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Biochemical and Functional Properties of Lysine-Specific Cysteine Proteinase (Lys-Gingipain) as a Virulence Factor of *Porphyromonas gingivalis* in Periodontal Disease[1]

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The oral anaerobic bacterium *Porphyromonas gingivalis* has been implicated as a major etiologic agent of progressive periodontal disease. A novel lysine-specific cysteine proteinase, termed "Lys-gingipain," was purified from the culture supernatant of the Arg-gingipain-deficient mutant of *P. gingivalis* (KDP112) by a simple method including immunoaffinity chromatography. The purified enzyme was found to be composed of a single polypeptide of M_r=51,000. Analysis of the enzymatic properties revealed several distinctive features of this enzyme. The proteolytic activity was remarkably activated by thiol-reducing agents and inhibited by idoacetamide, idoacetic acid, and leupeptin. The enzyme was also inhibited by the chloromethyl ketones of tosyl-L-lysine and tosyl-L-phenylalanine. However, internal protease inhibitors, such as cystatins and α1-antichymotrypsin, had no effect on the activity, suggesting its resistance to normal host defense systems *in vivo*. Despite its narrow specificity for synthetic substrates containing Lys in the P1 site, the enzyme extensively degraded human type I collagen and immunoglobulins G and A (both serum and secretory types). Most important, the enzyme was able to disrupt the functions of polymorphonuclear leukocytes, as shown by its inhibitory effect on the generation of active oxygen species from the activated cells. These results suggest that Lys-gingipain, like Arg-gingipain, plays a crucial role as a virulence factor from *P. gingivalis* in the development of periodontal disease *via* the direct destruction of periodontal tissue components and the disruption of normal host defense mechanisms.

Key words: Arg-gingipain, Lys-gingipain, lysine-specific cysteine proteinase, periodontal disease, *Porphyromonas gingivalis*.

Porphyromonas gingivalis, a Gram-negative anaerobic bacterium, is thought to be a major etiologic bacterium associated with several periodontal diseases including chronic adult periodontitis, generalized juvenile periodontitis, periodontal abscess, and refractory periodontitis (*1-4*). This bacterium is known to produce a variety of virulence factors in both the cell-associated and secretory forms, such as fimbriae, hemagglutinins, lipopolysaccharides, and hydrolytic enzymes (*2, 5*). Among these factors, proteolytic enzymes have attracted intense interest and led to a vigorous search for agents to prevent or attenuate the virulence of the bacterium, since they are believed to be involved in a wide range of pathologies of progressive periodontal disease (*6, 7*). Most of the proteinases that have been referred to as trypsin-like enzymes have been found to be cysteine proteinases that are activated by thiol-reducing agents in an unusual order of preference and have unusual susceptibilities to various protease inhibitors (*8-15*). Recently, the trypsin-like activity associated with the bacterium was found to be attributable to either Arg-X- or Lys-X-specific proteinases (*16*). These enzymes have now been termed Arg-gingipain (gingipain-R, RGP) and Lys-gingipain (gingipain-K, KGP) on the basis of their peptide cleavage specificity after arginine and lysine residues, respectively. Although early attempts to isolate and characterize Arg-X-specific cysteine proteinases corresponding to Arg-gingipain are considered to be completed, detailed information on purification and characterization of Lys-X-specific cysteine proteinase corresponding to Lys-gingipain is not yet available because of the difficulty of isolating the enzyme, which seems to be less stable than Arg-gingipain.

So far, three genes encoding Lys-X-specific proteinases have been described (*17-19*). These genes are highly

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Abbreviations: CL, chemiluminescence; E-64, [L-3-*trans*-carboxyoxiran-2-carbonyl]-L-leucyl-agmatin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMN, polymorphonuclear leukocytes; TPCK, tosyl-L-phenylalanine chloromethyl ketone; TLCK, tosyl-L-lysine chloromethyl ketone.

homologous to one another, but have significant differences in the carboxy-terminal hemagglutinin domain. The nucleotide sequence of *kgp* gene from *P. gingivalis* strain 381 has a 5,169-bp open reading frame encoding 1,723 amino acids with a calculated molecular mass of 218 kDa (*17*). The deduced amino acid sequence suggested that the precursor of Lys-gingipain comprises at least four domains: the signal peptide, the amino-terminal propeptide, the catalytic proteinase domain, and the carboxy-terminal hemagglutinin domain. The deduced amino acid sequences of the amino-terminal proregion and the proteinase domain had no significant similarity with any other protein sequences, although the sequence of the carboxy-terminal hemagglutinin domain exhibited significant similarity to that of Arg-gingipain (*7, 17, 20-22*). Thus, it has been considered that Lys-gingipain, together with Arg-gingipain, represents a new class of the cysteine proteinases. However, as compared with the structural information based on cloning and sequencing of its gene, information on the biochemical and functional properties of Lys-gingipain at the protein level is very limited, because of the difficulty of preparing a sufficient amount of the enzyme as described above.

In the present study, we have used an Arg-gingipain-deficient mutant of *P. gingivalis* (KDP112) (*23*), which is completely devoid of Arg-gingipain activity, for efficient isolation of Lys-gingipain, because the coexistence of Arg-gingipain makes it difficult to obtain an appropriate amount of Lys-gingipain. We report here the unusual catalytic features of Lys-gingipain purified from the culture supernatant of KDP112, including its narrow specificity for synthetic substrates containing Lys in the P1 site and its unique inhibition profiles for various protease inhibitors, and discuss possible mechanisms by which the enzyme functions as a periodontal virulence factor.

EXPERIMENTAL PROCEDURES

Materials—Arg-gingipain was purified from *P. gingivalis* strain 381 by the method of Kadowaki *et al.* (*13*). All the synthetic chromogenic substrates and protease inhibitors (leupeptin, chymostatin, elastatinal, antipain, E-64, phosphoramidon) were purchased from Peptide Institute, Osaka. Acid-soluble human type I collagen was from Seikagaku Kogyo, Tokyo. Human immunoglobulin G (IgG) was from Jackson Immuno Research Lab., West Grove, PA. Both secretory and serum types of immunoglobulin A (IgA) were from Cappel™, Durham, NC. Human cystatin S was a kind gift from Dr. E. Saito (Department of Oral Biochemistry, Nippon Dental University at Niigata. Zymosan A and Brij 35 were from Sigma Chemical. All other chemicals were of reagent grade or better.

Antiserum Production and Characterization—Partially purified Arg-gingipain fraction from *P. gingivalis* 381, which contains both Arg-gingipain and Lys-gingipain and corresponds to the fraction obtained after the isoelectric focusing step of Kadowaki *et al.* (*13*), was emulsified with an equal volume of complete Freund's adjuvant and injected intramuscularly into a male rabbit. The injection was repeated three times at 2-week intervals. Blood was obtained from the ear vein of the rabbit at monthly intervals a week after the last booster immunization. The IgG fraction was isolated by ammonium sulfate fractionation followed by protein A-Sepharose 4B (Pharmacia) affinity chromatography. Immunoblot and immunoprecipitation analyses revealed that the antibodies immunoreacted with both Arg-gingipain and Lys-gingipain. The IgG was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. This IgG-Sepharose 4B column had the ability to bind Lys-gingipain as well as Arg-gingipain.

Bacterial Strains and Culture Conditions—The wild-type *P. gingivalis* strain 381 and the *rgpA rgpB* double mutant (Arg-gingipain-null mutant, KDP112) (*23*) were used and maintained on blood agar plates in anaerobic conditions (10% CO_2, 10% H_2, 80% N_2). The microorganisms were grown in 100 ml of broth containing 3.7 g of brain heart infusion broth (BHI) (Difco), 0.5 g of yeast extract (Difco), 0.1 g of L-cysteine, 0.5 mg of hemin, and 0.1 mg of menadione to the early phase, then transferred to 2,000 ml of the same broth and cultivated anaerobically at 37°C for 2 days. Erythromycin (10 μg/ml) and tetracycline (1 μg/ml) were added to the culture media of KDP112.

Purification of Lys-Gingipain—The Arg-gingipain-null mutant KDP112 was prepared as previously described (*23*). The cell-free culture supernatant of KDP112 was obtained by centrifugation at 10,000 × *g* for 20 min at 4°C. Ammonium sulfate was added to the culture supernatant to give 70% saturation. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 20 min and resuspended in 20 mM sodium phosphate buffer, pH 8.0, containing 0.05% Brij 35 and 0.1 M NaCl. After dialysis against the same buffer at 4°C overnight, insoluble materials were removed by centrifugation at 25,000 × *g* for 30 min. The dialysate was applied to the IgG-Sepharose 4B affinity column equilibrated with the buffer. After washing the column with the buffer, the adsorbed proteins were eluted with 6 M urea solution (pH 8.4) and concentrated by ultrafiltration (Amicon PM-10 membrane). The denatured proteins were then renatured by gradual removal of urea through dialysis against a large amount of 10 mM sodium phosphate buffer, pH 7.0, containing 0.05% Brij 35. The dialyzed sample was subjected to cation-exchange chromatography on a Mono S column that had been equilibrated with 10 mM sodium acetate buffer, pH 5.5, containing 0.05% Brij 35 and eluted with a linear gradient of NaCl (0-500 mM). The enzyme-active fractions were eluted between 50-150 mM NaCl. The pooled active fractions were concentrated and dialyzed against 10 mM sodium phosphate buffer, pH 7.0, containing 0.05% Brij 35.

Polyacrylamide Gel Electrophoresis (PAGE)—Sodium dodecyl sulfate (SDS)-PAGE was performed according to Laemmli (*24*). The proteinase inhibitors leupeptin (1 mM) and TPCK (200 μM) were included in the solubilizing buffer to avoid autoproteolysis of the enzyme.

NH_2-Terminal Sequence Analysis—The purified enzyme separated by SDS-PAGE was transferred onto polyvinylidene difluoride membranes and stained with Coomassie Blue R-250. The stained band corresponding to Lys-gingipain was excised, and the adsorbed protein was subjected to automatic sequencing (Applied Biosystems Model 473A).

Determinations—Lys-gingipain activity was determined with the synthetic substrate *t*-butyl-oxycarbonyl-L-valyl-L-leucyl-L-lysine-4-methyl-7-coumarylamide (Boc-Val-Leu-Lys-MCA) (final conc. 10 μM) in 20 mM sodium phosphate buffer, pH 7.5, containing 5 mM cysteine in a total volume of 1 ml. After incubation at 40°C for 10 min, the reaction

was terminated by adding 1 ml of 10 mM iodoacetamide (pH 5.0), and the released 7-amino-4-methylcoumarin was measured at 460 nm (excitation at 380 nm). One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of 7-amino-4-methylcoumarin/ml under the conditions.

Degradative activity against human placental type I collagen was determined by incubation at 20°C for 1 h with Lys-gingipain or Arg-gingipain in 20 mM sodium phosphate buffer, pH 7.5, containing 10 mM dithiothreitol in a total volume of 50 μl. The reaction was terminated by adding 5 μl of the proteinase inhibitor cocktail (leupeptin, TPCK, EDTA-Na_2, 10 mM each). After heating at 100°C for 5 min in a solubilizing buffer containing 1% SDS, 0.16 mM 2-mercaptoethanol, and 2.2% sucrose, the cleavage products were analyzed by SDS-PAGE in 5-12% gradient polyacrylamide gels. Hydrolyses of human immunoglobulins G and A were also analyzed by SDS-PAGE after incubation at 37°C for 1 h with the respective enzymes in 20 mM sodium phosphate buffer, pH 7.5, containing 10 mM dithiothreitol in a total volume of 50 μl.

Measurement of Luminol-Dependent Chemiluminescence (CL) Response—Sterilized 0.2% oyster glycogen (Sigma) in saline was intraperitoneally injected into guinea pigs. At 14 h after the injection, the peritoneal exudate cells were collected, washed twice with Hanks' balanced salt solution, and suspended in the same medium. The cell suspension (1×10^7 cells/ml, more than 90% = PMNs) was preincubated at 37°C for 30 min with the indicated concentrations of purified Lys-gingipain and Arg-gingipain. PMNs were then washed with phosphate-buffered saline (PBS) and resuspended in PBS at a final cell concentration of 2×10^7 cells/ml.

Zymosan A (Sigma) suspended in PBS (20 mg/ml) was boiled for 5 min and washed prior to being opsonized to reduce clumping. The zymosan suspension was incubated with an equal volume of guinea pig serum at 37°C for 30 min. The particles were washed twice with PBS and suspended in the original volume in PBS. The cuvette for the reaction mixture consisting of 0.1 ml of freshly diluted luminol solution (0.2 mM), 0.1 ml of PMN suspension (2×10^7 cells/ml), and 0.1 ml of the opsonized zymosan (20 mg/ml) was maintained at 37°C in Automatic Luminescence Analyzer LB 9505 AT (Berthold, Bad Wildbad, Germany). The intensity of light emitted in the cuvette was recorded automatically. The values were expressed as the percentage of the maximal CL response.

TABLE I. **Purification of Lys-gingipain from the culture supernatant of Arg-gingipain-deficient *P. gingivalis* mutant (KDP112).** The starting material was 2 liters of the culture supernatant of KDP112. The Lys-gingipain activity was determined at pH 7.5 using Boc-Val-Leu-Lys-MCA as a substrate. One unit is defined as the amount of enzyme required to release 1 nmol of 7-amino-4-methylcoumarin per min at 40°C.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Ammonium sulfate	368	2174	5.9	100	1
Immunoaffinity column	2.5	526	210.4	24	35.7
Mono S column					
Non-adsorbed	0.8	236	295.0	11	50.0
50-150 mM NaCl	0.3	107	356.7	5	60.7

RESULTS

Purification of Lys-Gingipain—The purification scheme for Lys-gingipain from the culture supernatant of Arg-gingipain-null mutant (KDP112) is summarized in Table I. The culture medium of KDP112 was used as the starting material of purification of Lys-gingipain. The Arg-X-specific proteolytic activity was not detected in the medium. After ammonium sulfate fractionation followed by dialysis, the enzyme solution was loaded onto a IgG-Sepharose affinity column (1.5×3.0 cm) equilibrated with 20 mM sodium phosphate buffer, pH 8.0, containing 0.05% Brij 35 and 0.1 M NaCl. As Brij 35 protects the enzyme from inactivation, the following purification steps were carried out in the presence of 0.05% Brij 35. The column was washed with the same buffer, and the proteins bound to the column were eluted with 6 M urea solution (pH 8.4). Immediately after concentration by ultrafiltration, the eluted proteins were gradually dialyzed against a large amount of 10 mM sodium phosphate buffer, pH 7.0, containing 0.05% Brij 35 using a linear gradient system. In the IgG-Sepharose 4B affinity chromatography step, the recovery of the Lys-gingipain activity in the urea eluate was about 24%. This immunoaffinity chromatography provided a rapid and efficient method for removing most of contaminating proteins, although the yield was not high. The complete elimination of contaminating proteins was accomplished by cation-exchange chromatography on a Mono S column (0.5×5 cm). The pooled enzyme active fractions which were eluted from the column with NaCl concentrations between 50 and 150 mM were concentrated and dialyzed against 10 mM sodium phosphate buffer, pH 7.0, containing 0.05% Brij 35. By this simple procedure, the enzyme was purified about 60-fold over the ammonium sulfate fraction, the total recovery of the activity being greater than 5%.

The final enzyme preparation showed a single protein band with an apparent molecular mass of 51 kDa by SDS-PAGE (Fig. 1). Since the molecular mass of the enzyme by gel filtration on TSK gel G2000SW was estimated to be about 52 kDa (not shown), the enzyme appeared to be a

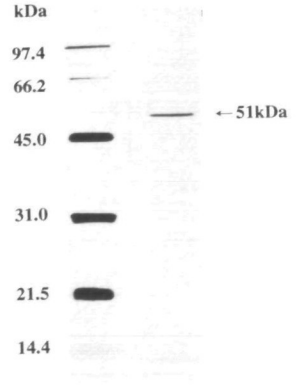

Fig. 1. **SDS-PAGE under reducing conditions of the purified Lys-gingipain.** SDS-PAGE was performed with a 10% polyacrylamide gel in Tris-HCl buffer, pH 8.9. After electrophoresis, the proteins were visualized by silver staining. The left column indicates the profile of the molecular markers.

single polypeptide chain with a molecular mass of 51 kDa. The purified enzyme separated by SDS-PAGE under reducing conditions was transferred to a polyvinylidene difluoride membrane. The NH_2-terminal amino acid sequence of the 51-kDa polypeptide was found to be FNRDVYTD---, which corresponded to the sequence F^{226}NRDVYTD^{233} predicted from the cDNA sequence (*17*). As the amino acid sequence of the mature Lys-gingipain is known to start with the 229th Asp (*17*), the purified enzyme from KDP112 appears to have three additional residues at the amino-terminal end.

Effect of Activators and Inhibitors—The purified enzyme was found to be require the presence of thiol-reducing agents for its proteolytic activity. Cysteine, 2-mercaptoethanol, and dithiothreitol resulted in a remarkable increase in the activity. Maximal activation by these agents was accomplished with a final concentration of 1 mM. The extent of activation of the enzyme by these agents was much greater than those of other cysteine proteinases so far known. Powerful activation by thiol-reducing agents is also clearly consistent with the classification of Lys-gingipain as a cysteine proteinase.

The enzyme was strongly inhibited by iodoacetamide and the two chloromethyl ketones TPCK and TLCK (Table II). The enzyme was also considerably inhibited by iodoacetic acid. It should be noted that Lys-gingipain was less susceptible to leupeptin and E-64 than Arg-gingipain. Lys-gingipain was strongly inhibited by leupeptin at concentrations of more than 10^{-4} M, while Arg-gingipain was completely inhibited by 10^{-8} M leupeptin. Also, only about 20% of Lys-gingipain was inhibited by E-64 at concentrations of 10^{-8}-10^{-4} M, while Arg-gingipain was strongly inhibited by this agent at 10^{-5}-10^{-4} M. In contrast, about 90% of Lys-gingipain activity was inhibited by iodoacetamide at a concentration of 10^{-3} M, while more than 70% of Arg-gingipain activity remained at the same concentration of the agent. The general serine protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP) had little or no effect on Lys-gingipain, or on Arg-gingipain (Table II). Elastatinal and chymostatin also were not inhibitory to Lys-gingipain. The metal chelators EDTA, EGTA, and phosphoramidon had little or no inhibitory effect on Lys-gingipain. The enzyme was partially inhibited

TABLE II. **Effects of various compounds on the activities of Lys-gingipain and Arg-gingipain.** The purified enzyme was preincubated with various compounds at the indicated concentrations at 37°C for 5 min, then incubated for 10 min with the synthetic substrate Boc-Val-Leu-Lys-MCA. The values are expressed as percentage of the activity determined in the presence of 5 mM cysteine without these compounds.

Compound	Concentration	% of activity remaining	
		Lys-gingipain	Arg-gingipain[a]
None	—	100	100
Chymostatin	50 μg/ml	115	2
TPCK	1 mM	1	5
TLCK	1 mM	4	20
PMSF	1 mM	94	76
Elastatinal	50 μg/ml	89	83
DFP	1 mM	134	111
Leupeptin	43 nM	105	2
	43 μM	57	0
	430 μM	17	0
E-64	43 nM	80	94
	43 μM	87	45
	430 μM	88	10
Antipain	50 μg/ml	84	7
Iodoacetic acid	1 mM	24	33
Iodoacetamide	10 μM	100	104
	100 μM	80	97
	1 mM	8	71
	10 mM	0	0
Pepstatin	50 μg/ml	87	112
EDTA	1 mM	110	18
EGTA	1 mM	105	45
Phosphoramidon	1 mM	112	46
$CaCl_2$	1 mM	98	133
$MgCl_2$	1 mM	90	139
$FeCl_3$	1 mM	58	87
$ZnCl_2$	1 mM	50	68
$CuSO_4$	1 mM	21	2
$MnSO_4$	1 mM	50	111
Cystatin (egg white)	5.6 μg/ml	99	(118)[b]
Cystatin S (human)	6.8 μg/ml	92	(106)[b]
α_1-Antichymotrypsin	29 μg/ml	98	(114)[b]

[a]Data from Ref. *13*. [b]The values in parentheses were obtained at the concentrations of 500 μg/ml of each compound.

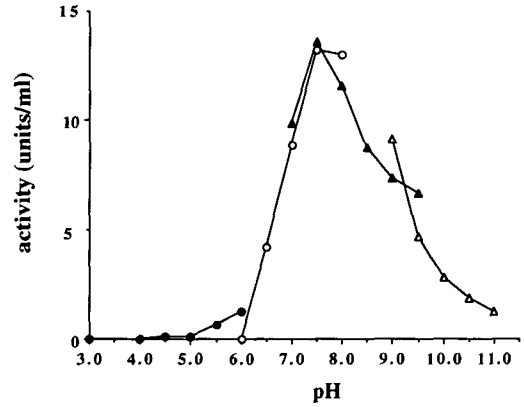

Fig. 2. **Optimal pH of Lys-gingipain.** The purified enzyme was incubated with the substrate solution at 40°C for 10 min in four buffer systems: 0.1 M sodium acetate buffer, pH 3.0-6.0; 0.02 M sodium phosphate buffer, pH 6.0-8.0; 0.02 M barbital buffer, pH 7.0-9.5; and 0.1 M glycine-NaOH buffer, pH 9.0-11.0.

TABLE III. **Substrate specificity of Lys-gingipain toward synthetic peptidyl amidomethylcoumarins.** Final concentrations of the respective substrates were 10 μM.

Synthetic substrate	Activity (nmol/mg/min)	% of maximum activity
Boc-Val-Leu-Lys-MCA	56	100
Boc-Glu-Lys-Lys-MCA	23	41
Z-Phe-Arg-MCA	0	0
Boc-Phe-Ser-Arg-MCA	0	0
Arg-MCA	0	0
Lys-MCA	0	0
Leu-MCA	0	0
Ala-MCA	0	0
Lys-Ala-MCA	2	4
Suc-Leu-Leu-Val-Tyr-MCA	0	0
Suc-Ala-Ala-Pro-Phe-MCA	0	0
Suc-Ala-Pro-Ala-MCA	0	0
Suc-Gly-Pro-MCA	2	4
Boc-Arg-Val-Arg-Arg-MCA	0	0
Boc-Glu-Arg-Arg-MCA	1	2
Z-Lys-MCA	0	0

by the heavy metal ions Fe^{3+}, Zn^{2+}, Cu^{2+}, and Mn^{2+} at 1 mM concentration. Importantly, the internal protease inhibitors, namely, the serine protease inhibitor α_1-antichymotrypsin and the cysteine protease inhibitors cystatins (egg white cystatin and human cystatin S), had no effect on the activity. These results indicate that Lys-gingipain, like Arg-gingipain, is a novel type of cysteine proteinase having the catalytic features of a serine-type proteinase.

Other Enzymatic Properties—The optimal pH for hydrolysis of the synthetic substrate Boc-Val-Leu-Lys-MCA by Lys-gingipain was examined in the presence of 5 mM cysteine. The optimal pH was found to be around 7.5 (Fig. 2). In addition, the enzyme exhibited the relatively high activity even at pH values between 6.5 and 7.0 and between 8.0 and 9.5. The cleavage specificity was investigated by digestion of various synthetic peptidyl amidomethylcoumarins with the purified enzyme. Table III summarizes the relative rates of hydrolysis of these substrates by the enzyme. The best substrate was found to be Boc-Val-Leu-Lys-MCA. Lys-gingipain was also capable of splitting Boc-Glu-Lys-Lys-MCA, although less efficiently than the optimal substrate. The enzyme exhibited little or no activity toward the other substrates, and it had no detectable aminopeptidase activity. Thus, the enzyme exhibited a strong preference for substrates containing Lys in the P1 site.

Lys-gingipain was also capable of hydrolyzing protein substrates, such as bovine hemoglobin, bovine serum albumin, and casein, as revealed by measuring acid-soluble products from them (not shown). In comparison with Arg-gingipain, it hydrolyzed bovine hemoglobin to a similar extent, but bovine serum albumin and casein less efficiently. As determined by SDS-PAGE, Lys-gingipain degraded acid-soluble human placental type I collagen and human IgG and IgA (both serum and secretory types) (Fig. 3). When the type I collagen was incubated with Lys-gingipain at 20°C for 1 h, significant protein bands were barely detectable for this protein, indicating that it had been extensively hydrolyzed by the enzyme. The degradation of type I collagen was completely dependent on the presence of cysteine. Human IgG and IgA (both types) were also efficiently degraded by the enzyme in the presence of dithiothreitol. Compared with collagen, these immunoglobulins were limitedly degraded during the incubation period. Arg-gingipain also similarly degraded these proteins, although the degradative profiles were different between Arg-gingipain and Lys-gingipain.

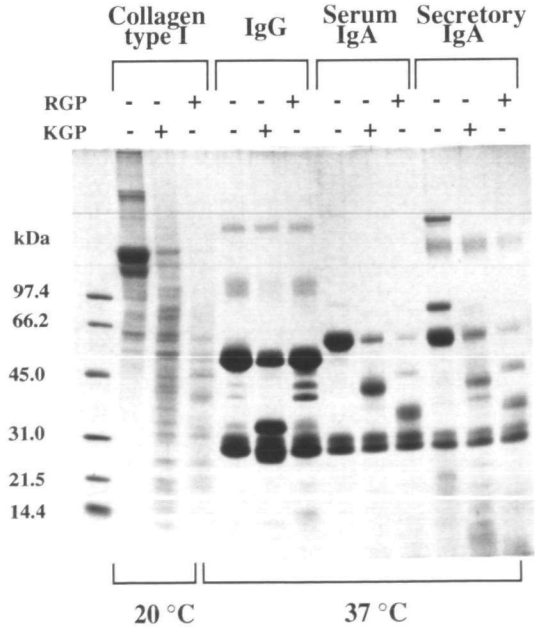

Fig. 3. **Degradation patterns of human type I collagen, human IgG, and IgA by Lys-gingipain and Arg-gingipain.** Human placental type I collagen and human IgG and IgA (15 μg each) were incubated in 20 mM sodium phosphate buffer containing 10 mM dithiothreitol with or without Lys-gingipain (KGP) or Arg-gingipain (RGP) (0.5 μg each) at pH 7.5 for 1 h at 20 and 37°C, respectively. The reaction was terminated by addition of the protease inhibitor cocktail (leupeptin, 100 μg/ml; TPCK, 1 mM; and EDTA, 1 mM). SDS-PAGE was performed under reducing conditions in a 5-12% gradient gel in Tris-HCl buffer, pH 8.9, after denaturation at 100°C for 5 min. The gel was stained with Coomassie Brilliant Blue. The left column indicates the profile of the molecular markers.

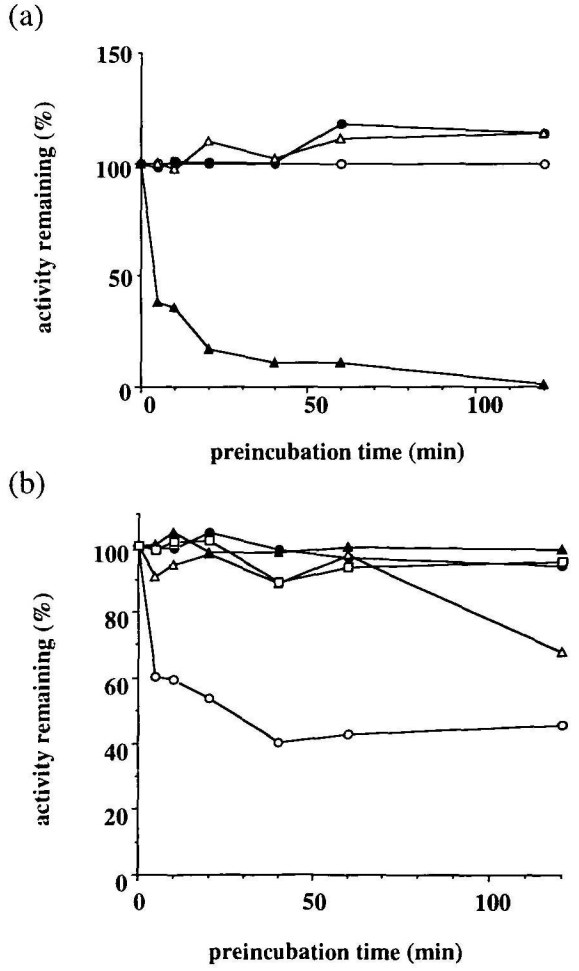

Fig. 4. **The thermal and pH stability of Lys-gingipain.** (a) The purified enzyme was preincubated in 10 mM sodium phosphate buffer containing 0.05% Brij 35 at pH 7.0 at 0 (○), 37 (●), 45 (△), and 60°C (▲). At the indicated times, samples were taken and the remaining activity was determined. (b) The enzyme was preincubated at 0°C at various pH values. At the indicated times, samples were taken and the remaining activity was determined. Buffers used were 0.1 M sodium acetate (pH 3.5, ○ and 5.5, ●), 20 mM sodium phosphate (pH 7.0, △ and 8.0, ▲), and 0.1 M glycine-NaOH (pH 10.5, □).

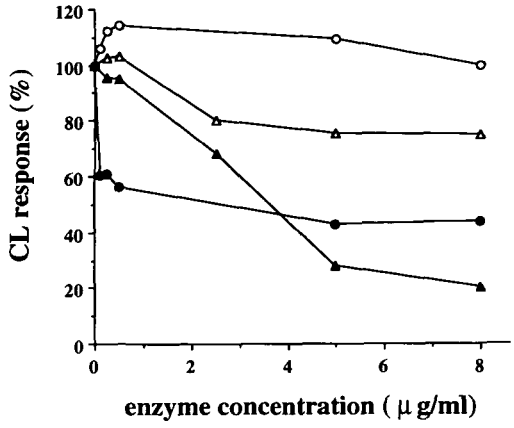

Fig. 5. **Effects of Lys-gingipain and Arg-gingipain on the CL response of PMNs.** Guinea pig PMNs (1×10^7 cells/ml) were preincubated with various concentrations of Lys-gingipain or Arg-gingipain at 37°C for 30 min with or without 1 mM cysteine, then subjected to the CL response assay. Lys-gingipain only (○), with cysteine (●); Arg-gingipain only (△), with cysteine (▲).

The thermal stability of Lys-gingipain at pH 7.5 was tested by preincubation for up to 2 h at 37, 45, and 60°C followed by assay under the standard conditions with Boc-Val-Leu-Lys-MCA as a substrate. No loss of the enzyme activity was observed at between 0 and 45°C (Fig. 4a). However, the enzyme was rapidly inactivated at 60°C, with about 40% of the initial activity remaining after 10 min of incubation. The pH stability was also tested by preincubation for up to 2 h at pH 3.5, 5.5, 7.0, 8.0, and 10.5 at 0°C, followed by assay under the same conditions as above (Fig. 4b). No significant loss of activity was observed at pH 5.5, 7.0, 8.0, and 10.5. However, the enzyme was inactivated after 5 min of incubation at pH 3.5 by 60% of the initial activity.

Suppression of the Function of PMNs—Attempts to demonstrate the pathological functions of Lys-gingipain in normal host defense mechanisms were carried out by using guinea pig PMNs stimulated by serum-activated zymosan. The PMNs were preincubated with the enzyme in the presence or absence of 1 mM cysteine, then the CL response of PMNs, which is known to be closely related to the bactericidal activity, was determined. As shown in Fig. 5, the enzyme had minimal or no effect on the CL response of PMNs in the absence of cysteine. However, the thiol-activated enzyme resulted in a marked decrease in the CL response. By treatment with Lys-gingipain at concentrations up to 0.5 μg/ml, the CL response was rapidly decreased to about 55% of the initial response. This value appeared to remain constant even at increasing concentrations of the enzyme up to 8 μg/ml. Under the same conditions, Arg-gingipain suppressed the CL response more intensely than Lys- gingipain did. In contrast to Lys-gingipain, Arg-gingipain suppressed the CL response to about 80% of the initial level even in the absence of cysteine.

DISCUSSION

The proteolytic activities produced by *P. gingivalis* have been thought to play a key role as major virulence factors in the progression of periodontal disease, and much work has been done to identify and characterize the pathogenic proteinases involved. We have shown previously that Arg-gingipain is one of the most potent virulence factors of *P. gingivalis*, a part of which is secreted extracellularly, and have suggested that the enzyme is directly involved in the destruction of periodontal tissues and the disruption of normal host defense mechanisms (*13, 23*). Arg-gingipain has also been suggested to be involved in the processing and translocation of secretory and cell surface proteins of the organism (*7, 22, 25*). In addition, *P. gingivalis* is also known to produce Lys-specific cysteine proteinase as another trypsin-like cysteine proteinase. Recently, it has been suggested that the single enzyme Lys-gingipain is responsible for the trypsin-like activity with lysine-X-specificity associated with this organism (*16*). Compared with the structural information based on the cloning and sequencing of its gene, detailed information about the biochemical and functional features of Lys-gingipain at the protein level has not yet been obtained.

In this paper, we have purified Lys-gingipain from the Arg-gingipain null mutant of *P. gingivalis* (KDP112) by a simple method using immunoaffinity chromatography and clarified several of its unique catalytic features. SDS-PAGE showed that Lys-gingipain from the culture medium of KDP112 was composed of a single polypeptide of 51 kDa. The amino acid residue of the NH_2-terminus could not be identified by direct sequence analysis, but the adjacent sequence was determined and found to be identical with the sequence starting with Phe^{226} of Lys-gingipain precursor predicted from its nucleotide sequence (*17*). As the NH_2-terminal amino acid sequence of the enzyme from the wild-type strain started with the 229th Asp residue, the present enzyme had three additional amino acid residues at the NH_2-terminal end. This is mainly due to the deficiency of Arg-gingipain in the starting source, since Arg-gingipain is a major processing enzyme for the secretory proteins such as Arg-gingipain itself, as well as the cell surface proteins such as fimbrilin and the 75-kDa protein (*7, 22*), and since the NH_2-terminal Asp residue of the wild-type mature Lys-gingipain resided in the most probable site for cleavage by Arg-gingipain. The purified Lys-gingipain, like Arg-gingipain, was not completely typical of the papain superfamily of cysteine proteinases, and this was consistent with the structural data indicating they have no sequence similarity with any other known cysteine proteinases (*17*). Studies with synthetic peptide substrates showed that the enzyme exhibited one of the most restricted specificities known for the cysteine proteinase family: only peptide bonds containing Lys, not Arg, in the P1 site were cleaved. The selected substrates for the enzyme were Boc-Val-Leu-Lys-MCA and Boc-Glu-Lys-Lys-MCA, which are considered to be the preferred substrates for plasmin. However, since the K_m values of Lys-gingipain for these substrates were larger than those of Arg-gingipain for Z-Phe-Arg-MCA and Boc-Phe-Ser-Arg-MCA (6.7×10^{-5} M *vs.* 5.8×10^{-6} M) under the same assay conditions, it appears to be necessary to develop more preferential substrates for this enzyme. The susceptibility to various protease inhibitors was characteristic of the enzyme. The enzyme was absolutely thiol-dependent, as indicated by the unusual stimulation of activity by thiol-reducing agents and by its inhibition by iodoacetamide and iodoacetic acid (Table II). However, Lys-gingipain was

much less susceptible to leupeptin and E-64 than was Arg-gingipain.

On the other hand, Lys-gingipain was similar to Arg-gingipain in some enzymatic properties. The optimal pH for hydrolysis of both synthetic and protein substrates by both enzymes was around 7.5. Neither Lys-gingipain nor Arg-gingipain was inhibited by internal protease inhibitors, such as cystatins and α_1-antichymotrypsin, suggesting that Lys-gingipain, as well as Arg-gingipain, diffused in host tissues and cells could evade inactivation by these inhibitors. These findings together suggest that Lys-gingipain is readily activated *in vivo*, since the periodontal crevice is known to be anaerobic and to contain various components derived from body fluids and destructive host tissues and cells. The CL response of PMNs was significantly suppressed by Lys-gingipain in the presence of cysteine. The profile of suppression appeared to be different from that of Arg-gingipain. The CL response of PMNs was greatly suppressed by Arg-gingipain in a concentration-dependent manner, falling to 20% of the initial value with 8 μg/ml of the enzyme, while the maximal suppression by Lys-gingipain (about 50%) was obtained with concentrations in the region of 0.5 μg/ml and above (Fig. 5). The difference between Lys-gingipain and Arg-gingipain in the suppression profile for the CL response of PMNs may be due to the difference in their substrate specificities. Namely, Arg-gingipain may degrade PMN proteins progressively and widely, while Lys-gingipain may hydrolyze these proteins rapidly and limitedly. The ability to suppress the CL response of PMNs seems important in relation to the progression of periodontal diseases, since impaired PMN functions appears to be closely related to disruption of the normal host defense mechanisms. The importance of PMNs in periodontal diseases is also supported by the finding that individuals with impaired PMN functions have a high prevalence of periodontitis (*26*) and that most patients with juvenile periodontitis show defective neutrophil chemotaxis (*26, 27*). Therefore, Lys-gingipain, as well as Arg-gingipain, is most likely to be involved in disruption of normal host defense mechanisms *via* the impairment of PMN functions. Further, the suppressive activity of Lys-gingipain toward the CL response of PMNs was absolutely dependent on its proteolytic activity, and its ability to evade inactivation by mammalian internal protease inhibitors, such as cystatins and α_1-antichymotrypsin, may allow it to disrupt PMN functions. Furthermore, since Lys-gingipain was capable of degrading human IgG and IgA (both serum and secretory types) (Fig. 3), the enzyme is also likely to contribute to disruption of normal host defense mechanisms *via* the impairment of the humoral immunity system.

In addition to these abilities, Lys-gingipain had another important ability related to the virulence of *P. gingivalis*. The enzyme showed strong activity to degrade human type I collagen (Fig. 3), a major component of the connective tissue in gingiva, periodontal ligaments, and the alveolar bone. Therefore, Lys-gingipain is likely to play an important role in the direct destruction of periodontal tissue components, which is one of the most important pathological conditions in progressive periodontal disease. The present study thus suggests that secretory Lys-gingipain, as well as Arg-gingipain, plays a key role in the pathogenesis of periodontal disease *via* destruction of physiologically important periodontal proteins and disruption of normal host defense mechanisms.

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