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Characterization of Sodium Dodecyl Sulfate-Stable *Bacteroides gingivalis* Proteases by Polyacrylamide Gel Electrophoresis

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Profiles of the proteolytic activities found in *Bacteroides gingivalis* culture supernatants, outer membranes, vesicles, and cell extracts were analyzed in sodium dodecyl sulfate-polyacrylamide gels containing covalently bound bovine serum albumin. A total of eight distinct bands of proteolytic activity could be detected. Four of these were found in the culture supernatant (P1, P2, P3, and P4). The outer membranes, vesicles, and the cell extract each contained seven major proteolytic bands (P1, P3, P4, P5, P6, P7, and P8). No activity was found in the membrane-free extract, suggesting that the proteases were associated with the cell envelope. With the exception of P7 and P8, all the proteolytic bands were dependent on reducing agents for activity. The eight proteolytic bands were distributed in an identical manner in all four strains of *B. gingivalis* studied. The effects of protease inhibitors, pH, and heat were determined. Sulfhydryl group reagents and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone reduced proteolytic activity. The optimum pH was found to be between 7 and 8. A 30-min preincubation at 50°C inactivated the P6, P7, and P8 proteolytic bands. All proteolytic activity was lost after the samples were heated at 75°C for 30 min.

A number of studies indicate that *Bacteroides gingivalis* is an important etiologic agent of periodontitis (16, 19, 21). This putative pathogen produces a number of suspected virulence factors, including cell-associated and secreted proteases (1, 6, 10, 18, 20). A number of proteins, including collagen (7, 15), immunoglobulins (13, 17), iron-binding proteins (3), and complement factors C3 and C5 (17), are hydrolyzed by the organism. There are a number of reports on the isolation of serine and thiol proteases from culture supernatant, outer membranes, and cell extracts of *B. gingivalis* (1, 6, 10, 18, 20). These reports indicate that the organism synthesizes a number of different proteolytic enzymes, but to date there has not been a comprehensive look at the number and diversity of the proteases which are located in various subcellular fractions or which are secreted by the organism. Presumably the distribution and form of the proteolytic activity will be important in pathogenesis. Some enzymes may be released by the cell, whereas others will remain associated with vesicles or cells.

Electrophoretic procedures provide an opportunity to simultaneously look at a number of proteolytic activities in a single sample. The proteases can be detected by laying the gel onto an agarose gel containing a protein, after electrophoretic separation (4, 5), or by covalently linking the protein substrate into the polyacrylamide gel before electrophoresis (11, 12).

The aim of the study was to determine and compare the proteolytic profiles in specific fractions of *B. gingivalis*, by using polyacrylamide gels containing covalently bound bovine serum albumin (BSA). The effects of protease inhibitors as well as pH and heat pretreatments were also evaluated.

MATERIALS AND METHODS

Bacteria and growth conditions. *B. gingivalis* ATCC 33277, HW11D-5, W83, and RB46D-1 were used in the present investigation. The bacteria were grown in Trypticase (1.7%; BBL Microbiology Systems, Cockeysville, Md.)-yeast extract (0.3%) medium containing potassium phosphate (0.25%), sodium chloride (0.5%), hemin (10 μg/ml), and vitamin K (1 μg/ml). In some experiments, the Trypticase content was lowered to 0.8%, and the medium was supplemented with either 1% casein or 1% Casamino Acids (Difco Laboratories, Detroit, Mich.). Cultures were incubated at 37°C in an anaerobic chamber with an atmosphere of N_2-H_2-CO_2 (85:10:5) for 72 h, unless noted otherwise. Except where noted, studies were done with strain 33277.

Preparation of fractions. Various fractions of *B. gingivalis* 33277 were prepared in order to determine their proteolytic patterns. These fractions were (i) culture supernatant, obtained by centrifugation at 80,000 × *g* for 1 h and concentrated 15 times by ultrafiltration on a membrane with a 5,000 molecular weight cutoff; (ii) outer membranes, as prepared by Boyd and McBride (2); (iii) vesicles, prepared according to the protocol of Grenier and Mayrand (8); as well as (iv) cell and (v) membrane-free extracts prepared as follows. The cells were broken by ultrasonic treatment for 1 min (30% duty cycle, output 5; Sonifier cell disrupter; Branson Sonic Power Co., Danbury, Conn.), and then the cellular debris and unbroken cells were removed by centrifugation at either 10,000 × *g* for 20 min (cell extract) or 100,000 × *g* for 3 h (membrane-free extract). The comparison of protease patterns for the different strains of *B. gingivalis* was carried out by using a culture supernatant concentrated 15 times by ultrafiltration as well as a cell extract.

Preparation of polyacrylamide-BSA conjugate. The method has been previously described by Kelleher and Juliano (12). Briefly, 400 mg of linear polyacrylamide (BDH Chemicals Ltd., Poole, England) was dissolved in 20 ml of 0.2 M sodium phosphate buffer (pH 6.8) and mixed with 8 ml of glutaraldehyde (25% in water), and the mixture was incubated at 37°C for 24 h. The nonconjugated glutaraldehyde was removed by exhaustive dialysis at 4°C against 10 liters of distilled water for 4 days (the water was changed three times per day). Then, 2 ml of a 10% BSA solution (0.2 M sodium

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TABLE 1. Locations, molecular weights, and peptidase activities[a] of the proteolytic bands seen in polyacrylamide gels containing covalently bound BSA

Proteolytic bands	Mol wt	Protease profile present in fraction[b]:						Peptidase activity with:		
		Supernatant	Crude cell extract	High-speed supernatant of crude cell extract	High-speed pellet of crude cell extract	Outer membranes	Vesicles	BAPNA	GPPNA	SAAPNA
P1	110,000	+	+	−	+	+	+	+	−	−
P2	100,000	+	−	−	−	−	−	+	−	−
P3	90,000	+	+	−	+	+	+	+	−	−
P4	80,000	+	+	−	+	+	+	+	−	−
P5	65,000	−	+	−	+	+	+	+	−	−
P6	50,000	−	+	−	+	+	+	+/−	−	+/−
P7	30,000	−	+	−	+	+	+	−	+	+/−
P8	29,000	−	+	−	+	+	+	−	+	+/−

[a] −, No activity; +/−, weak activity; +, strong activity.
[b] Fractions were prepared as described in Materials and Methods.

phosphate buffer [pH 7.2]) was added and allowed to react for 24 h at 25°C. The reaction experiment was stopped by the addition of 250 mg of glycine–3 mg of sodium azide. After incubation for 24 h at 4°C, the mixture was dialyzed (4°C for 2 days) against 10 liters of distilled water containing 0.05% sodium azide. The polyacrylamide-BSA conjugate was stored at 4°C. Other substrates, including immunoglobulins A and G, α_1-antitrypsin, fibrinogen, and transferrin, were also conjugated to linear polyacrylamide and assayed for proteolytic degradation.

Electrophoresis. Sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) was carried out by the Laemmli method (14), using a Mini PROTEAN II cell (0.075 cm thick) (BioRad Laboratories, Richmond, Calif.). Before the gels were cast, the acrylamide was mixed with BSA-polyacrylamide conjugate to give a concentration of 200 μg of protein per ml of gel. Samples were solubilized in SDS buffer (4% SDS, 20% glycerol, 0.125 M Tris hydrochloride [pH 6.8]) for 30 min at 37°C. Electrophoresis was conducted at room temperature at a constant 200 V. Reference proteins (and their molecular weights) were phospholipase *b* (97,400), BSA (68,000), ovalbumin (43,000), and α-chymotrypsinogen (25,700). The protein content of boiled and nonboiled samples was also analyzed by SDS-PAGE, using a BSA-free gel and silver nitrate staining.

Development of proteolytic bands. After electrophoresis, the gel was gently shaken in 100 mM Tris hydrochloride buffer (pH 7.0) containing 2% Triton X-100 for 30 min, rinsed twice with water, and then shaken for a further 30 min in 100 mM Tris hydrochloride buffer (pH 7.0). The gel was transferred into development buffer, which unless noted otherwise, was 100 mM Tris hydrochloride (pH 7.0) containing 2.5 mM $CaCl_2$ and 50 mM cysteine. The gel was incubated at 37°C for 2 h and stained for protein with Coomassie brilliant blue R-250. After the gel was destained, proteolytic activity was visualized as a clear band against a dark blue background.

Effects of inhibitors. The effect of a number of putative compounds on the proteolytic pattern was determined by incubating the gels with development buffer containing the compound of interest. Inhibitors tested were: *p*-chloromercuribenzoic acid, 8 mM; iodoacetic acid, 8 mM; phenylmethylsulfonyl fluoride, 4 mM; *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 4 mM; EDTA, 40 mM; $HgCl_2$, 10 mM; $ZnCl_2$, 10 mM; $MgCl_2$, 10 mM; $CaCl_2$, 10 mM; pepstatin, 4 μg/ml; and aprotinin, 4 μg/ml. In all cases, the gel was preincubated for 1.5 h in the presence of these compounds before the development of proteolytic bands.

Effect of pH. The effect of pH was determined by preincubating gels for 1.5 h in 200 mM sodium citrate buffer (pH 5 and 6), 200 mM Tris hydrochloride buffer (pH 7 and 8), or 200 mM sodium carbonate buffer (pH 9 and 10). Cysteine (50 mM final concentration) was then added to activate the proteases.

Heat stability of proteases. Temperature lability was evaluated by incubating the samples at 50, 65, and 75°C for 30 min before electrophoresis on BSA-polyacrylamide gel.

Detection of peptidase activity in proteolytic bands. The samples (culture supernatant and cell extract) were electrophoresed on SDS-PAGE-BSA gels (1.5 mm thick) as described above. A narrow vertical strip was cut from one side of the gel and developed for proteolytic bands. The sections of the remaining gel corresponding to the proteolytic activity were removed and finely chopped in 10 mM Tris hydrochloride buffer (pH 7.0). The proteins were eluted overnight with constant agitation and then dialyzed at 4°C against distilled water for 8 h and lyophilized. The proteolytic fractions were suspended in 1 ml of 50 mM Tris hydrochloride (pH 7.0) and kept at −70°C. The peptidase activities were measured as previously described (10), using *N*-α-benzoyl-DL-arginine-pNA (BAPNA), *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-pNA (SAAPNA), and glycyl-L-proline-pNA (GPPNA). After diazotization, semiquantitative determinations were made according to the following scale: −, no color reaction; +/−, faint red color; and +, strong red color.

Chemicals. Unless indicated otherwise, all chemicals, buffers, and reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Protease profiles. A number of proteolytic bands were detected in *B. gingivalis* fractions when the gels were incubated for 2 h at 37°C in the presence of a reducing agent (Table 1). Incubating the gels for a longer time in the development buffer did not result in the appearance of more bands but did lead to a loss of resolution. The culture supernatant which had been concentrated 15 times contained four proteolytic bands (Fig. 1, lane A), P1 (R_f = 0.07), P2 (R_f = 0.09), P3 (R_f = 0.17), and P4 (R_f = 0.25). These activities were not removed by centrifugation at 100,000 × *g* for 3 h. The crude cell extract showed the presence of seven major proteolytic bands (Fig. 1, lane B); the P1, P3, and P4 bands migrated to the same positions as the proteolytic bands seen in the culture supernatant, whereas bands P5 (R_f = 0.36), P6 (R_f = 0.56), P7 (R_f = 0.89), and P8 (R_f = 0.93) were unique

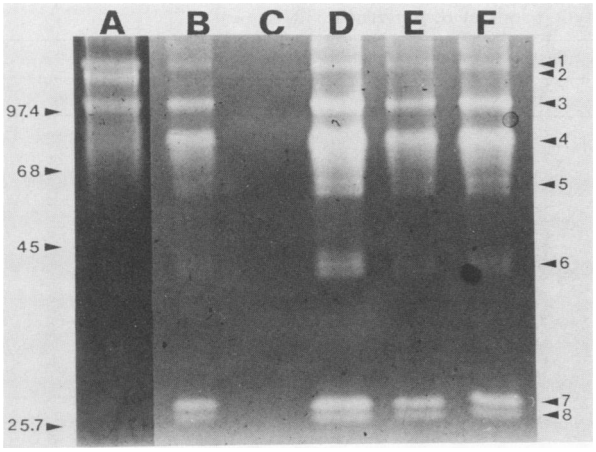

FIG. 1. *B. gingivalis* protease profiles on BSA-conjugated polyacrylamide gels developed for 2 h at 37°C in the presence of 50 mM cysteine. Lanes: A, 15-time concentrated supernatant (10 μl); B, crude cell extract (2 μl containing 5 μg of protein); C, high-speed supernatant of crude cell extract (2 μl); D, outer membrane preparation (2 μg); E, vesicle preparation (2 μg); F, high-speed pellet of crude cell extract (2 μl).

to the cell extract. Centrifuging the cell extract at 100,000 × *g* for 3 h removed all the proteolytic bands, suggesting that the activity in this fraction was associated with the cell envelope (Fig. 1, lane C). The seven bands found in the pellet obtained by ultracentrifugation confirmed this result (Fig. 1, lane F). The outer membrane and vesicle preparations (Fig. 1, lanes D and E, respectively) contained the seven major proteolytic bands previously found in the crude cell extract. In the absence of cysteine, the proteolytic bands P7 and P8 were visualized in the cell extract, the outer membranes, and the vesicles. No proteolytic bands were detected in the cell-free culture supernatant in the absence of a reducing agent. The identical proteolytic pattern was observed in all preparations of *B. gingivalis* 33277 and in the three other strains examined (*B. gingivalis* HW11D-5, W83, and RB46D-1).

Silver nitrate staining of the various samples solubilized at either 37 (30 min) or 100°C (5 min) can be seen in Fig. 2. The proteolytic fractions contained several protein bands. When the samples were solubilized at 37°C, it was impossible to associate the protein bands with the sharp proteolytic bands, indicating the relatively small amount of proteolytic protein and the high sensitivity of the in situ protein hydrolysis method.

The growth conditions appeared to have no effect on the protease profiles. No modifications in the patterns were observed even after three subcultures of *B. gingivalis* in a growth medium containing 1% casein or 1% Casamino Acids. Finally, the same proteolytic bands were detected when either immunoglobulin G, immunoglobulin A, fibrinogen, α_1-antitrypsin, or transferrin was used as substrate.

The peptidase activity was assayed by using the proteases eluted from SDS-PAGE gels (Table 1). The trypsinlike enzyme substrate BAPNA was degraded by the proteolytic bands P1 to P5. The P6 to P8 bands weakly degraded the chymotrypsinlike enzyme substrate SAAPNA. The synthetic peptide GPPNA was degraded by the P7 and P8 bands.

Preliminary experiments showed that different factors could affect the detection and the sharpness of the proteolytic bands. Optimum resolution occurred when both the electrophoresis and the incubation times were reduced to a minimum. The mini-slab gel system with 0.075-cm-thick gels proved to give the best results. The electrophoretic separation took only 45 min, and the thin gel allowed rapid diffusion of SDS out of the gel and cysteine into the gel, thus reducing the time between electrophoresis and development. The activity of most of the proteolytic bands was markedly reduced when SDS was not removed by washing the gels with Triton X-100. Cysteine was a more effective reducing agent than either dithiothreitol or 2-mercaptoethanol.

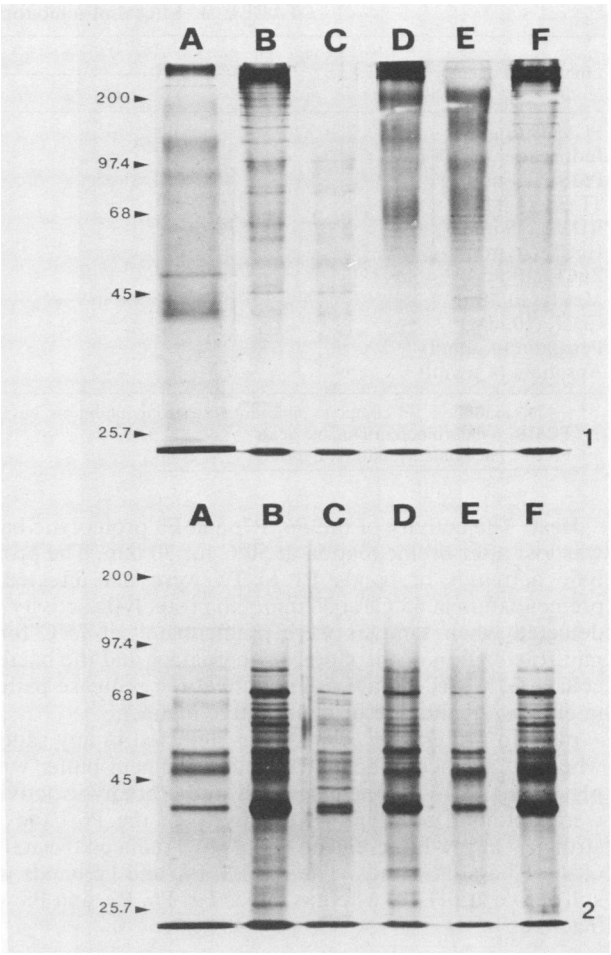

FIG. 2. Silver nitrate staining of *B. gingivalis* proteolytic samples. (Gel 1) Samples solubilized in SDS buffer for 30 min at 37°C. (Gel 2) Samples solubilized in SDS buffer (containing 2-mercaptoethanol) for 5 min at 100°C. Lanes: A, 15-time concentrated supernatant (10 μl); B, crude cell extract (2 μl containing 5 μg of protein); C, high-speed supernatant of crude cell extract (2 μl); D, outer membrane preparation (2 μg); E, vesicle preparation (2 μg); F, high-speed pellet of crude cell extract (2 μl).

Inhibitors. The results of the inhibition studies are summarized in Table 2. TLCK inhibited the activity of all proteolytic bands. The activity of the eight bands was reduced to various extents by either of the sulfhydryl group blocking reagent *p*-chloromercuribenzoic acid or iodoacetic acid. The absence of inhibition observed in some cases with these agents may be due to the necessity of incorporating high concentrations of cysteine into the development buffer in order to stimulate proteolysis by bands P1 to P6. The divalent cations Hg^{2+} and Zn^{2+} greatly reduced proteolytic activity. The P6 proteolytic band was inhibited by the chelator EDTA.

TABLE 2. Effects of inhibitors on proteolytic bands of *B. gingivalis* 33277

Inhibitor (concn or amt)	Effect[a] on proteolytic band:							
	P1	P2	P3	P4	P5	P6	P7	P8
PCMB[b] (8 mM)	−	−	−	−	−	±	±	±
Iodoacetic acid (8 mM)	±	±	±	+	+	+	−	−
PMSF[c] (4 mM)	−	−	−	−	±	±	±	±
TLCK (4 mM)	+	+	+	+	+	+	+	+
EDTA (40 mM)	−	−	−	−	−	±	−	−
$HgCl_2$ (10 mM)	±	±	±	+	+	+	+	+
$ZnCl_2$ (10 mM)	+	+	+	+	+	+	+	+
$MgCl_2$ (10 Mm)	−	−	−	−	−	−	−	−
$CaCl_2$ (10 mM)	−	−	−	−	−	−	−	−
Pepstatin (4 μg/ml)	−	−	−	−	−	−	−	−
Aprotinin (4 μg/ml)	−	−	−	−	−	−	−	−

[a] −, No inhibition; +, complete inhibition; ±, strong inhibition, but some residual activity.
[b] PCMB, *p*-chloromercuribenzoic acid.
[c] PMSF, Phenylmethysulfonyl fluoride.

Heat. The activity of the P6, P7, and P8 proteolytic bands was lost after preincubation at 50°C for 30 min. The proteolytic activities of bands P1 to P5 were not affected by preincubation at 65°C for 30 min. No proteolytic activity was detected when samples were preincubated at 75°C for 30 min. Incubation of the culture supernatant and the bacterial cells at 37°C for 20 days did not affect the protease patterns in either a qualitative or quantitative manner.

pH. No proteolytic activity was detected in any samples when the gels were incubated in development buffer with a pH of 5 or 11. With one exception, the proteolytic activities were optimum at pH 7. The activity of the P6 band was strongest at pH 8. Increasing the pH to 9 reduced the activity of the P3 and P6 bands. The P1, P2, P4, and P5 bands were still active at pH 10, whereas bands P3, P6, P7, and P8 were inactive.

DISCUSSION

We have described a simple, sensitive, and rapid method to characterize the proteolytic system found in *B. gingivalis*. Different subcellular bacterial fractions as well as various strains of *B. gingivalis* were analyzed. We feel this is a valuable technique for the rapid analysis and preliminary characterization of SDS-stable proteolytic enzymes present in crude preparations containing a number of different proteolytic activities. This procedure provides the opportunity to simultaneously study the effect of a variety of experimental conditions on a number of proteases by simply changing the composition of the development buffer.

In order to preserve biological activity, samples were prepared for electrophoresis by incubation in 2% SDS at 37°C. In the absence of SDS, the proteolytic activity appeared as a smear, mainly located in the upper part of the resolving gel. It is probable that the activities associated with the membranes are not solubilized in the absence of SDS and therefore do not migrate as discrete entities but rather as pieces of outer membrane. It could also mean that the proteases bind to the substrate in the absence of SDS or that they are associated with other molecules. In the absence of SDS, the low-molecular-weight proteases which do not require reducing conditions for activity may actively digest the substrate as they migrate through the gel.

Since a large number of proteases including *B. gingivalis* proteases (3) degrade BSA, this general substrate was found to be suitable for the determination of protease patterns. A number of other protein substrates, including immunoglobulins G and A as well as α_1-antitrypsin, transferrin, and fibrinogen were tested and found to give similar protease profiles. This result indicates a broad range of activity for the *B. gingivalis* proteases.

Four proteolytic bands were detected in a concentrated culture supernatant, whereas seven major bands were associated with a crude cell extract of *B. gingivalis*. The activity in the cell extract could be removed by ultracentrifugation, suggesting that the proteolytic activity was due to the presence of outer membrane fragments and was not due to soluble proteases. An outer membrane preparation showed the same seven major bands, P1 and P3 to P8. The growth of *B. gingivalis* is dependent on amino acids and peptides; therefore, it appears reasonable that proteases required for the breakdown of the primary substrate are located within the outer membrane or are released from the cell. The protease pattern of the vesicle preparation was identical to that of the outer membranes, confirming that these vesicles are derived from the outer membrane. The proteolytic band P2 was found in the culture supernatant and not in outer membranes. This was the case even when the gel was heavily overloaded with membranes. On the basis of heat stability, sensitivity to inhibitors, peptidase activity, and migration in SDS-PAGE gels, we are assuming that the P1, P3, and P4 enzymes found in culture supernatants and membranes are the same. Final proof will depend on isolation and comparison of the various enzymes.

The R_fs assigned to the various activities were identical in every preparation. However, the R_fs should not be considered as necessarily reflecting the molecular weights of the monomeric forms of the enzymes, because the samples were not boiled in SDS before electrophoresis.

The sulfhydryl groups appear important for proteolytic activity since (i) cysteine was required for the detection of all but the two lowest-molecular-weight proteolytic bands found in the outer membrane preparation and (ii) TLCK, iodoacetic acid, and Hg^{2+} were inhibitory. The inhibition observed in the case of the TLCK could also indicate that the proteolytic activity is due to trypsinlike enzymes. Similar inhibition results have been obtained in studies of purified and partially purified *B. gingivalis* proteases (6, 10, 18, 20).

The possibility that the different zones of hydrolysis are the result of autodigestion products or of two or three proteases appearing in an aggregated form or complexed with other cell components cannot be ruled out by the experiments described here. However, the protease patterns

were consistent and stable in all preparations of the same strain and in preparations of different strains of *B. gingivalis* (33277, HW11D-5, W83, and RB46D-1). Even prolonged incubation (20 days) of culture supernatant and cells in an anaerobic environment at 37°C did not alter the proteolytic profile. Furthermore, there is evidence to suggest that the enzymes are different. (i) The proteolytic band P2 is found exclusively in the supernatant, whereas bands P5 to P8 are found only in the particulate fraction. (ii) The proteolytic bands P7 and P8 are slightly inhibited by cysteine, whereas the activity of the P1 to P6 bands are dependent on the reducing agent. (iii) The P6 band is the only one inhibited by EDTA. (iv) The P6, P7, and P8 bands are sensitive to being heated at 50°C for 30 min, whereas the other bands are unaffected. (v) Three peptidase activities can be associated with the different bands, P1 to P5 bands hydrolyze the trypsinlike arginine-containing substrate BAPNA but do not hydrolyze the glycyl-proline substrate (GPPNA) or the chymotrypsinlike substrate (SAAPNA); the P6 band shows activity toward both BAPNA and SAAPNA; the P7 and P8 bands hydrolyze the glycyl-proline substrate (GPPNA) and show some activity toward SAAPNA but do not hydrolyze BAPNA. (vi) The P8 band has been isolated in a previous study (10) and has been shown to be antigenically and enzymatically different from the other bands.

Grenier and Mayrand (9) have demonstrated that *B. gingivalis* W83 and RB46D-1 were highly virulent in the guinea pig animal model, whereas *B. gingivalis* 33277 and HW11D-5 did not induce lesions in the same model. Our results indicate that the protease profile cannot be used to differentiate between the virulent and avirulent strains, since no major differences could be found. However, this does not exclude the possibility that there are other potentially important proteases not detected in this study which are associated with virulence or that the relative distribution of the activities may change when the organisms are growing in situ.

Attempts to correlate the proteolytic activities seen in this study with proteases described by others are difficult because molecular weights cannot be compared. The trypsinlike protease purified by Yoshimura et al. (20) could represent the P1, P3, P4, P5, or P6 band in view of its location in the outer membrane, sensitivity to TLCK, dependence on reducing agents, and BAPNA-degrading activity. The P8 proteolytic band, which is bound to the outer membrane fragments represents the glycylprolyl protease isolated by Grenier and McBride (10). The P1, P2, P3, or P4 band may be the high-molecular-weight, cell-free, TLCK-sensitive trypsinlike protease characterized by Fujimura and Nakamura (6). Since the *B. gingivalis* vesicles are strongly proteolytic and are released into the culture medium, it is essential to distinguish between vesicle-associated proteases and proteases which exist free in culture supernatant.

The technique used here is a powerful tool to study systems in which there are a number of different proteolytic activities. However, it should be appreciated that the method will fail to detect proteases which are not active against BSA or those that are irreversibly inhibited by SDS.

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