Purification and Characterization of a Novel Cysteine Proteinase (Periodontain) from Porphyromonas gingivalis

EVIDENCE FOR A ROLE IN THE INACTIVATION OF HUMAN α1-PROTEASE INHIBITOR®

(Received for publication, December 11, 1998, and in revised form, February 10, 1999)

Daniel Nelson, Jan Potempa‡, Tomasz Kordula§, and James Travis¶

From the Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602 and the Departments of ‡Microbiology and Immunology and §Animal Biochemistry, Jagiellonian University, 31-120 Krakow, Poland

Periodontal disease is characterized by inflammation of the periodontium manifested by recruitment of neutrophils, which can degranulate, releasing powerful proteinases responsible for destruction of connective tissues, and eventual loss of tooth attachment. Although the presence of host proteinase inhibitors (serpins) should minimize tissue damage by endogenous proteinases, this is not seen clinically, and it has been speculated that proteolytic inactivation of serpins may contribute to progression of the disease. A major pathogen associated with periodontal disease is the Gram-negative anaerobe Porphyromonas gingivalis, and in this report, we describe a novel proteinase that has been isolated from culture supernatants of this organism that is capable of inactivating the human serpin, α1-proteinase inhibitor, the primary endogenous regulator of human neutrophil elastase. This new enzyme, referred to as periodontain, belongs to the cysteine proteinase family based on inhibition studies and exists as a 75-kDa heterodimer. Furthermore, periodontain shares significant homology to streptopain, a proteinase from Streptococcus pyogenes, and prtT, a putative proteinase from P. gingivalis. Clearly, the presence of this enzyme, which rapidly inactivates α1-proteinase inhibitor, could result in elevated levels of human neutrophil elastase clinically detected in periodontal disease and should be considered as a potential virulence factor for P. gingivalis.

The anaerobe Porphyromonas gingivalis has been strongly implicated as a major causative organism of adult onset periodontal disease (1–4). Enzymes from this organism have been found to degrade collagen, fibrinogen, immunoglobulins, complement proteins, and fibronectin, among others (for reviews, see Refs. 5–8). Recent evidence has shown that three proteinases released from P. gingivalis, referred to as gingipains R (Rgp A and Rgp B) and gingipain K because of their ability to cleave specifically after arginine and lysine residues, respectively, may have a more physiologically relevant role in modulating the human immune system rather than in their general ability to degrade proteins. Working in concert, these proteinases have already been shown to produce bradykinin from high molecular weight kinogen, either directly or indirectly (kallikrein activation), resulting in vascular permeability enhancement (9). This mechanism, which is used to provide nutritional components for the growth and proliferation of P. gingivalis, is presumed to be responsible for both the increased gingival crevicular fluid (GCF) and edema noted clinically in the periodontal pockets of patients with advanced periodontitis (10).

The interaction of P. gingivalis with the host innate immune response has been paradoxical, with both pro- and anti-inflammatory responses reported. For example, P. gingivalis lipopolysaccharide has been shown to increase mRNA levels of interleukin 8 in neutrophils (11), whereas gingipains R have been shown to increase neutrophil chemotaxis by release of C5a from C5 of the complement system (12). However, these same proteinases are also capable of cleaving the C5a receptor from infiltrating neutrophils (13), effectively neutralizing their localized chemotactic activity. Additionally, P. gingivalis cells have the ability to inhibit both interleukin 8 accumulation in gingival epithelial cells (14) as well as transepithelial migration (15). These contradictions may be explained by an apparent compartmentalization in the periodontal cavity, whereby distal activation of chemotactic components and proximal paralysis of these same factors create a “leukocyte wall” between the periodontal plaque and gingival epithelium (16). Indeed, it has recently been reported that soluble gingipains can stimulate interleukin 8 activity, whereas membrane-bound gingipains, with a limited ability to diffuse beyond the plaque surface, completely degrade interleukin 8 (17).

The recruitment of neutrophils to the leukocyte wall through both the increased leakage of blood vessels and a chemotactic gradient would at first seem suicidal to P. gingivalis. However, this is not likely to be the case, as this organism has evolved mechanisms to survive in the presence of neutrophils. P. gingivalis proteinases have been shown to degrade C3 of the complement system and immunoglobulins (18, 19), thereby averting opsonization and subsequent detection by the host. Furthermore, gingipain R has been shown to have an inhibitory effect on the oxidative burst utilized by neutrophils to kill microorganisms (20). Similarly, the bacterial outer membrane of P. gingivalis can act as an antioxidant sink due to the incorporation of large amounts of heme (21).

Activated neutrophils in the leukocyte wall undergo degranulation, due to the inability to phagocytize foreign organisms, thereby expelling large quantities of human neutrophil elastase (HNE) and cathepsin G. Although these proteinases can...
cause abnormal connective tissue destruction, the presence of human plasma proteinase inhibitors (serpins) should minimize this process as they would complex with the endogenous proteinases and be taken up by the liver for degradation. In fact, high protein levels of α,-proteinase inhibitor (α,-PI) have been detected in GCF samples from patients with severe periodontal disease (22); however, despite the presence of this inhibitor, there also remains a high HNE activity (23, 24), indicating that the former must be present in either complexed, oxidized, or proteolytically inactivated forms. This is supported by evidence showing that less than 35% of available α,-PI in the GCF is active as an inhibitor (25). Furthermore, it has been shown that individuals with α,-PI deficiencies have a significantly higher frequency of periodontal pocket depths ≥5 mm, thereby predisposing them to manifestations of periodontal disease (26).

Recently, we provided preliminary data that indicated that *P. gingivalis* elicited a proteinase that rapidly inactivated α,-PI (27). This enzyme is believed to be at least partially responsible for the altered balance between the levels of HNE and functional inhibitor in the GCF. In this report, we describe the purification and properties of this enzyme, which we refer to as periodontain, not only because of its function as a cysteine proteinase but also because it may act as a putative factor in the dysregulation of α,-PI function in the periodontal cavity. In addition, we provide the deduced protein sequence of periodontain as determined by both partial peptide sequencing of the purified protein and analysis of the *P. gingivalis* genome.

**EXPERIMENTAL PROCEDURES**

**Materials Used**

Disopropyl fluorophosphate, leupeptin, and 3,4-dichloroisocoumarin were purchased from Calbiochem. All other materials used were obtained from Sigma, unless otherwise indicated, and were of at least analytical grade.

**Methods**

**Bacteria Cultivation**—The strain of *P. gingivalis* (HG66) that was used for the purification of periodontain was a gift of Dr. Roland Arnold (University of North Carolina, Chapel Hill, NC). Cells were grown in 5 liters of broth containing 150 g of trypticase soy broth, 25 g of yeast extract (both from Difco), 25 mg of hemin, 2.5 g of cysteine, 0.5 g of diithiothreitol, and 5 mg of menadione (all from Sigma), anaerobically, at 37 °C for 24 h in an atmosphere of 8% N2, 10% CO2, 5% H2. The seed culture was used to inoculate 100 liters of the same broth, and the bacterium was then grown in a 130-liter fermentor (W.B. Moore, Inc.) at the University of Georgia Fermentation Plant. Cells were grown for 24 h until late stationary phase of bacterial growth (A660 > 2.0). Additional strains, 33277, W50 (both from ATCC), W12, and 381 (both gifts of Dr. Caroline A. Genco, Boston University Medical School), were grown under the same conditions in 1-liter volumes.

**Cellular Localization**—Whole cell culture mixtures were fractionated to determine localization of periodontain. First, a low speed centrifugation (6,000 × g for 20 min at 4 °C) was used to pellet the cells, after which the supernatant was subjected to high speed ultracentrifugation (100,000 × g for 120 min at 4 °C) to separate vesicles from the supernatant, which contained all soluble proteins. For quantitation of proteolytic activity all samples were brought to an equal volume by the addition of 50 mM Tris, pH 7.4. The mass of the native enzyme was determined by following its ability to specifically inactivate α,-PI, using α-chymotrypsin as a target proteinase exactly as described previously (27). Briefly, native α,-PI (0.15 nmol) was mixed with samples containing a putative inactivating activity in assay buffer (50 mM Tris, 10 mM cysteine, pH 7.8) and allowed to incubate for a desired time, after which an equimolar amount of α-chymotrypsin (0.15 nmol) was added to complex any remaining functional α,-PI. The chymotrypsin assay mixtures were then incubated for an additional 30 min at 37 °C. After the incubation, the assay mixtures were clarified by centrifugation (40,000 × g for 30 min), concentrated by ultrafiltration (Amicon PM-10 membrane), and analyzed by SDS-PAGE in 20-ml fractions, each representing 5 liters of starting supernatant, to a Sephadex G-150 column (5 × 105 cm) equilibrated with Buffer A, supplemented with protection against HNE at 30,000 ppm (25). The dialyzed fraction was clarified by centrifugation (40,000 × g for 30 min), concentrated by ultrafiltration (Amicon PM-10 membrane), and analyzed by SDS-PAGE in 20-ml fractions, each representing 5 liters of starting supernatant, to a Sephadex G-150 column (5 × 105 cm) equilibrated with Buffer A, at a flow rate of 30 ml/h. The activity was pooled, dialyzed against 50 mM Tris, pH 7.4 (Buffer B), and further purified by ion exchange chromatography on a Mono-Q column (Amersham Pharmacia Biotech, fast protein liquid chromatography system), with elution in a linear gradient of 0–500 mM NaCl in Buffer B. Activity was concentrated and final purification obtained by separation on a TSK-GEL G3000SW (Toso-Haas) column using 50 mM Tris, 200 mM NaCl, pH 7.4.

**Electrophoresis—** Enzyme purification and visualization of the heavy and light chains was monitored by SDS-PAGE of a 10% separating gel using the TAA-3/-Tricine buffer system, according to Schagger and von Jagow (28). Non-denaturing PAGE (29) in a 4–20% gradient gel was used to show the native protein as a single band.

**Molecular Mass Determination**—The mass of the native enzyme was determined by gel filtration using a TSK-GEL G3000SW (Toso-Haas) calibrated with gel filtration standards (Bio-Rad). The mass of the separated heavy and light chains from SDS-PAGE were estimated by scanning the gel using the Eagle Eye II imaging system (Stratagene) and calculating a linear regression of low molecular weight electrophoresis standards (Amersham Pharmacia Biotech) as reference. Accurate molecular mass measurements of the digestion products of α,-PI after enzymatic inactivation employed the use of matrix-assisted laser desorption ionization, with mass spectra acquired using a Vesta matrix assisted laser desorption ionization linear time-of-flight mass spectrometer (Perspective Biosystems) at the Mass Spectroscopy Facility (University of Georgia, Athens, GA) according to the manufacturer’s instructions.

**Protein Sequence Analysis**—For amino-terminal sequence analysis, proteins resolved by electrophoresis were electrophoresed onto a polyvinylidene difluoride membrane according to Matsudaiva (30). Sequence analysis was performed with an Applied Biosystems 4760A gas-phase sequencer at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA) according to the manufacturer’s recommendations.

**Inhibition Studies**—For inhibition studies, periodontain was used at a concentration that was capable of completely inactivating 0.15 nmol of α,-PI in our standardized assay (27) after exactly 1 h of incubation.

---

**Fig. 1. Periodontain is a heterodimer as a native protein.** A. Non-denaturing PAGE of 1 μg pure periodontain (lane 1) and 5 μg of albumin (lane 2). Note that albumin forms dimers and, to a lesser extent, trimers when subjected to electrophoresis under non-denaturing conditions. B. 10% SDS-PAGE of 1 μg of pure periodontain indicating the presence of heavy and light chains.
Representatives of the various classes of proteinase inhibitors, at indicated concentrations, were preincubated with enzyme for 5 min prior to the addition of α1-PI. The cleavage of inhibitor (%) was normalized to a native inhibitor control in order to give the relative percent inactivation for each compound or protein tested against periodontain. The compound, t-trans-epoxysoyuccinyl-leucylamide-(4-guanidino)-butane (E-64), which stoichiometrically inhibits cysteine proteinases of the papain family, was used to titrate periodontain. This allowed us to quantitate stock solutions of periodontain in terms of active enzyme and make dilutions to the desired concentrations for the degradation experiments described below.

**Protein and Peptide Degradation by Periodontain**—The degradation of proteins was followed by using either native or reduced, carboxymethylated, maleylated lysozyme (30 μM). Either protein was incubated with periodontain (30 nM) in a final volume of 20 μl of assay buffer for specific time intervals. The reaction was stopped by addition of 20 μl of SDS sample buffer (4% SDS, 20% glycerol, 0.125% Trit-2-HCl, pH 6.8), followed by boiling for 5 min, after which the entire sample was electrophoresed on a 12% gel and stained with 0.1% Coomassie Brilliant Blue to visualize the protein bands.

To determine the cleavage sites within the reactive site loop (RSL) of α1-PI, inhibitor (20 μg) was incubated with 1 μg of periodontain for 4 h, after which the sample was subjected to 16% SDS-PAGE to separate the ~3-kDa fragment obtained by cleavage within the loop. The fragment was analyzed for both amino-terminal sequence and molecular mass using an automated protein sequencer and mass spectroscopy, respectively, as described above.

For analysis of the fragments obtained through digestion of the insulin β-chain, periodontain (8 nM) was incubated with this peptide substrate (40 μM) in assay buffer in a final volume of 90 μl for desired time intervals. After stopping the reaction by addition of 10 μl of 1 N HCl, samples were centrifuged (10,000 × g for 2 min), and the entire supernatant (100 μl) was subjected to reverse-phase high-pressure liquid chromatography. Sample application to a Beckman Ultrasphere 5 μm ODS column (4.6 × 250 mm) equipped with an Ultrasphere 5 μm ODS guard column (4.6 × 45 mm) was carried out in 0.1% trifluoroacetic acid in water (solvent A) and separations were performed with a linear gradient of 0.08% trifluoroacetic acid in 80% acetonitrile/water (solvent B) over 40 min at a flow rate of 1 ml/min. The peptide elution was monitored at 215 nm.

**Gelatin Zymograph**—Zymography analysis on gelatin gels was performed on pure samples of periodontain in the presence of 5 mM cysteine, with or without 100 μM E-64. After the addition of SDS sample buffer, the samples were subjected to electrophoreses at 4 °C on a 10% gel. The gel was washed twice with 2.5% (w/v) Triton X-100 to remove the SDS and then incubated in activation buffer (50 mM Tris, 20 mM cysteine, pH 7.4) at 37 °C for 2 h. The zymogram was developed in 0.1% Amido Black, with clearing zones indicating proteolytic digestion of the incorporated gelatin.

**Cloning of Gene Fragment Encoding the Amino Terminus of Periodontain**—Based on the amino-terminal sequence (23 residues) of the native inhibitor control in order to give the relative percent inactivation for each compound or protein tested against periodontain. The compound, t-trans-epoxysoyuccinyl-leucylamide-(4-guanidino)-butane (E-64), which stoichiometrically inhibits cysteine proteinases of the papain family, was used to titrate periodontain. This allowed us to quantitate stock solutions of periodontain in terms of active enzyme and make dilutions to the desired concentrations for the degradation experiments described below.

**RESULTS**

**Enzyme Purification**—Previous experience in purifying enzymes from culture supernatants of *P. gingivalis* indicated that ice-cold acetone precipitation as an initial step was successful in separating active proteinases from the bulk of peptides and proteins present in or released into the growth medium. Similarly, G-150 gel filtration as an early step was also utilized (32, 33), both to resolve proteins into rough molecular weight fractions and to remove the excess heme and phytoheme that coprecipitate during acetone treatment. Subsequent anion exchange chromatography (Mono Q) and gel filtration (TSK) allowed periodontain to be purified to near homogeneity (Fig. 1A). Our yield of 11% corresponded to over 1 mg of pure protein per each 5 liters of starting culture fluid (Table I).

**Physical Properties**—TSK gel filtration of the pure enzyme yielded a single protein peak that eluted with a molecular mass of ~75 kDa, based on a linear regression data analysis from standards (data not shown). However, SDS-PAGE yielded two distinct bands at 55 and 20 kDa, suggesting the presence of a heterodimer (Fig. 1B). Amino-terminal sequence analysis of each of these subunits yielded the sequences of TEGPATE-VHAI MDNQHPANDPMR and DEWKIGSVSVK for the heavy (55 kDa) and light (20 kDa) chains, respectively. Analysis of the single protein species isolated from a nondenaturing gel (Fig. 1A) gave two new amino-terminal sequences in equimolar quantities, which corresponded to those described above, confirming that periodontain is a heterodimer. It should also be noted that either heating of the sample and/or presence of SDS in the gel buffer was sufficient to separate the heavy and light chains on electrophoresis, suggesting that the native heterodimer was stabilized by ionic-hydrophobic interactions rather than a disulfide bridge or covalent bonds. Isoelectric focusing yielded a pI of 5.3 for the native protein (data not shown), whereas gelatin zymography indicated that the 55-kDa heavy chain contained the catalytic active site, but the 20-kDa light chain was devoid of enzymatic activity (Fig. 2).

**Stability**—Periodontain activity was detected over a broad pH range of 6.0–9.0, with the optimum being 7.5–8.0. The
Periodontain, a Novel P. gingivalis Endopeptidase

enzyme was stable at 37 °C overnight, and at 4 °C for several weeks, when stored in the absence of cysteine. The presence of reducing agent resulted in a 50% loss of activity at 37 °C overnight, presumably because of autodigestion. Heating to 60 °C caused complete loss of activity. Samples were routinely stored at −80 °C for several months with less than a 10% loss in activity.

**Activation and Inhibition**—Periodontain was completely inactive in the absence of reducing agents, whereas full activity was achieved with either free cysteine, β-mercaptoethanol, dithiothreitol, or dithioerythritol at 0.1 mM concentration. Unlike indicated that cleavage took place after the glutamic acid (P 5), we examined the activity of periodontain on native proteins. Furthermore, increasing concentrations of these reagents (up to 10 mM) did not cause any additional stimulation of periodontain activity. Finally, Ca²⁺ did not have a stabilizing effect, and glycyl-glycine did not stimulate activity, indicating additional differences between periodontain and the gingipains (data not shown). Based on its requirement for a reducing environment to become active, periodontain can be classified as a cysteine proteinase, and this is confirmed by the fact that it is readily inhibited by common cysteine proteinase inhibitors (Table II).

**Enzyme Specificity**—Periodontain was originally identified as a unique proteinase that was able to inactivate α₁-PI through proteolytic cleavage. From the 3-kDa size difference of cleaved versus native α₁-PI noted on SDS-PAGE (Fig. 3A), we speculated that periodontain caused hydrolysis within the exposed carboxyl-terminal RSL of this molecule. Sequencing of the peptide generated by incubation of α₁-PI with periodontain indicated that cleavage took place after the glutamic acid (P 5), and to a lesser extent, phenylalanine (P 7), of the α₁-PI RSL (Fig. 3B). However, screening numerous synthetic para-nitroanilide substrates with either Glu or Phe specificity in the analogous position yielded no detectable cleavage by periodontain. Indeed, even when we used the synthetic substrate Phe-Leu-Glu-para-nitroanilide, which mimics the P7, P6, and P5 residues within the RSL of α₁-PI, no hydrolysis was detected, indicating that a specific amino acid residue at the site of hydrolysis does not dictate the specificity of this enzyme.

We then investigated the activity of periodontain on protein and peptide substrates to further elucidate cleavage specificity. However, the insulin β-chain was hydrolyzed to such an extent that individual cleavages could not be mapped, even at low E:S molar ratios (1:5000). Rather, the high pressure liquid chromatography analysis revealed no less than 10 peptides were generated from this 30-amino acid polypeptide within 15 min, and complete digestion occurred in 60 min (data not shown). Next, we examined the activity of periodontain on native proteins. However, the enzyme was unable to degrade azocasein, casein,

![FIG. 2. Periodontain heavy chain contains the catalytic active site.](image)

Gelatin zymography of the pure enzyme was performed as described under "Experimental Procedures." Zones of enzymatic activity are indicated by negative staining. The four left lanes contain a serial dilution of purified periodontain (1–0.125 μg), and the four right lanes contain identical dilutions of enzyme preincubated with 100 μM E-64 for 5 min prior to electrophoresis.

![FIG. 3. Inactivation of α₁-PI by periodontain.](image)

A, α₁-PI (2 μM) was incubated with periodontain (2 nM) for 0, 15, 30, 45, and 60 min before the reaction was stopped by the addition of SDS sample buffer. Right lane, sample was preincubated with 100 μM E-64 for 5 min prior to 60 min of incubation with α₁-PI. The samples were electrophoresed on a 12% separating gel. B, samples were separated on a 16% peptide gel to isolate the 3-kDa fragment produced by this cleavage. Both aminoterminal amino acid sequence analysis and mass spectroscopy of the isolated fragment revealed that the major cleavage site was after the P5 glutamic acid (thick arrow), and a minor cleavage site was after the P7 phenylalanine (thin arrow), relative to the P1-P3 methionine-serine bond that forms the "bait" region of the RSL and is attacked by HNE at the position indicated by an asterisk.

![FIG. 4. Effect of periodontain on lysozyme and reduced, carboxymethylated, or maleylated lysozyme.](image)

Periodontain (30 nM) was incubated with 30 μM of either lysozyme or reduced, carboxymethylated, maleylated lysozyme (lysozyme-RCM) for the indicated times, and the reaction was stopped by SDS sample buffer and electrophoresed on a 12% gel.

| Inhibitor Class | Inhibition | Concentration | Inhibition |
|-----------------|------------|---------------|------------|
| EDTA            | Metallo    | 25 mM         | 0          |
| Dichloroisoucumin | Serine     | 0.5 mM        | 2          |
| Disopropylfluorophosphate | Serine | 0.5 mM        | 5          |
| Leupeptin       | Serine/Cysteine | 50 μM        | 88         |
| Tosyl-L-lysine chloromethyl ketone | Serine/Cysteine | 5 mM       | 91         |
| Phe-Pro-Arg chloromethyl ketone | Serine/Cysteine | 10 μM       | 99         |
| Z-Phe-Lys benzyloxy methyl ketone | Cysteine | 10 μM        | 100        |
| Iodoacetamide   | Cysteine   | 5 mM          | 100        |
| E-64            | Cysteine   | 100 μM        | 100        |
lysozyme, collagen, fibrin, plasminogen, and fibrinogen (data not shown). In contrast, when lysozyme was reduced, carboxymethylated, or maleylated, complete digestion was noted in less than 10 min (Fig. 4). These results, together with activity detected on the gelatin zymograph, indicate that periodontain cleaves denatured or easily accessible polypeptide chains, but it cannot cleave whole proteins with defined secondary or tertiary structure, α₁-PI being the exception.

Structure of the Periodontain Gene—The region encoding for the amino-terminal sequence of the 55-kDa catalytic subunit of periodontain was amplified by PCR using degenerate primers and P. gingivalis strain W83 genome, and streptopain, from S. pyogenes, were aligned according to homology modeling (black boxes indicate identity, and gray boxes indicate similarity). The putative catalytic cysteine and histidine residues of streptopain are marked (+). The single underlined residues indicate the obtained amino-terminal sequence of the light chain of periodontain, whereas the double underlined residues indicate the sequence obtained from both amino-terminal sequencing of the heavy chain and DNA sequencing of the 69 base pair long PCR product. Arrows indicate the putative cleavage sites necessary to form the mature periodontain heterodimer from the nascent polypeptide.

FIG. 5. Sequence alignment of periodontain, PrtT, and streptopain. The putative gene products of prtT and periodontain, both deduced from P. gingivalis strain W83 genome, and streptopain, from S. pyogenes, were aligned according to homology modeling (black boxes indicate identity, and gray boxes indicate similarity). The putative catalytic cysteine and histidine residues of streptopain are marked (+). The single underlined residues indicate the obtained amino-terminal sequence of the light chain of periodontain, whereas the double underlined residues indicate the sequence obtained from both amino-terminal sequencing of the heavy chain and DNA sequencing of the 69 base pair long PCR product. Arrows indicate the putative cleavage sites necessary to form the mature periodontain heterodimer from the nascent polypeptide.

would have a predicted molecular mass of 76,727 Da, composed of a 52,981-Da catalytic heavy chain and a 23,764-Da carboxy-terminal light chain, with a calculated pI of 5.18. This is virtually in complete agreement with our experimental findings of a native protein of 70–80 kDa by gel filtration, composed of a catalytic subunit of 55 kDa and a noncatalytic subunit of 23 kDa by SDS-PAGE, with a pI of 5.3. All of these data suggest a potential role in the processing of the pro-form of periodontain by both gingipains R and gingipain K, all of which are abundantly present in P. gingivalis.

Distribution of Periodontain—Using the cloned 69-base pair catalytic amino-terminal fragment as a probe, Southern blot analysis of the W50 strain revealed a single hybridizing band with each of the restriction enzyme digests (data not shown). Furthermore, an α₁-PI inactivating activity was detected in all P. gingivalis strains tested. Interestingly, periodontain was most frequently associated with the membrane and outer membrane vesicles in strains 33277, W50, W12, and 381, despite the fact that it was soluble in strain HG66 (Fig. 7). This is also in agreement with the distribution of other P. gingivalis proteinases (32, 33).

DISCUSSION

The multiple trypsin-like proteolytic activities have a significant contribution to the virulence of P. gingivalis, for both
invasion and host defense evasion. In support of this concept, deletion of the two genes (rgpA and rgpB) encoding various forms of gingipains R has been shown to attenuate in vitro virulence of the knockout strain (34). In addition, the use of antibodies to gingipains R polypeptide chain-derived fragments has shown in vivo protection against *P. gingivalis* infection in a mouse model system (35).

Despite the importance of these bacterial proteinases, the abundance of host-derived metalloproteinases and serine proteinases, including active HNE, in the gingival crevicular fluid of individuals with severe periodontitis, is still believed to be the primary factor responsible for much of the extracellular matrix destruction that occurs in this disease (36). The concentration of HNE within the neutrophil is near 3.0 μM, and it is likely to be as high as 268 μM at sites of inflammation (37), such as those that occur during the development of another major connective tissue disease, pulmonary emphysema.

The regulating inhibitor of HNE is α1-PI, a plasma protein that forms a complex with this proteinase and is rapidly removed from the circulation and degraded (38). The inhibitor, however, can itself be inactivated by either oxidation at its reactive site or by proteolytic cleavage by nontarget proteinases within the RSL region (27, 39), and it is believed that both mechanisms occur during the development of emphysema. Certainly, the high levels of active HNE in the GCF, despite the presence of α1-PI, would suggest that parallel mechanisms for inhibitor inactivation may be also occurring in periodontal disease (37). In this respect, it has been reported that whole cells or culture supernatants from *P. gingivalis* are capable of proteolytically inactivating α1-PI (40, 41), although it is clear that this is not due to any of the gingipain R or K forms because the inhibitor contains no basic residues within its RSL (42). Thus, another proteinase(s) must be involved in this process, and it is likely that periodontain, the enzyme described in this report, serves this purpose.

Periodontain was easily purified from *P. gingivalis* culture fluids and was found to be a cysteine proteinase that is apparently produced as a heterodimer. This enzyme is similar to the other characterized cysteine proteinases from *P. gingivalis* in that all are primarily secreted in strain HG66, yet are present on membranes and vesicles in all other strains. Each has a signal peptide sequence, a long propeptide, a large catalytic domain (∼50 kDa), and an additional carboxyl-terminal extension (20–40 kDa). In contrast, whereas the gingipains have a highly restrictive specificity, periodontain is characterized by an apparently nonspecific proteolysis of peptides or denatured proteins. Although the ability of this enzyme to hydrolyze α1-PI, a native protein, is contrary to our premise of its inability to cleave proteins with defined structure, this can be explained by the fact that the RSL is present in a flexible, extended conformation protruding above the protein core (43) and, as such, may mimic a denatured protein or peptide.

From analysis of the amino terminus of the catalytic subunit and subsequent cloning of this fragment, we were able to elucidate the entire gene sequence from the partially completed genome sequence for *P. gingivalis* obtained from NCBI. Searching the data base of known sequences has revealed that periodontain is highly homologous to both PrtT, a putative cysteine proteinase from *P. gingivalis*, and streptopain (EC 3.4.22.10), a secreted cysteine proteinase from *Streptococcus pyogenes* (Table III and Fig. 5). The PrtT gene, which has recently been cloned and sequenced (44, 45), is believed to encode a putative proteinase of 96–99 kDa. It is not surprising that periodontain and PrtT have highly homologous sequences, being from the same organism. Indeed, rgpA and rgpB are two completely separate genes that code for almost identical proteins with significantly overlapping specificity. However, until the gene product for PrtT has been purified and characterized, functional similarities between PrtT and periodontain cannot be assessed.

Periodontain and PrtT, containing 843 and 840 amino acid residues, respectively, are more than twice the size of the streptopain (398 residues) (Fig. 5). The function of the light chain of periodontain is presently unknown; however, the carboxyl-terminal domain for the putative PrtT gene product has recently been shown to be identical to the putative product for a hemin-regulated gene, hemR (46). Due to the high homology of the catalytic domains of all three genes, we believe that periodontain should be classified with *PrtT* and streptopain as
an additional member of the C10 family of cysteine proteinases, as recently outlined (47).

Because *P. gingivalis* is an asaccharolytic organism, it must acquire both carbon and energy predominantly from proteinaceous sources. Recent evidence using radiolabeled substrates has shown that although this organism is very efficient at taking up dipeptides, it is incapable of transporting single amino acids (which may endogenously be present in GCF) across bacterial cell membranes (48). This is supported by the fact that despite a large body of research performed on extracellular endopeptidases from *P. gingivalis*, no aminopeptidases or carboxypeptidases have been described to date. Obviously, the gingipains, which only have a specificity for either Lys-X or Arg-X bonds, would be restricted in the ability to degrade large proteins to the size of di- or tripeptides, which could then be transported into the bacterium. Therefore, it is possible that a number of broadly specific proteinases and peptidases, including periodontain, may be physiologically important to *P. gingivalis*, not necessarily as virulence factors, but rather for nutrient acquisition. This may be accomplished by three pathways. First, because periodontain is the only peptidase in *P. gingivalis*, may be physiologically important to transport into the bacterium. Therefore, it is possible that a number of broadly specific proteinases and peptidases, including periodontain, may be physiologically important to *P. gingivalis*, not necessarily as virulence factors, but rather for nutrient acquisition. This may be accomplished by three pathways. First, because periodontain is the only peptidase in *P. gingivalis* so far described that possesses the ability to inactivate α1-PI, this may be a mechanism for increasing the levels of HNE, a nonspecific host proteinase that might be utilized in protein degradation. Second, in combination with HNE, periodontain may augment the degradation of peptides produced by the actions of the gingipains. Third, there is evidence that *P. gingivalis* produces a prolyl dipeptidyl peptidase (49), a prolyl tripeptidyl peptidase (50), and an uncharacterized collagenase. This proteolytic milieu in the GCF could aid in the final production of peptides capable of being taken up by *P. gingivalis*.

REFERENCES

1. Moore, W. E. C., Holdenman, L. V., Smibert, R. M., Hash, D. E., Burmeister, J. A., and Ranney, R. R. (1982) Infect. Immun. 38, 1137–1148
2. Seeransky, S. S., and Haffajee, A. D. (1992) J. Periodontol. 63, 322–331
3. Sista, J., Bragd, L., Wikstrom, M., and Dahlén, G. (1986) J. Clin. Periodontol. 13, 570–577
4. White, D., and Mayrand, J. (1981) J. Periodontol. Res. 16, 259–265
5. Potempa, J., and Travis, J. (1996) Acta Biochim. Pol. 43, 455–466
6. Potempa, J., Pike, R., and Travis, J. (1995) Perspect. Drug Discov. Design 2, 445–458
7. Travis, J., Pike, R., Imamura, T., and Potempa, J. (1997) J. Periodontal. Res. 32, 120–125
8. Kuramitsu, H. K. (1998) Oral Microbiol. Immunol. 13, 263–270
9. Imamura, T., Potempa, J., Pike, R. N., and Travis, J. (1995) Infect. Immun. 63, 1999–2003
10. Darany, D. G., Beck, F. M., and Walters, J. D. (1992) J. Periodontal. Res. 63, 743–747

**D. Nelson, J. Potempa, T. Kordula, and J. Travis, unpublished observation.**