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Purification and Characterization of Two Forms of a High-Molecular-Weight Cysteine Proteinase (Porphypain) from *Porphyromonas gingivalis*

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Porphyromonas gingivalis*, an organism implicated in the etiology and pathogenesis of human periodontal diseases, produces a variety of potent proteolytic enzymes, and it has been suggested that these enzymes play a direct role in the destruction of periodontal tissues. We now report that two cell-associated cysteine proteinases of *P. gingivalis* W12, with molecular masses of approximately 150 kDa (porphypain-1) and 120 kDa (porphypain-2), as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, have been separated and purified to apparent homogeneity. These proteinases appear to be SDS-stable conformational variants of a 180-kDa enzyme, and they are the largest cysteine proteinases yet purified from *P. gingivalis*. The purified proteinases hydrolyze fibrinogen, tosyl-Gly-L-Pro-L-Arg *p*-nitroanilide, and tosyl-Gly-L-Pro-L-Lys *p*-nitroanilide. While hydrolysis of both synthetic substrates by porphypain-1 and -2 requires activation by reducing agents, is inhibited by EDTA, and is stimulated in the presence of derivatives of glycine, the Arg-amidolytic activity is sensitive to leupeptin and H-D-tyrosyl-L-prolyl-L-arginyl chloromethyl ketone, whereas the Lys-amidolytic activity is sensitive to tosyl-L-lysyl chloromethyl ketone and insensitive to leupeptin. These data suggest that porphypains contain two types of active sites. These cell-associated *P. gingivalis* proteinases may contribute significantly and directly to periodontal tissue destruction.

Porphyromonas gingivalis is a gram-negative anaerobe that has been strongly implicated as an etiologic agent of human periodontal diseases (review in reference 11). *P. gingivalis* produces an array of potent proteolytic enzymes that have recently been under intensive investigation. These proteinases, which are found associated with the bacteria, as components of membrane-derived vesicles as well as in culture supernatants, degrade a number of host cell adhesion proteins present in the extracellular matrix and basement membranes, as well as a number of plasma proteins important in the clotting cascade and host defense (13, 14, 18, 27, 29). Three main proteolytic activities have been detected in *P. gingivalis* strains: a so-called trypsinlike activity, a glycylprolyl peptidase activity, and a collagenolytic activity (1, 31, 32). It is unclear at this time how many distinct proteinases are produced by this organism, how variable individual strains are with respect to the numbers and types of proteolytic activities that they express, and whether the three activities are completely independent (2). Several groups have recently reported purification of trypsinlike enzymes of *P. gingivalis*, all of which are thiol dependent and which range in size from 44 to 85 kDa (6, 7, 9, 19, 20, 28). Additionally, genes encoding three apparently unrelated proteinases, *prtT*, which encodes a 54-kDa thiol-dependent proteinase (22), *tpr*, which encodes a 64-kDa cysteine proteinase (5), and *prtA*, which encodes a 110-kDa proteinase (GenBank accession number L27483), have been cloned and sequenced.

Previous studies from our laboratory (14) have shown that *P. gingivalis* W12 cells degrade human fibrinogen in a stepwise fashion, yielding primarily a large fragment with a relative molecular mass of 97 kDa and smaller fragments in the range of 45 to 50 kDa. Fibrinogen lysis is mediated by cell-associated, thiol-dependent proteinases with relative molecular masses of 150 and 120 kDa. In this study, the 150- and 120-kDa proteinases have been purified to apparent homogeneity and biochemically characterized.

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MATERIALS AND METHODS

Chemicals. Plasminogen-free human fibrinogen, Triton X-100, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), *N*-α-tosyl-L-lysyl chloromethyl ketone (TLCK), tosyl-L-phenylalanyl chloromethyl ketone (TPCK), *N*-ethylamaleimide (NEM), diisopropyl fluorophosphate (DFP), EDTA, *o*-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), iodoacetamide, benzamidine, 1-amino-4-guanidinobutane (agmatine), methylguanidine, leupeptin, chymostatin, elastatinal, tosyl-Gly-L-Pro-L-Arg *p*-nitroanilide (GPR-pNA), tosyl-Gly-L-Pro-L-Lys *p*-nitroanilide (GPK-pNA), L-cysteine, β-mercaptoethanol, dithiothreitol (DTT), Tris base, and Tris-HCl were purchased from Sigma. H-D-tyrosyl-L-prolyl-L-arginyl chloromethyl ketone (YPRCK) was obtained from Bachem Biosciences (Philadelphia, Pa.). All reagents for electrophoresis were purchased from Bio-Rad. DEAE-Sephacel was obtained from Pharmacia. All other chemicals were of reagent grade and obtained from commercial sources.

Bacteria. *P. gingivalis* W12 cells were grown in basal anaerobic broth (30) to mid-logarithmic phase (2×10^8 to 5×10^8 cells per ml after 16 h of growth) as previously described (14) in 1-liter batch cultures. Two of these cultures were then inoculated into 9 liters of sterile basal anaerobic broth and grown for 16 to 24 h at 37°C. Bacteria were harvested by filtration (Pellicon cassette system, with a 0.22-μm-pore-size Durapore filter; Millipore Corp., Bedford, Mass.) and centrifugation (13,000 × *g*, 20 min), washed three times (4°C) with Tris buffer (TB; 50 mM Tris-HCl, 3 mM sodium azide [pH 7.4]), after the final wash resuspended (5×10^{10} cells per ml) in the same buffer, and stored at 4°C.

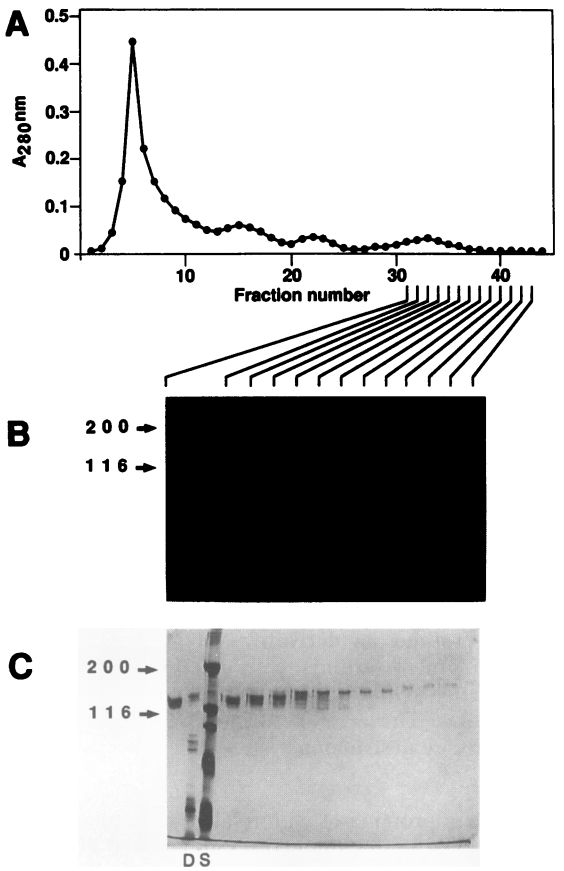

FIG. 1. Separation of the 150- and 120-kDa proteinases by preparative electrophoresis. (A) Elution profile of protein from the column. Protein was determined by A_{280} (●). (B) Zymogram of the indicated fractions in a fibrinogen-containing gel. (C) SDS-PAGE of the indicated fractions stained with silver. Electrophoresis was performed under nonreducing conditions in 6% polyacrylamide gels. Lane D contains the DEAE-Sephacel pool, and lane S contains standard proteins. The arrows and numbers at the left of panels B and C indicate the migration distances and relative molecular masses (in kilodaltons) of standard proteins in the region of interest.

Purification of the proteinases. Our previous studies (14) demonstrated that cell-associated fibrinogenolytic proteinases of 150 and 120 kDa can be solubilized in active form from *P. gingivalis* by using the anionic detergent sodium deoxycholate. Moreover, these enzymes were consistently observed over a wide range of detergent/protein ratios, suggesting that they are discrete molecular species. We were able to use CHAPS (10) in place of deoxycholate to achieve extraction of a large portion of the high-molecular-weight proteinases from the cells with minimal cell disruption. Following harvesting and washing as described above, bacteria were stored overnight at 4°C to permit oxidative inactivation of most of the proteolytic activity. All subsequent steps were performed at 4°C. The bacteria were extracted with CHAPS in TB (10^{10} cells per ml, 0.5% CHAPS; total volume, 500 ml) with gentle mixing for 2 h. Bacteria were separated from the detergent extract by centrifugation (13,000 × *g*, 20 min), and the supernatant was passed over a DEAE-Sephacel (Pharmacia) column (2.6 by 40 cm; bed volume, 212 ml) equilibrated with TB containing 0.5% CHAPS at a flow rate of 90 ml/h. After loading, the column was washed with 500 ml of TB containing 1% CHAPS at the same flow rate. The column was eluted with the same buffer containing a linear sodium chloride gradient (0 to 1 M, 1 liter of buffer, flow rate of 90 ml/h), and 7.8-ml fractions were collected. Chromatography on DEAE-Sephacel concentrated the 150- and 120-kDa proteinases into a few fractions, and as determined by zymography, most of the proteolytic activity was associated with proteins migrating at 150 and 120 kDa (data not shown). Fractions containing the 150- and 120-kDa proteinases were pooled, supplemented with TLCK (1 mM, final concentration), and stored at 4°C. Addition of TLCK to the pooled fractions greatly enhanced the stability of the partially purified enzymes and permitted prolonged storage of the pooled fractions. Since TLCK reacts only with the reduced form of the enzymes, and most of the enzymes were in the oxidized form, we believe that TLCK scavenged any active enzyme present and protected the enzymes against autolytic degradation. TLCK was removed in the final purification step.

TABLE 1. Purification of the 150- and 120-kDa proteinases from *P. gingivalis* W12

Step	Total activity (ΔA_{405} h^{-1})	Total protein (mg)[a]	Sp act (ΔA_{405} h^{-1} mg^{-1})	Purification (fold)	Yield (%)
CHAPS extract	13,125	2,160	6.08	1	100
DEAE-Sephacel pool	4,699	181	26	4.3	35.8
Preparative electrophoresis					
150-kDa proteinase	112	0.8	140	23	0.9
120-kDa proteinase	640	1.7	376	62	4.9

[a] Determined by A_{280}.

Final purification was achieved by preparative electrophoresis using a Bio-Rad Prep Cell 491 as recommended by the manufacturer. A 6% polyacrylamide gel was cast in the small Prep Cell column (28-mm internal diameter). The gel was overlaid with water-saturated 2-butanol and polymerized at room temperature for 2 h. The gel was stored at room temperature overnight to ensure complete polymerization. The stacking gel (4% polyacrylamide) was polymerized immediately prior to electrophoresis. For sample preparation, one-third of the pooled DEAE-Sephacel fractions (15 to 25 ml) was concentrated approximately 20-fold and desalted at 4°C in an Amicon filtration concentrator (W. R. Grace and Co., Danvers, Mass.) fitted with a YM-10 membrane. The sample was prepared so that it contained approximately 10 mg of protein in a final volume of 1.0 ml. The sample was mixed 3:1 with sample buffer (160 mM Tris base, 0.02% bromphenol blue, 60% sucrose, 7% sodium dodecyl sulfate [SDS], adjusted to pH 8.8 with HCl) and loaded onto the column. Electrophoresis was performed at 4°C with a constant current in Tris-glycine-SDS buffer (25 mM Tris base, 0.192 mM glycine, 0.1% SDS), and 1.5-ml fractions were collected. Separation and purification of the 150- and 120-kDa proteinases was achieved by this procedure (Fig. 1). Proteinase purification was monitored by using GPR-pNA as the substrate (Table 1). The 150-kDa proteinase was purified 23-fold and the 120-kDa proteinase was purified 62-fold by these procedures, with yields of 0.9 and 4.9%, respectively.

Enzyme assays. The 150- and 120-kDa proteinases both hydrolyzed GPR-pNA and GPK-pNA, synthetic peptide substrates that have been reported to be highly selective for

plasmin and thrombin, respectively (15). GPR-pNA was hydrolyzed by intact bacteria and by the purified proteinases more rapidly than was GPK-pNA. The rates of hydrolysis of the substrates were determined by measuring the increase in A_{405} (at pH 7.9 and 25°C) as a function of time (15). The rate of hydrolysis of the substrates by *P. gingivalis* cells, as well as by the purified proteinases, showed an absolute dependence on addition of reducing agents such as DTT, β-mercaptoethanol, or L-cysteine to incubation mixtures. There was little dependence of the rate of reaction on pH between 7.5 and 8.5; however, pH 7.9 was optimal. Addition of Triton X-100 to the substrate solution was necessary to sequester SDS that coelutes with the purified proteinases following preparative electrophoresis (4); otherwise, the substrates precipitated on addition of the enzyme solution.

From these preliminary experiments, we established the colorimetric assay conditions for the enzymes as follows. GPR-pNA or GPK-pNA was used as the substrate, a reducing agent and Triton X-100 were included in the assay mixture, and the hydrolysis of substrate was allowed to proceed at pH 7.9 and 25°C. The rate of hydrolysis was measured on the basis of the increase in A_{405}, using an Ultrospec II spectrophotometer (LKB) with a thermostated cuvette holder and a chart recorder. During enzyme purification, the synthetic peptide substrates detected enzyme activity in the same fractions as did the zymograms (data not shown).

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (12) under nonreducing conditions, using a Protean II or Mini-Protean II (Bio-Rad) electrophoresis system. Protein samples were mixed with sample buffer (3:1) (0.02% bromphenol blue, 160 mM Tris-HCl, 60% sucrose, 7% SDS [pH 8.8]) and, except as noted in the figure legends, were not boiled prior to electrophoresis. Electrophoresis was performed at 4°C with constant voltage (175 V per gel). Gels were stained with Coomassie brilliant blue R-250 or with silver (16).

Zymography. Proteinases were detected by using fibrinogen-containing gels as previously described (14). Briefly, plasminogen-free human fibrinogen (1 mg/ml, final concentration) was added to acrylamide solutions before the gels were cast, and the protein was copolymerized with the gel. After electrophoresis, gels were washed first with 2.5% Triton X-100 in distilled water, then with 2.5% Triton X-100 in TB, and finally with TB. The washing procedure was performed over a 30-min period to remove SDS. Proteolytic bands were activated in the washed gels by incubation for 1.5 h in TB containing 50 mM L-cysteine at 37°C. Finally, the gels were fixed in 50% methanol–7% acetic acid and stained with Coomassie brilliant blue R-250 in 30% methanol–5.8% acetic acid. Lytic bands were visualized as clear zones against the blue background of the gel after destaining.

Determination of the isoelectric points. The isoelectric points of the proteinases were determined using a Rotofor Preparative Isoelectric Focusing Cell (Bio-Rad). Fractions containing the peak of proteolytic activity following chromatography on DEAE-Sephacel were pooled and dialyzed against 0.5% CHAPS in distilled water until the salt concentration was below 10 mM. Pharmalyte (pH 3 to 10; Pharmacia) was added to the dialyzed sample to give a final concentration of 2%. The Rotofor chamber was filled with a solution containing 0.5% CHAPS, 5 mM β-mercaptoethanol, and Pharmalyte (pH 3 to 10; 2%, final concentration), and the solution was prefocused for 1 h at 4°C to establish the pH gradient. The prepared sample was added to the Rotofor cell and focused for 4 h at 4°C, after which fractions were collected as instructed by the manufacturer. The fractions were assayed for proteolytic activity by zymography in fibrinogen-containing gels.

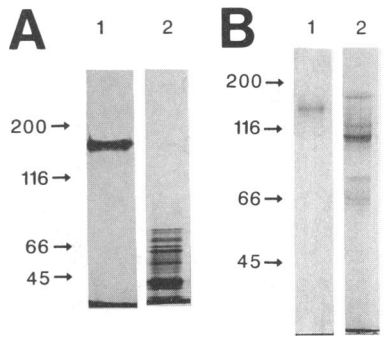

FIG. 2. (A) Effect of heating on the electrophoretic mobility of the 150-kDa proteinase. Samples of the 150-kDa proteinase were analyzed by SDS-PAGE in 3 to 9% polyacrylamide gradient gels under nonreducing conditions and stained with silver. Lane 1, 150-kDa proteinase (approximately 10 μg of protein); lane 2, same as lane 1 except boiled for 3 min in the sample buffer and concentrated prior to electrophoresis. (B) Effects of proteinase inhibitors on the electrophoretic mobility of the 150-kDa proteinase. Samples were analyzed by SDS-PAGE in a 7% polyacrylamide gel. Samples of the 150-kDa proteinase were incubated at room temperature with L-cysteine (50 mM) for 5 min prior to addition of YPRCK (0.1 mM, final concentration) and TLCK (0.1 mM, final concentration), after which incubation was continued for an additional 10 min. Samples were then mixed with the sample buffer and loaded onto the gel (lane 1) or boiled for 3 min prior to electrophoresis (lane 2). The arrows and numbers at the left indicate the migration distances and molecular masses (in kilodaltons) of standard proteins.

N-terminal amino acid sequencing and peptide mapping. Peptides to be sequenced were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.), and stained with Coomassie brilliant blue R-250. N-terminal amino acid sequencing was performed with a Porton 2090E protein sequencer. Phenylthiohydantoin derivatives were identified by on-line reverse-phase high-performance liquid chromatography (HPLC) with diode array detection. For peptide mapping, samples of the proteinases were reduced, alkylated, and digested with trypsin directly in the gel matrix. Tryptic peptides were extracted from the gel with 2 M urea in 0.1 M NH_4HCO_3 and dried. Analytical reverse-phase HPLC of the tryptic digests was performed with a Vydac C-18 column (5 μm, 2.1 by 25 cm). The trypsin digestions and tryptic peptide mapping were performed at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, Conn.

Protein determination. Protein concentration was determined by measuring the A_{280}, using bovine serum albumin as a standard.

RESULTS

Determination of the sizes, structures, and relationship between the 150- and 120-kDa proteinases. (i) Effects of reducing agents and heating on electrophoretic mobilities of the proteinases. The standard approach for determining whether a protein is composed of disulfide-linked subunits (boiling under reducing conditions and subsequent alkylation prior to SDS-PAGE) turned out to be problematic for these proteinases. Both reducing agents and heating activated the enzymes during sample preparation. As shown in Fig. 2A, boiling the 150-kDa enzyme in the electrophoresis sample

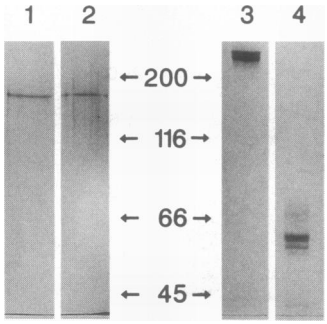

FIG. 3. Effect of DTT on the size of the 150-kDa proteinase. Samples of the 150-kDa proteinase were supplemented with TLCK (1 mM) and incubated for 15 min at 25°C in the electrophoresis sample buffer, alone (lane 1) or with 100 mM DTT (lane 2). Samples of fibrinogen treated in the same manner were incubated in the sample buffer alone (lane 3) or with 100 mM DTT (lane 4). The arrows and numbers in the center indicate the migration distances and molecular masses (in kilodaltons) of standard proteins.

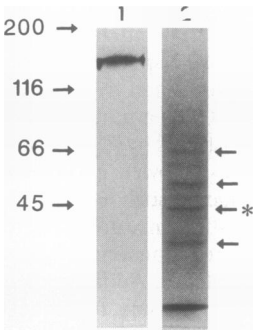

FIG. 4. Autolysis of the 150-kDa proteinase. Proteins were separated by SDS-PAGE (8% polyacrylamide gel). Lane 1, 150-kDa proteinase (10 μg) (untreated protein stained with silver); lane 2, 150-kDa proteinase, heated to 60°C for 15 min in the presence of 100 mM DTT and then concentrated approximately 10-fold prior to electrophoresis (stained with Coomassie brilliant blue R-250). The arrows and the numbers on the left indicate the migration distances and molecular masses (in kilodaltons) of standard proteins. The arrows on the right indicate the migration distances of four major peptides in the autolytic digest. The asterisk denotes the 43-kDa peptide for which amino acid sequence was obtained.

buffer in the absence of reducing agents and subsequent concentration of the boiled sample to facilitate staining resulted in generation of many smaller peptides (lane 2). When reducing agents were added prior to boiling of the sample, identical peptides were visualized; however, visualization of the peptides by silver staining required that the sample be even more highly concentrated after boiling (data not shown). These results suggest that whether or not reducing agents are added, autolysis occurs in the presence of SDS during the brief time before the samples reach an inactivating temperature. Identical results were obtained for the 120-kDa proteinase (data not shown).

To block the apparent proteolysis during heating, the experiment described in Fig. 2A was repeated as follows. The 150-kDa proteinase was activated briefly (5 min) by 50 mM L-cysteine, after which YPRCK and TLCK were added (each at a final concentration of 0.1 mM) prior to boiling of the sample. The results are shown in Fig. 2B. Addition of reducing agents and proteinase inhibitors had no effect on the electrophoretic mobility of the proteinase (lane 1) unless the sample was completely denatured by heating to 100°C (lane 2). After boiling, several discrete bands were observed. One protein, migrating at approximately 180 kDa, was seen, as were several discrete proteins in the ranges of 100 to 120 and 60 to 70 kDa. These data suggest that the 150-kDa proteinase preparation is composed of a 180-kDa protein and several smaller proteins ranging from 100 to 120 and 60 to 70 kDa in size. Proteins of identical size were obtained when the 120-kDa proteinase was treated in the same manner (data not shown). We propose that the 180-kDa protein represents the completely unfolded, intact proteinase. The sizes of the smaller proteins, 100 to 120 and 60 to 70 kDa, suggest that they may have been derived from the 180-kDa protein by internal proteolytic (autolytic) cleavage at highly preferred sites, possibly during enzyme extraction and purification. The proteolytically derived fragments of the enzyme appear to remain associated during purification and SDS-PAGE unless the preparation is boiled.

To further confirm that the proteins present in the 150-kDa proteinase preparation are not held together by disulfide bonds alone, the proteinase preparation was incubated in the sample buffer, in the presence of TLCK, under strong reducing conditions (100 mM DTT), and no change in size was observed (Fig. 3, lanes 1 and 2). Under identical conditions, fibrinogen, used here as an extensively disulfide-linked model protein, was completely reduced to its three nonidentical component polypeptide chains without boiling of the sample (Fig. 3, lanes 3 and 4). The 150-kDa proteinase undergoes limited autolysis when heated at temperatures below 100°C.

When the enzyme was incubated in sample buffer under reducing conditions at 60°C for 15 min, autolysis occurred (Fig. 4). The peptides in the sample were catalytically inactive and had to be concentrated in order to be detected by Coomassie brilliant blue R-250 staining. Four major autolysis peptides were identified (Fig. 4, lane 2). Direct N-terminal sequencing revealed that three of the four peptides were mixtures; however, one of the peptides, migrating in the range of 43 kDa (Fig. 4, lane 2, asterisk), was pure and yielded the sequence ANEAKVVLAADN. This peptide was obtained reproducibly in several different autolytic digests. The data presented thus far suggest that both the 150- and 120-kDa proteinases are SDS-stable conformational variants of a 180-kDa proteinase that are likely formed when the latter is released from the cell surface by detergents. This model also explains why multiple amino acids were released at each cycle when the purified 150- and 120-kDa proteinases were subjected to direct N-terminal amino acid sequencing from polyvinylidene difluoride membranes.

(ii) Isoelectric point and comparative HPLC tryptic mapping. The close relationship of the 150- and 120-kDa proteinases to each other is also supported by three other lines of evidence. First, the two proteinases have the same isoelectric point, 6.1, so it is highly unlikely that their amino acid compositions vary significantly. Second, the relationship of the 150- and 120-kDa proteinases was explored by comparative HPLC tryptic mapping. As can be seen in Fig. 5, in nearly every instance where there is a major peak in the 150-kDa proteinase chromatogram, there is a corresponding major peak in the chromatogram of the 120-kDa proteinase. While there are a few peaks that are unique to each digest, the fact that the profiles are nearly identical confirms their derivation from the same protein. Third, as detailed below, they are catalytically identical.

Characterization of the proteinases. (i) Verification of the

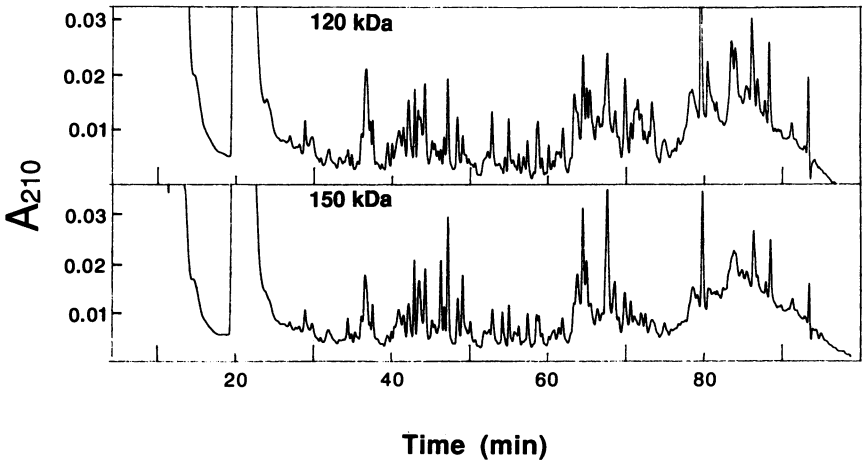

FIG. 5. Comparative HPLC tryptic peptide mapping of the 150-kDa proteinase peptides (bottom) and 120-kDa proteinase peptides (top). Tryptic peptide digests dissolved in water were filtered (0.22-μm-pore-size UltraFree Millipore filter unit) and injected onto a reverse-phase C-18 column equilibrated in a mixture containing 98% solvent A (0.06% aqueous trifluoroacetic acid) and 2% solvent B (0.052% aqueous trifluoroacetic acid in 80% [vol/vol] acetonitrile) at a flow rate of 150 μl/min. Peptides were eluted with increasing concentrations of solvent B as follows: 0 to 63 min (63% A and 37% B), 63 to 95 min (25% A and 75% B), and 95 to 105 min (2% A and 98% B).

specificity of fibrinogen lysis by *P. gingivalis* W12 cells. We have previously shown that *P. gingivalis* W12 cells degrade ^{125}I-fibrinogen in a stepwise fashion, yielding primarily a large fragment with a relative molecular mass of 97 kDa and other, less well defined fragments in the range of 50 kDa, via cell surface-associated 150- and 120-kDa proteinases (14). The 97- and 50-kDa fragments are similar in size to the major fragments, fragments D and E, generated from fibrinogen by the action of plasmin. To explore the possibility that the cell-associated proteinases degrade fibrinogen in a manner similar to plasmin, we incubated freshly harvested *P. gingivalis* W12 cells with unlabeled fibrinogen at 37°C. Under these conditions, fibrinogen was rapidly degraded by the bacteria (Fig. 6), yielding a major cleavage product migrating in the range of 97 to 100 kDa and smaller products migrating in the range of 45 to 50 kDa. Digests containing mainly the 97-kDa cleavage product were separated from bacteria by centrifugation and subsequently subjected to reduction and alkylation in preparation for SDS-PAGE, electrotransfer to polyvinylidene difluoride membranes, and N-terminal sequencing. No peptides larger than 54 kDa were detected. Estimates of the molecular masses of the peptides were determined graphically, and eight peptides were analyzed by N-terminal amino acid sequencing. Two major peptides, 43 kDa, with N-terminal sequence DN ENVVNEYSSELE, and 38 kDa, with the N-terminal sequence SRKMLEEIMKYEAS, were readily identified. These peptides derive from cleavage of the fibrinogen molecule after Lys-132 of the β chain and Lys-85 of the γ chain, respectively. They correspond to the β- and γ-chain fragments found in plasmin-generated fibrinogen D_1 fragments (8). Several of the bands sequenced contained mixtures of peptides. The other peptide clearly identified (15 kDa) had YQISVNKYRG as an N-terminal sequence and derives from cleavage of the β chain following Lys-338. All of the peptides identified were generated by cleavage within the fibrinogen molecule on the carboxyl side of a lysine residue, suggesting that the fibrinogenolytic *P. gingivalis* W12 proteinases degrade fibrinogen via an endopeptidase with a P_1 specificity for lysine (nomenclature according to reference 3).

(ii) Effects of reducing agents on hydrolysis of GPR-pNA and GPK-pNA by the purified proteinases. The purified 150- and 120-kDa proteinases hydrolyzed both GPR-pNA and GPK-pNA. Hydrolysis of both substrates by both enzymes showed an absolute dependence on the presence of reducing agents in the incubation mixture. The effects of reducing agents on the rate of hydrolysis of GPR-pNA by the 150- and 120-kDa proteinases are shown in Fig. 7. Essentially identical results were obtained when GPK-pNA was used as the substrate (data not shown). Both proteinases were activated by L-cysteine, DTT, and β-mercaptoethanol; however, L-cysteine was the most effective activator. The preferential activation of

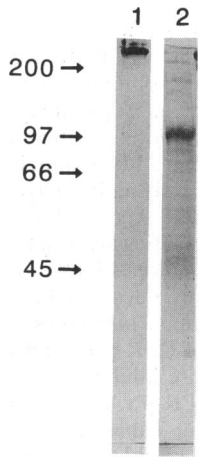

FIG. 6. Fibrinogen lysis by freshly harvested *P. gingivalis* W12 cells. Bacteria (5×10^8 cells) were incubated in TB with fibrinogen (140 μg) in a final volume of 0.5 ml for 30 min at 37°C. Bacteria were removed from the incubation mixture by centrifugation, and TLCK (10 mM, final concentration) was added to the supernatant fraction. The supernatant fraction was analyzed by SDS-PAGE (7.5% polyacrylamide gel) under nonreducing conditions. Lane 1, 10 μg of fibrinogen incubated without bacteria; lane 2, the supernatant fraction (33 μl) from the incubation with the bacteria. The gel is stained with Coomassie brilliant blue R-250. The arrows and numbers on the left indicate the migration distances and relative molecular masses (in kilodaltons) of standard proteins.

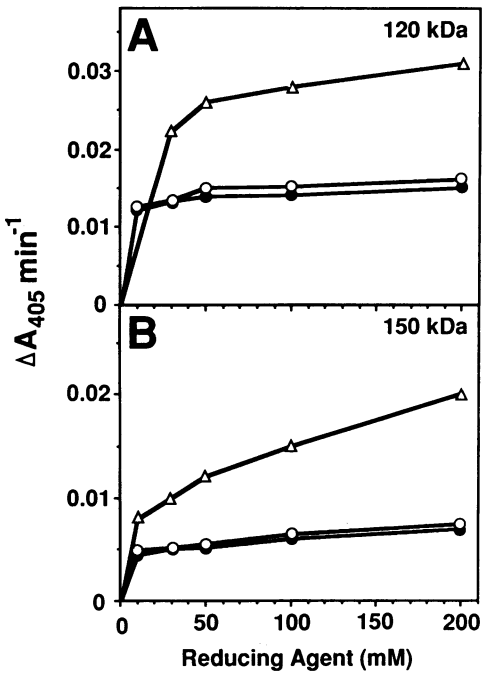

FIG. 7. Effects of reducing agents on hydrolysis of GPR-pNA by the 150- and 120-kDa proteinases. Samples of enzyme were incubated in 1 ml (total volume) of a solution containing 50 mM Tris-HCl buffer (pH 7.9), 0.2 mM GPR-pNA, 0.1% Triton X-100, and a reducing agent (β-mercaptoethanol [●], DTT [○], or L-cysteine [△]). Hydrolysis of the substrate at 25°C was monitored with an LKB Ultrospec II equipped with a thermostated cuvette holder and a chart recorder. Initial velocities were calculated from reaction progress curves recorded continuously for the first 5 min of the reaction.

the proteinases by L-cysteine may be due to the ability of L-cysteine to act both as a reducing agent and as a stimulatory agent for amidolytic activity of these proteinases. This unusual effect of L-cysteine on proteinase and amidase activity of a 50-kDa proteinase (gingipain) purified from *P. gingivalis* (6, 7) has been described in detail. Gingipain is an Arg-specific amidase and proteinase, and its amidolytic activity is stimulated by L-cysteine as well as by derivatives of glycine. Our proteinases possess both Arg- and Lys-amidolytic activities, and both amidolytic activities are stimulated by L-cysteine and derivatives of glycine. The stimulation of both amidolytic activities by glycylglycine is described in detail below (Table 2).

(iii) Effects of proteinase inhibitors, metal ions, and glycylglycine on hydrolysis of GPR-pNA and GPK-pNA. The effects of a variety of proteinase inhibitors, metal ions, and glycylglycine on hydrolysis of GPR-pNA and GPK-pNA by the 120-kDa proteinase are shown in Table 2. Essentially identical results were obtained for the 150-kDa proteinase (data not shown). Both the Arg- and Lys-amidolytic activities of the 150- and 120-kDa proteinases were inhibited by TLCK and YPRCK; however, the Lys-amidolytic activity was significantly more sensitive to inhibition by TLCK than by YPRCK, and the Arg-amidolytic activity was significantly more sensitive to inhibition by YPRCK than by TLCK. These data suggest that both the 150- and 120-kDa proteinases contain two discrete active sites, one more sensitive to YPRCK and one more sensitive to TLCK. The YPRCK-sensitive active site is also sensitive to inhibition by leupeptin, whereas the TLCK-sensitive site is not. The YPRCK-sensitive site is sensitive to inhibition by the arginine analogs agmatine and methylguanidine, whereas the TLCK-sensitive site is not. Neither the Arg-amidolytic activity nor the Lys-amidolytic activity was sensitive to the serine proteinase inhibitor DFP or PMSF, nor were they sensitive to inhibition by *o*-phenanthroline. The thiol-blocking agents iodoacetamide and NEM were slightly to moderately inhibitory; however, it is difficult to assess their inhibitory activities in the presence of reducing agents needed for enzyme activation. Hydrolysis of both substrates was inhibited by EDTA, and in both cases the inhibition could be reversed by adding Ca^{2+} ion to the reaction mixture. Since Ca^{2+} by itself did not activate the enzymes, we conclude that Ca^{2+} is tightly bound to the enzymes and is probably required for conformational stabilization.

Both the Arg- and Lys-amidolytic activities of the 150- and 120-kDa proteinases were stimulated to about the same extent, in a concentration-dependent manner, by glycylglycine. This unusual property—stimulation of amidolytic activity of a proteinase by glycylglycine—has been reported for only one other enzyme, gingipain, an Arg-specific 50-kDa cysteine proteinase purified from the culture medium of another strain of *P. gingivalis* (6, 7). In the case of our proteinases, when DTT was used instead of L-cysteine as the reducing agent, more pronounced stimulation of Arg- and Lys-amidolytic activities, five to seven times the level of controls, could be achieved. When the proteinases were reduced with DTT (100 mM) and stimulated by glyclyglycine (200 mM), the rate of hydrolysis of both substrates approached the level achieved by L-cysteine (100 mM) in the presence of glycylglycine (200 mM), suggesting that the ability of L-cysteine to preferentially activate the proteinases is a function of its ability to both reduce the enzymes and activate them in a manner similar to glycylglycine.

Results of the biochemical characterization of the 150- and 120-kDa proteinases suggest that they are cysteine proteinases with two types of active sites. One type of active site (YPRCK and leupeptin sensitive) requires arginine in the P_1 position; the other type of active site (TLCK sensitive and leupeptin insensitive) requires lysine in P_1. In other respects, i.e., sensitivity to inhibition by EDTA and stimulation by glycine derivatives, these active sites are similar. The stability of the active sites of the purified proteinases was examined every 3 days for a period of 2 weeks following purification of the enzymes. There was no change in the absolute or relative amounts of Arg- or Lys-amidolytic activity of the purified enzymes over this period.

(iv) Endopeptidase activities of the purified proteinases. Both of the purified proteinases degrade fibrinogen into small peptides (data not shown). Since the thiol activation required by the purified enzymes has the simultaneous effect of reducing fibrinogen to fibrinogen chains, it is not possible to determine whether D-like fragments are generated from fibrinogen by the purified proteinases.

DISCUSSION

In a previous study (14), we identified two cell surface-associated proteinases, 150 and 120 kDa, of *P. gingivalis* W12 and provided evidence suggesting that these enzymes mediate fibrinogen lysis by *P. gingivalis* cells. In this study, we have purified these proteinases to apparent homogeneity. While we cannot rule out that minor differences arising from proteolytic processing exist between the 150- and 120-kDa proteinase preparations, our data suggest that these proteinases are SDS-stable conformational variants of the same 180-kDa proteinase. Our data also suggest that these enzymes undergo proteolytic (possibly autolytic) degradation during purification;

TABLE 2. Effects of proteinase inhibitors, metal ions, and glycylglycine on hydrolysis of GPR-pNA and GPK-pNA by the 120-kDa proteinase[a]

Additive	Concn (mM)	Residual activity (%)	
		GPR-pNA hydrolysis	GPK-pNA hydrolysis
Expt A			
TLCK	0.0001	100	77
	0.0005	100	47
	0.001	89	29
	0.01	69	9
	0.1	10	0
	1	0	0
YPRCK	0.0001	27	100
	0.0002	19	100
	0.0005	8	100
	0.001	3	100
	0.01	0	83
	0.1	0	53
	1	0	0
TPCK	0.1	67	88
PMSF	0.1	91	100
DFP	1	89	100
Iodoacetamide	5	79	29
NEM	5	80	80
Expt B			
L-Arginine	0.1	88	95
Benzamidine	0.01	92	100
Agmatine	0.01	40	92
Methylguanidine	0.01	63	83
Leupeptin	0.001	0	100
	0.1	0	93
Chymostatin	0.001	92	88
Elastatinal	0.001	94	100
Expt C			
o-Phenanthroline	1	95	100
$MgCl_2$	1	100	100
$CaCl_2$	1	100	100
Expt D			
EDTA	0.01	87	83
	0.1	0	7
	1	0	9
Expt E			
Glycylglycine	20	100	122
	200	250	200

[a] The initial velocity of substrate hydrolysis was measured at 25°C by using a chart recorder. The residual activity measured without additives was assumed to be 100% in each experiment. In experiment A, the additive solution (2 μl) was added to 98 μl of 25 mM Tris-HCl buffer (pH 7.9) containing 0.05% Triton X-100, 50 mM L-cysteine, and enzyme. After preincubation for 5 min, 1 ml of substrate solution (50 mM Tris-HCl buffer [pH 7.9] containing 0.1% Triton X-100, 100 mM L-cysteine, and 0.22 mM substrate) was added. The concentrations of the additives in the preincubation mixture are shown. In experiments B and C, enzyme (40 μl) was added to 1 ml of 50 mM Tris-HCl buffer (pH 7.9) containing 0.1% Triton X-100, 100 mM L-cysteine, 0.05 mM (experiment B) or 0.2 mM (experiment C) substrate, and an additive at the concentration shown. Experiment D was performed like experiment A except that EDTA, at the indicated concentration, was present in both the preincubation mixture and the substrate solution. In experiment E, 100 μl of 25 mM Tris-HCl buffer (pH 7.9) containing 0.05% Triton X-100, 50 mM L-cysteine, and enzyme was preincubated at 25°C for 5 min, after which 1 ml of substrate solution (50 mM Tris-HCl buffer [pH 7.9] containing 0.1% Triton X-100, 100 mM L-cysteine, and 0.22 mM substrate) containing glycylglycine at the concentration shown was added.

however, the proteolytically derived peptides remain associated by noncovalent interactions unless the proteinases are denatured by heating to 100°C. We propose the name "porphypain" for these proteinases, (porphypain-1 for the 150-kDa proteinase and porphypain-2 for the 120-kDa proteinase) to indicate that like clostripain, they are cysteine proteinases with a specificity for a basic amino acid in P_1 (17) and to indicate that they are derived from the genus *Porphyromonas*.

The fact that porphypains are inactivated by chloromethyl ketones and some thiol-reactive agents and are unaffected by serine proteinase inhibitors such as DFP and PMSF suggests that they are cysteine proteinases. They are the largest cysteine proteinases yet purified from *P. gingivalis*. The proteinases appear to possess two types of active sites, a YPRCK- and leupeptin-sensitive active site that requires arginine in P_1 and a TLCK-sensitive and leupeptin-insensitive active site that requires lysine in P_1. These active sites are identical in sensitivity to EDTA and in the unusual property of being stimulated by glycylglycine. From a slightly different view, the two active sites differ only in that the S_1 subsite of one requires a delocalized positive charge (arginine) in P_1, whereas the S_1 subsite of the other requires a localized positive charge (lysine)

in P_1. From the data obtained thus far, it is not possible to determine whether two distinct active sites are present in each porphypain molecule or whether two classes of proteinase molecules exist, each with one or the other of the active sites. It may be that the two active sites are stable, conformational variants of the same active site. Further studies will be required to distinguish between these possibilities.

The enzymes we have purified in this study (porphypains) have similarities with other proteinases purified from *P. gingivalis* (6, 9, 21, 23, 24, 28). In particular, the Arg-amidolytic activity of porphypains appears to be identical to that of gingipain. From the similarity of the properties of gingipain and porphypains, it is highly likely that gingipain is a proteolytically derived fragment of prophypains. Pike et al. (24) have reported that a peptide with an N-terminal sequence identical to that of the 43-kDa peptide we have identified (ANEAKV VLAADN) is found associated with both high-molecular-weight gingipain and a 60-kDa lysine-specific proteinase, lys-gingipain. These authors consider this ANEAKVVLAADN peptide to be a hemagglutinin complexed with discrete arginine-specific (gingipain) and lysine-specific (lys-gingipain) proteinases; however, our data suggest that this peptide is a proteolytically derived, integral part of the proteinase molecule. The proteinases of *P. gingivalis* purified and described by Pike et al. (24) were obtained from spent culture medium. From our results, it is highly likely that proteinases obtained from culture media have undergone proteolytic (autolytic) degradation, and this may account for the consistent finding of enzymes similar to porphypains but smaller in size, being detected in and isolated from culture medium.

It is clear that *P. gingivalis* strains possess several cysteine proteinases. Several apparently unrelated *P. gingivalis* proteinases have recently been cloned and sequenced (*prtA*, GenBank accession number L27483 [5, 22]). While it is probably premature to speculate on the relationship of these enzymes to porphypains, the ANEAKVVLAADN sequence is not present in the deduced amino acid sequence of *prtT* or *tpr*; however, it is present in the deduced amino acid sequence of *prtA*. The ANEAKVVLAADN sequence is also present in the deduced amino acid sequence of a gene cloned from *P. gingivalis* W12 in this laboratory that appears to encode a protein larger than that encoded by *prtA* (14a).

It is highly significant that an organism proposed as an etiologic agent of human periodontitis has on its surface a cysteine proteinase which behaves like plasmin. Moreover, *P. gingivalis* may release this enzyme, in a variety of forms, into the surrounding tissues. The activity of the host proteinase plasmin is under intricate, multilevel control (26), suggesting that in order for the host to maintain homeostasis, the activity of plasmin must be highly restricted to act locally and must be inactivated rapidly when its activity is no longer required. Plasmin, together with the matrix metalloproteinases that it activates, is responsible for the turnover of most of the proteins in basement membranes and the extracellular matrix. In addition to its role in the lysis of fibrin, plasmin has been implicated as a mediator of cell migration during both normal and pathological processes (25). A bacterial cell that has a plasminlike activity on its surface could mediate direct damage to host tissues as well as evade specific and nonspecific host defense systems. It could also interfere with the regulation of the plasminogen system, and it may activate latent matrix metalloproteinases. The possession of cell surface lysyl endopeptidase activity renders *P. gingivalis* highly adapted for survival in inflammatory lesions like the periodontal pocket and may facilitate bacterial invasion of host tissues. Because of its location on the cell surface, porphypain may be a prime mediator of *P. gingivalis*-host interactions.

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