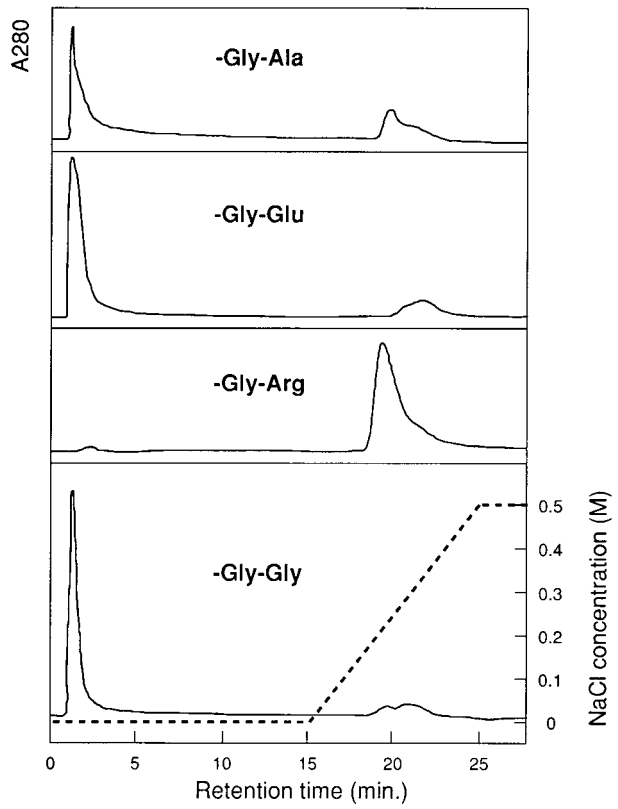

FIG. 5. Binding of fimbriae to various dipeptidyl ligands coupled with biotin-*N*-hydroxysuccinimide-activated HiTrap columns previously equilibrated with 10 mM Tris-HCl (pH 7.8). After application of fimbriae, the columns were washed with the same buffer; this was followed by elution with a linear gradient concentration of 0 to 0.5 M NaCl in the same buffer. The elution profile of the fimbriae was monitored by a UV detector at 280 nm.

Taken together, *P. gingivalis* proteases may contribute to the initiation of periodontal disease by enhancing the attachment of *P. gingivalis* cells to the host cell surface by the mechanism described above and by the inactivation of components involved with host defense systems, stimulation of inflammatory reaction, and destruction of periodontal connective tissue, which eventually would permit deep penetration of *P. gingivalis* and other subgingival microbes into the connective tissue of the periodontium. Further work is required to clarify the relationship between the proteases produced by *P. gingivalis* and other periodontal pathogens and protease-modified host cells in the development of adult periodontitis.

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