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Purification of an 80,000-M_r Glycylprolyl Peptidase from *Bacteroides gingivalis*

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An enzyme from *Bacteroides gingivalis* SUNYAB A7A1-28 that hydrolyzes the synthetic peptide glycyl-L-proline 4-methoxy-β-naphthylamide was purified 1,040-fold by urea extraction, gel filtration, ion-exchange chromatography, and fast protein liquid chromatography. The molecular weight of the enzyme was 80,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 75,000 as determined by gel filtration. The optimum pH for the hydrolysis of glycyl-L-proline 4-methoxy-β-naphthylamide was 7.5 to 8.5. The enzyme activity was inhibited by the serine protease inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride by 82.5 and 78%, respectively. The activity was also inhibited by Hg^{2+} (55.6%) and Zn^{2+} (45%).

Bacteroides gingivalis is an important microorganism that has been proposed to play a major role in the pathogenesis of periodontal disease (25). It has also been identified in abscesses of odontogenic origin (6, 31). A wide variety of virulence factors have been associated with the microorganism (19), which include the proteolytic enzymes. The substrates capable of being degraded by *B. gingivalis* include collagen, gelatin (4, 18), fibrin (15), immunoglobulin A (13), and a number of synthetic substrates (28). Further characterization of the enzymatic activity has revealed that the enzymes of *B. gingivalis* include both thiol proteases (4, 21, 32) and serine proteases (1), with the latter capable of degrading the synthetic substrate glycyl-L-proline 4-methoxy-β-naphthylamide (Gly-Pro-NA) (10, 29). The objective of this study was to purify the glycylprolyl peptidase from *B. gingivalis*.

MATERIALS AND METHODS

Source and culture conditions of *B. gingivalis*. *B. gingivalis* SUNYAB A7A1-28 was provided by J. J. Zambon (Department of Oral Biology, State University of New York at Buffalo, Buffalo). This strain was originally isolated from a human periodontitis patient with non-insulin-dependent diabetes mellitus (33) and can produce a spreading infection in the mouse abscess model (5). To obtain the material for purification, the strain was grown in 100 ml of half-strength brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.5% yeast extract, 5 μg of hemin per ml, and 0.2 μg of menadione per ml at 37°C under anaerobic conditions (10% H_2, 5% CO_2, 85% N_2) for 20 h. The 100 ml was then used to inoculate another 1,500 ml of half-strength brain heart infusion broth. After 36 h this culture was used to inoculate 18 liters of half-strength brain heart infusion broth in a fermentor (model MF 128-S, New Brunswick Scientific Co., Inc., Edison, N.J.). Anaerobiosis was maintained in the fermentor by keeping a positive N_2 gas pressure (20 lb/in^2). Cells were cultivated at 37°C with agitation at 200 rpm for 22.5 h until the late exponential phase and a final optical density of 0.6 at 660 nm. Cells were harvested using a Durapore filter (0.5 μm; Millipore Corp., Bedford, Mass.). The concentrated cell suspension was centrifuged at 22,000 × *g* (model J2-21 centrifuge, Beckman Instruments, Inc., Palo Alto, Calif.) for 30 min to obtain the cell pellet. The cells were washed once with cold 50 mM Tris hydrochloride buffer (pH 7.4) containing 0.15 M NaCl and centrifuged again at 22,000 × *g*. Approximately 120 g (wet weight) of cell pellet was obtained.

Enzyme assay. The enzyme activity was measured during the course of purification by using Gly-Pro-NA as the substrate by a modification of the methods described by Hopsu et al. (12) and Umezawa et al. (30) as modified by Suido et al. (28). For samples that contained urea, the enzyme assay was performed after dialysis against 50 mM Tris hydrochloride (pH 7.4) buffer containing 0.15 M NaCl. The reaction mixture was prepared by mixing 0.3 ml of 1 mM substrate, 0.9 ml of 0.1 M Tris hydrochloride buffer (pH 7.4), and 0.3 ml of distilled water or an appropriate concentration of test solution. The mixture was incubated for 30 min at 37°C. A 0.6-ml volume of stabilized diazonium salt garnet GBC (0.5 mg/ml) in 1 M acetic acid buffer (pH 4.2) containing 10% Tween 20 was then added to the mixture. A diazo dye formed by coupling of the liberated naphthylamine with garnet GBC. After the mixture stood for 15 min at room temperature, the A_{525} was measured. One enzyme unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of β-naphthylamine per min under the assay conditions. The hydrolysis of three other synthetic substrates (glycyl-L-phenylalanine-naphthylamide [Gly-Phe-NA], *N*-benzoyl-DL-arginine-naphthylamide [BANA], and *N*-CBz-glycyl-glycyl-L-arginine-naphthylamide [*N*-CBz-Gly-Gly-Arg-NA]) was assayed by the same method. BANA and Gly-Phe-NA were dissolved in 0.1 ml of dimethyl sulfoxide and then diluted with 0.1 M Tris hydrochloride buffer (pH

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7.4). The synthetic substrates for the enzyme assays were obtained from Sigma Chemical Co., St. Louis, Mo. The collagenase assay was performed as described by Birkedal-Hansen, using type I collagen fibrils as the substrate (3).

Purification. All purification steps were carried out at 4°C. Each step was monitored with the assay for glycylprolyl peptidase.

It was found in initial experiments that glycylprolyl peptidase could be extracted from whole cells with urea. Pilot experiments were performed to determine the optimum conditions for the extraction of the enzyme with urea. A 100-mg (wet weight) cell pellet in 1 ml was extracted with 1, 2, 4, and 6 M urea in 50 mM Tris hydrochloride (pH 7.4) buffer containing 0.2 M NaCl for 3 h at 4°C. Cells and cell debris were removed by centrifugation at 80,000 × *g* (Beckman ultracentrifuge, model L8-80) for 30 min. The supernatant was dialyzed against distilled water and then against 50 mM Tris hydrochloride (pH 7.4) buffer containing 0.15 M NaCl. The dialyzed supernatant was assayed for the release of enzymes and protein from the cells.

(i) Preparation of crude extract. The crude extracts for purification were prepared by extracting 50 g (wet weight) of *B. gingivalis* SUNYAB A7A1-28 in 500 ml of 50 mM Tris hydrochloride (pH 7.4) containing 0.2 M NaCl and 6 M urea. The slurry was stirred for 3 h at 4°C. Cells were removed by centrifugation at 16,000 × *g* for 30 min (Beckman centrifuge, model J2-21). The resulting supernatant was concentrated by ultrafiltration (Amicon Corp., Lexington, Mass.) with a PM30 Diaflo membrane. The concentrated material was dialyzed against 50 mM Tris hydrochloride buffer (pH 7.4) containing 0.15 M NaCl and centrifuged at 80,000 × *g* for 30 min to remove the insoluble precipitate. The final volume of the concentrated material was 50 ml.

(ii) Sephadex G-200 gel filtration. Five milliliters of the concentrated material was applied to a Sephadex G-200 superfine (Pharmacia Uppsala, Sweden) column (1.6 by 70 cm) equilibrated with 50 mM Tris hydrochloride buffer (pH 7.4) containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 15 ml/h and 2.5-ml fractions were collected. The procedure was repeated 10 times and fractions containing the enzyme were pooled, concentrated by ultrafiltration with a PM30 Diaflo membrane, and dialyzed against 20 mM Tris hydrochloride buffer (pH 7.4).

(iii) Q Sepharose ion-exchange chromatography. The concentrated dialyzed material (10 ml) was passed through a Q Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) ion-exchange column (1.5 by 40 cm) equilibrated with 20 mM Tris hydrochloride buffer (pH 7.4). Elution was performed with a linear gradient of 0 to 1.5 M NaCl at a flow rate of 150 ml/h. The fractions containing the enzyme were pooled, concentrated, and dialyzed overnight against 20 mM Tris hydrochloride (pH 7.4).

(iv) Mono Q ion-exchange chromatography. A 2-ml volume of the concentrated sample obtained in Q Sepharose chromatography was applied to a Mono Q HR5/5 anion-exchange column and eluted by a linear gradient of NaCl (0 to 0.75 M NaCl) in 20 mM Tris hydrochloride buffer (pH 7.4) at a flow rate of 2.0 ml/min, using the Pharmacia fast protein liquid chromatography system. One-milliliter fractions were collected.

Protein assay. The protein concentrations were measured by the method of Lowry et al. (17) with bovine serum albumin as the standard.

Molecular weight determination. The molecular weight of the protease was estimated by gel filtration on a Sephadex G-200 column (1.6 by 70 cm) by the method of Andrews (2).

TABLE 1. Effect of urea concentrations on glycylprolyl peptidase extraction

Urea concn (M)	Gly-Pro peptidase (mU/ml)	Protein (mg/ml)	Sp act (mU/mg)	Enzyme release[a] (%)
0	0	0.10	0	0
1	15	0.12	125	23.43
2	15	0.12	125	23.43
4	24	0.24	100	37.5
6	56	0.24	233	87.5

[a] Total enzyme activity in 1 ml of bacterial suspension (100 mg of cells per ml) was 64 mU of glycylprolyl activity defined as 100%.

To examine the purity of the sample, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (14) with a 12.5% separation gel and a 3.5% stacking gel, using a discontinuous buffer system and a minigel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Samples were dissolved in sample buffer containing 4% SDS and were heated at 100°C for 2 min. A mixture of six protein standards (low-molecular-weight calibration kit, Pharmacia) was electrophoresed in parallel in the presence of 5% 2-mercaptoethanol for the estimation of molecular weight. Electrophoresis was performed at 5 mA for 5 h and the gels were stained with Coomassie brilliant blue R-250. The presence of lipopolysaccharides in the sample was detected in silver-stained gels by the method of Hitchcock and Brown (11). Reagents for gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, Calif.

Determination of optimum pH. The enzyme activity was determined at various pH values by using Gly-Pro-NA as the substrate with 0.1 M Tris hydrochloride buffer (pH 7.0 to 9.0), 0.1 M acetate buffer (pH 4.0 to 5.6), 0.1 M potassium phosphate buffer (pH 5.0 to 8.0), and 0.1 M carbonate buffer (pH 9.0 to 10.0). The relative activity was expressed as the percentage of activity under standard assay conditions (0.1 M Tris hydrochloride buffer, pH 7.4).

Effects of various protease inhibitors. The effect of various inhibitors and metal ions was determined at pH 7.4 in a 0.1 M Tris hydrochloride buffer. The purified enzyme preparation was preincubated with each inhibitor for 15 min at room temperature before the substrate solution was added to start the proteolytic reaction. The activity was expressed as a percentage of total activity in the control which did not contain any inhibitors. The protease inhibitors included *p*-aminophenylmercuric acetate, *N*-ethylmaleimide, iodoacetate, antipain, leupeptin, EDTA, 1,10-phenanthroline, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, 1-chloro-3-tosylamide-7-amino-L-2-heptanone hydrochloride, pepstatin, α_1-antitrypsin, and α_2-macroglobulin (Sigma). The effect of zinc chloride and mercuric chloride on the enzyme was also investigated. Stock solutions of inhibitors were prepared in TBS (50 mM Tris hydrochloride, 0.2 M NaCl; pH 7.4) with or without initial dilution in organic solvents and used in concentrations as described previously (4).

RESULTS

***B. gingivalis* degraded all four synthetic substrates used in this study and it also degraded reconstituted collagen.** Pilot experiments revealed that the optimum condition for the extraction of glyclyprolyl peptidase from *B. gingivalis*

TABLE 2. Purification of glycylprolyl peptidase from *B. gingivalis* SUNYAB A7A1-28

Purification step	Vol (ml)	Total protein (mg)	Total units (mU)	Sp act (mU/mg)	Purification (fold)	Yield (%)
6 M urea extraction	500	260.0	20,000	77	1	100
Extract concn (PM30)	50	49.0	18,666	381	4.9	93.3
Sephadex G-200	10	2.0	4,400	2,200	28.6	22
FPLC[a] Q Sepharose	2	0.3	2,332	7,773	101.05	11.5
Mono Q HR5/5	4	0.02	1,600	80,000	1,040	8

[a] FPLC, Fast protein liquid chromatography.

SUNYAB A7A1-28 whole cells was 6 M urea in 50 mM Tris hydrochloride–0.2 M NaCl (pH 7.4). This released 87.5% of the enzyme with a specific activity of 233 mU/mg of protein (Table 1). This concentration of urea was used to obtain starting material for subsequent experiments.

Extraction of 50 g (wet weight) of *B. gingivalis* SUNYAB A7A1-28 yielded 91% of total glycylprolyl peptidase activity. This extract also contained glycylphenyl peptidase activity. It did not degrade *N*-CBz-Gly-Gly-Arg-NA, BANA, or collagenase. The extract was viscous and contained 0.4% (wt/vol) carbohydrate as determined by the anthrone reaction (24).

Concentration of the extract by ultrafiltration on a PM30 membrane yielded an increase in the specific activity of the enzyme by 4.9-fold (Table 2). The enzyme eluted as a single peak in fractions 25 to 40 (Fig. 1) from a Sephadex G-200 column with an apparent molecular weight of 75,000. This step achieved a 28.6-fold increase in purity. When the active fractions from gel filtration chromatography were applied to a Sepharose anion-exchange column, the enzyme eluted in fractions 66 to 82 (Fig. 2), corresponding to a gradient of 0.4 to 0.8 M NaCl. The active fractions from Mono Q column chromatography eluted in fractions 11 to 13 (Fig. 3). This procedure resulted in a 1,040-fold increase in purity and an 8% yield (Table 2).

Samples from each purification step were analyzed on an SDS-polyacrylamide gel under nonreducing conditions (Fig. 4). The final enzyme preparation demonstrated two bands with a molecular weight of approximately 80,000. When examined after the purified sample is boiled in the presence

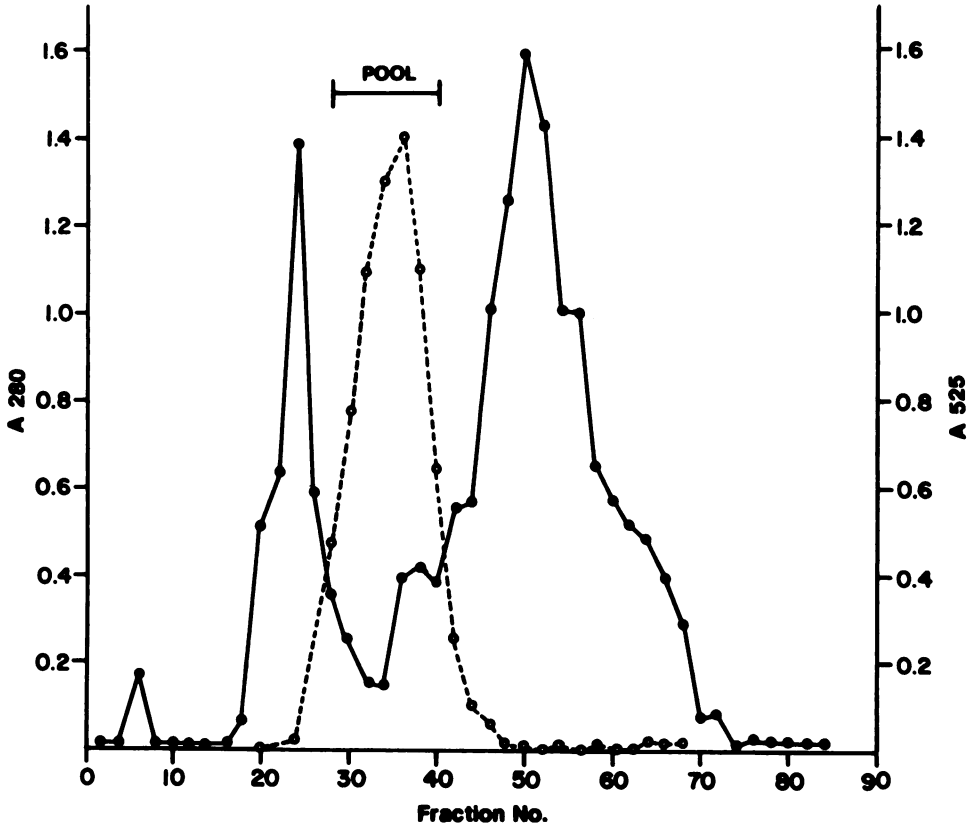

FIG. 1. Sephadex G-200 gel filtration chromatography of glycylprolyl peptidase. The PM30-concentrated enzyme was applied to the column and eluted as described in the text. Fractions 25 to 40 were pooled for further purification. Symbols: ●, protein (A_{280}); ○, glycylprolyl activity (A_{525}).

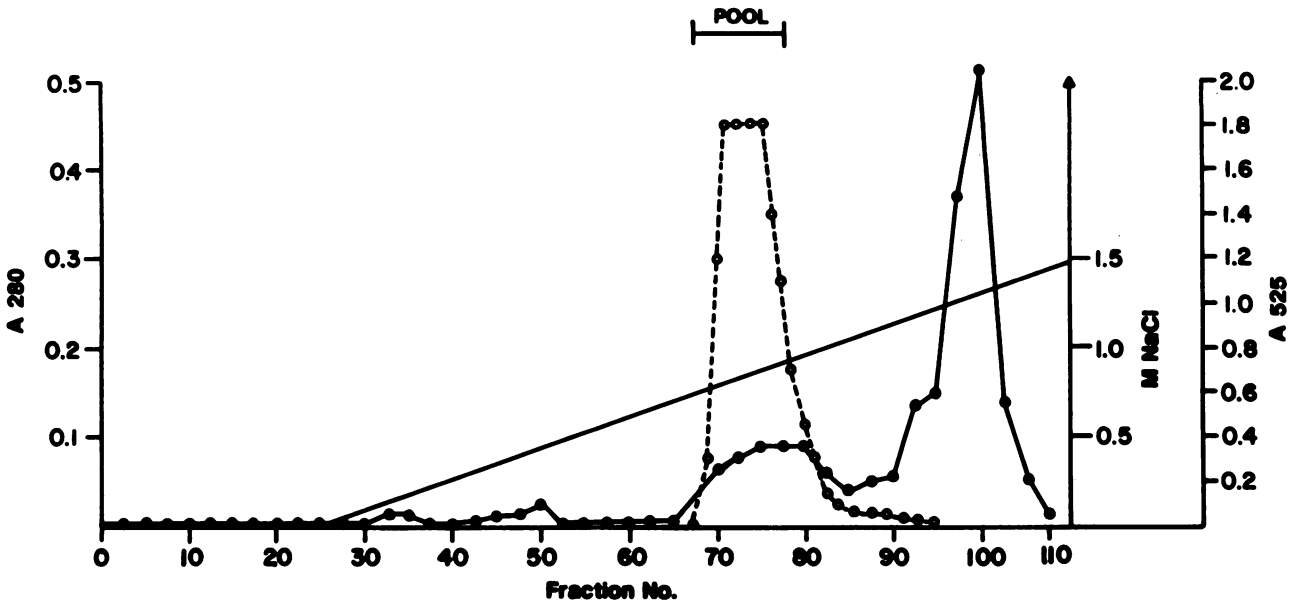

FIG. 2. Q Sepharose fast-flow ion-exchange column chromatography. The concentrated material from the Sephadex G-200 column was applied to the Q Sepharose column and eluted with a linear gradient of 0 to 1.5 M NaCl. Fractions 66 to 82 were pooled for further purification. Symbols: ●, protein (A_{280}); ○, glycylprolyl activity (A_{525}).

of a reducing agent, the apparent molecular weight does not change. At present we do not know whether both of the protein bands exhibit glycylprolyl activity. There is a trace protein contaminant of 94 kilodaltons in purified material, as demonstrated by Coomassie brilliant blue staining and also a trace of lipopolysaccharide contaminant at approximately 15 kilodaltons, as determined by silver staining. The optimum pH for hydrolysis of Gly-Pro-NA was 7.5 to 8.5. The enzyme activity decreased above pH 9.0 and below pH 6.5. The activity was completely lost at pH 5.0 (Fig. 5). In addition to Gly-Pro-NA, the purified enzyme degraded the substrate Gly-Phe-NA.

The effect of various protease inhibitors is shown in Table 3. Serine protease inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride showed 82.5 and 78% inhibition, respectively. Thiol blocking reagents such as *p*-aminophenylmercuric acetate and iodoacetate had no ef-

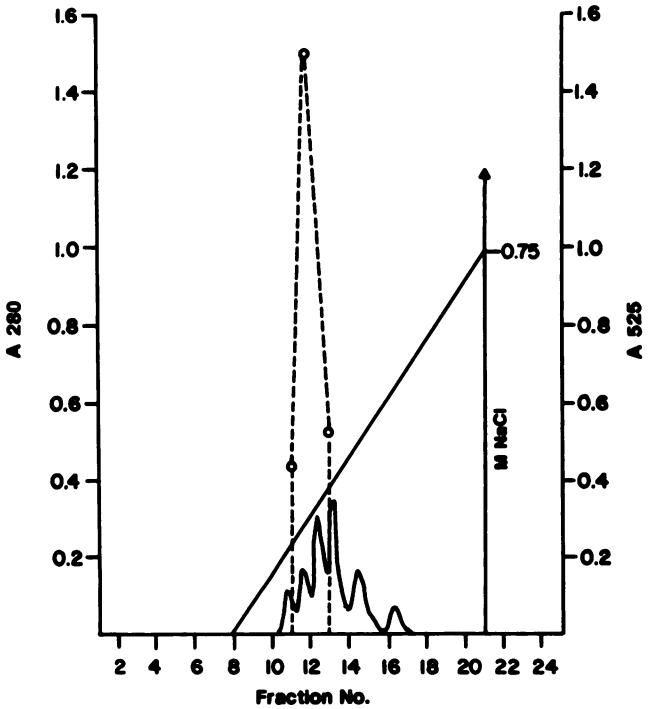

FIG. 3. Elution profile of the *B. gingivalis* glycylprolyl peptidase from fast protein liquid chromatography separation on a Mono Q HR5/5 prepacked ion-exchange column. ——, protein (A_{280}); – – –, glycylprolyl activity (A_{525}).

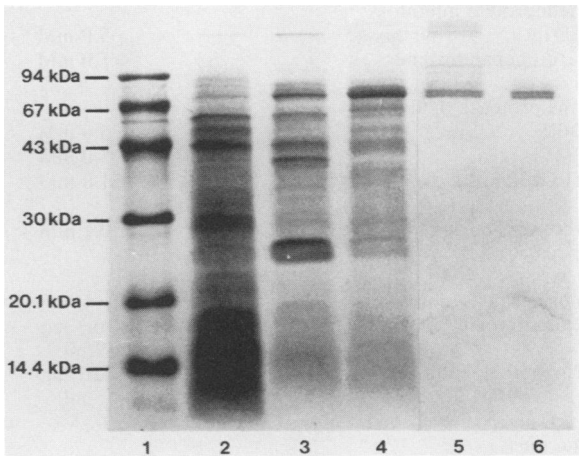

FIG. 4. Coomassie brilliant blue-stained 12.5% SDS–polyacrylamide gel, showing the glycylprolyl peptidase purification steps from *B. gingivalis* SUNYAB A7A1-28. Lane 1, Low-molecular-weight standards (Pharmacia), 10 μg; lane 2, 6 M urea extract, 20 μg; lane 3, PM30 membrane-concentrated urea extract, 15 μg; lane 4, active enzyme fractions from Sephadex G-200 column, 15 μg; lane 5, active enzyme fractions from Q Sepharose, 5 μg; lane 6, purified protein from Mono Q HR5/5 ion-exchange column, 2.5 μg. kDa, Kilodaltons.

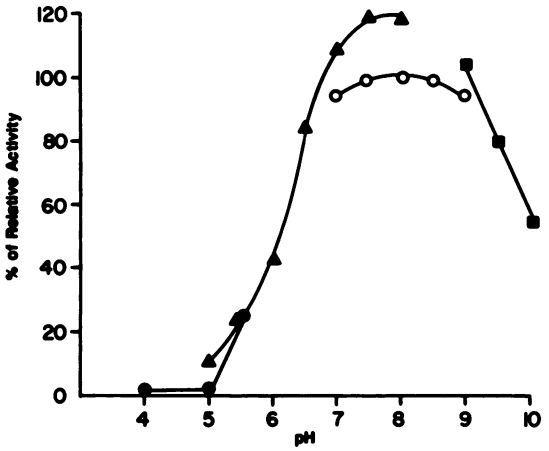

FIG. 5. Optimum pH for hydrolysis of Gly-Pro-NA by the *B. gingivalis* enzyme. The enzyme assay was performed at various pH values with the following buffers: ●, 0.1 M acetate, pH 4.0 to 5.6; ▲, 0.1 M phosphate, pH 5.0 to 8.0; ○, 0.1 M Tris hydrochloride, pH 7.0 to 9.0; ■, 0.1 M carbonate buffer.

fect on enzyme activity. The metal ions Hg^{2+} and Zn^{2+} inhibited the enzyme activity by 55.6 and 45%, respectively. Other protease inhibitors such as α_1-antitrypsin, α_2-macroglobulin, antipain, and leupeptin did not affect the enzyme activity. The enzyme activity was completely inactivated in the presence of 10 mM SDS.

DISCUSSION

On the basis of a variety of inhibitors, *B. gingivalis* enzymes can be classified as either thiol or serine proteases. Thiol proteases from *B. gingivalis* have been studied more extensively than serine proteases. *B. gingivalis* collagenolytic activity has been described by a number of investigators and has been identified as a thiol protease (4, 9, 18). A membrane-bound thiol protease was isolated and characterized by Yoshimura et al. (32) by using a synthetic substrate, benzoyl-L-arginine-*p*-nitroanilide. Several other investigators purified thiol proteases from *B. gingivalis* culture supernatants (7, 21) or from extracellular membrane vesicles (26). These enzymes were highly activated by thiol group reagents and inhibited by thiol protease inhibitors.

The serine proteases have been described less extensively. It has been shown that the synthetic substrate Gly-Pro-NA can be degraded by *B. gingivalis* (20, 28; K. C. Jin, P. K. Barua, J. J. Zambon, and M. E. Neiders, J. Endod., in press). This enzyme activity was shown to be inhibited by serine protease inhibitors and was found both in the supernatant and associated with the cell pellet (29). We purified the glycylprolyl peptidase of *B. gingivalis* cells, using a 6 M urea extract. The starting material did not contain the

TABLE 3. Effect of protease inhibitors and metal ions on the glycylprolyl peptidase activity of *B. gingivalis* SUNYAB A7A1-28

Inhibitor[a]	Concn	Solvent	% Residual activity
-SH blockers			
APMA	2.0 mM	TBS	133
N-Ethylmaleimide	5.0 mM	TBS	82.5
Iodoacetate	5.0 mM	TBS	105
Cathepsin inhibitors			
Antipain	0.1 mM	TBS	100
Leupeptin	0.1 mM	TBS	100
Metalloprotein inhibitors			
EDTA	5.0 mM	TBS	83.3
1,10-Phenanthroline	5.0 mM	TBS–1% DMSO[b]	100
Serine protease inhibitors			
DiPF	5.0 mM	TBS–0.1% isopropanol	17.5
PMSF	5.0 mM	TBS–1% DMSO	22.0
TLCK	1.0 mM	TBS–1% DMSO	100
Carboxyl protease inhibitor (pepstatin)	0.1 mM	TBS–1% ethanol	94.4
Protein protease inhibitors			
α_1-antitrypsin	0.1 mg/ml	TBS	122
α_2-macroglobulin	0.1 mg/ml	TBS	105
Metal ions			
$HgCl_2$	2.0 mM	TBS	44.4
$ZnCl_2$	2.0 mM	TBS	55.0
Detergent (SDS)	10 mM	TBS	0
Control			100

[a] APMA, *p*-Aminophenylmercuric acetate; DiPF, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; TLCK, 1-chloro-3-tosylamide-7-amino-L-2-heptanone hydrochloride.
[b] DMSO, Dimethyl sulfoxide.

"trypsinlike" enzyme activity detected by BANA (16), the enzyme that degrades *N*-CBz-Gly-Gly-Arg-NA (29) or collagenase (4). The purification steps yielded a 1,040-fold concentration of the glycylprolyl peptidase. Serine protease inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride showed distinct inhibition of enzyme activity, while other inhibitors did not decrease enzyme activity (Table 3). On the basis of inhibitor studies, we classified the enzyme as a serine protease.

There are reports on purification of two other serine proteases that break down Gly-Pro-NA from *B. gingivalis* (1, 10). Our enzyme has a molecular weight different from those of the other two *B. gingivalis* glycylprolyl peptidases described. Our enzyme had an apparent molecular weight of 80,000. Since the enzyme was extracted from cell pellets with urea, we can assume that the enzyme is located in the outer membrane or periplasm or both. Specific antibodies to the enzyme will be required in order to determine the precise location. Abiko et al. (1) purified a glycylprolyl peptidase from *B. gingivalis* 381 supernatant with a molecular weight of approximately 160,000. Grenier and McBride (10) purified a glycylprolyl peptidase from outer membranes of *B. gingivalis* ATCC 33277 with a molecular weight of 29,000 in the native state.

The question arises whether these proteases represent three separate enzymes or are breakdown products of one enzyme. It could be postulated that the enzyme that we obtained may be a breakdown product of the enzyme described by Abiko et al. (1). All of the described characteristics are similar except for the molecular size. However, it is unlikely that the enzyme described by Grenier and McBride (10) is a breakdown product of our enzyme. The optimal pH of their enzyme is 6.5, compared with pH 8 for our enzyme. Their enzyme was purified by SDS-polyacrylamide gel electrophoresis and showed activity after SDS-polyacrylamide gel electrophoretic analysis. The enzyme is only partially inactivated by 20 mM SDS, while our enzyme activity is completely abolished by 10 mM SDS. If these are different enzymes, then it suggests the production of several serine proteases by *B. gingivalis* that break down the same or different substrates. The production of several serine proteases has been described for *Escherichia coli* (22, 23). Further studies will be needed to determine whether the serine proteases of *B. gingivalis* are breakdown products or different enzymes.

The role of *B. gingivalis* serine proteases is not clear. So far very few native substrates that are degraded by purified serine proteases have been identified. The serine protease isolated by Grenier and McBride (10) breaks down azocasein and bovine serum albumin (8). The serine proteases of *E. coli* degrade *E. coli* membrane proteins (23), and *B. gingivalis* serine proteases may also have the ability to degrade membrane proteins. It is thought that such proteolytic activity may play a role in membrane turnover, metabolism, and biological regulation (22, 23, 27).

With the description of an increasing number of thiol and serine proteases, the implication of specific functions of these enzymes appears to be more complex. Further study of *B. gingivalis* enzymes is needed to determine the wide variety of roles that these enzymes may have in the metabolism, nutrition, and virulence of the organism.

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