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Characterization of the *Treponema denticola prtP* Gene Encoding a Prolyl-Phenylalanine-Specific Protease (Dentilisin)

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A chymotrypsin-like protease from *Treponema denticola* ATCC 35405 was purified by chromatographic techniques. The purified enzyme consisted of three polypeptides (38, 43, and 72 kDa). The protease exhibited specificity for peptide bonds containing phenylalanine and proline at the P1 and P2 positions, respectively, and was classified as a serine protease on the basis of inhibition studies. Naturally occurring protease inhibitors such as α1-antitrypsin and α1-antichymotrypsin had no effect on enzymatic activity. The enzyme degraded fibronectin, α1-antitrypsin, and gelatin while weakly degrading the immunoglobulin G heavy chain and type IV collagen. N-terminal amino acid sequences were determined for the 43- and 72-kDa proteins. On the basis of these sequences, the genes coding for the 43- and 72-kDa proteins were isolated and sequenced. The open reading frame which codes for the 72-kDa protein was designated *prtP*. This gene consists of 2,169 bp and codes for a protein with an M_r of 77,471. The protein appeared to be composed of a signal peptide region followed by a prosequence and the mature protein domain. The deduced amino acid sequence exhibited similarity with that of the *Bacillus subtilis* serine protease subtilisin. The deduced properties of the sequence suggest that the 72-kDa protein is a chymotrypsin-like protease. However, the nature and function of the 43-kDa protein have not yet been determined.

Treponema denticola is a helically shaped, motile bacterium which is found in the periodontal region of the human oral cavity. The levels of oral spirochetes, including *T. denticola*, increase dramatically in various types of human periodontal diseases (2, 15–17, 22, 32), and the microorganisms are considered to be putative pathogens in periodontitis. *T. denticola* displays several potential virulence properties, including the expression of attachment factors (4, 7, 44), resistance to host defense systems (10, 31), and proteases (41) which induce toxic effects on host cells. However, the absence of a suitable animal model for *T. denticola* has made it difficult to define the virulence factors of these organisms.

Various proteolytic enzymes which may be involved in the process of destruction of periodontal tissues have been reported for *T. denticola* strains (1, 6, 19–21, 24, 26, 39). Proteases of these microorganisms activate host latent procollagenase (34), exhibit cytopathic effects on porcine periodontal ligament cells (41), and degrade basement membrane collagen (40) as well as human bioactive peptides (20). For these proteolytic activities, trypsin-like enzymes (18, 24), chymotrypsin-like proteases (28, 39), a proline-specific oligopeptidase (19), and a proline iminopeptidase have been identified in this organism. The proline-specific oligopeptidase hydrolyzes bioactive peptides such as bradykinin (19). The chymotrypsin-like protease is located on the surface of *T. denticola* (6); it degrades host cell proteins such as fibronectin, fibrinogen, and immunoglobulin G (IgG) (39) and also hydrolyzes bioactive peptides such as bradykinin, substance P, and angiotensin I (20). It is possible that the degradation of host cell proteins and the inactivation of bioactive peptides by *T. denticola* have significant pathophysiologic consequences. These reports strongly suggested that the chymotrypsin-like protease may be an important virulence factor in these microorganisms. However, the structure of this enzyme has not yet been defined, although purification of the enzyme has been accomplished (20, 39). In the present study, we purified the chymotrypsin-like protease from *T. denticola* ATCC 35405 by conventional chromatography techniques and isolated and sequenced its corresponding gene.

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MATERIALS AND METHODS

Microorganisms and culture conditions. *T. denticola* and *Treponema vincentii* were maintained in TYGVS medium (24) under anaerobic conditions as described previously (9). For isolation of genomic DNA, *T. denticola* ATCC 35405 was grown in 100 ml of TYGVS medium, which was subsequently transferred to 4 liters of the same broth and cultivated at 37°C for 3 days.

Escherichia coli XL1-Blue and *E. coli* HB101 were used in subcloning and expression experiments. All *E. coli* strains were grown on Luria-Bertani agar plates or in Luria-Bertani broth in the presence of the appropriate antibiotics. Kanamycin was used at 25 μg/ml, ampicillin was used at 60 μg/ml, and tetracycline was used at 12.5 μg/ml.

Enzyme assays. The enzyme activity was measured by using the synthetic chymotrypsin substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (SAAPNA) (Sigma Chemical Company, St. Louis, Mo.). An aliquot of 5 μl of each enzyme preparation was mixed with 150 μl of 100 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM SAAPNA. The mixture was incubated at 37°C for 15 min, and then the reaction was stopped by adding 50 μl of 20% acetic acid. The release of *p*-nitroaniline was determined by measuring its A_{405}. One unit of the enzyme was defined as the amount of enzyme required to release 1.0 μmol of *p*-nitroaniline in 1 min at 37°C under these conditions. For evaluation of substrate specificity, the enzyme activity was measured with fluorescent substrates by the method of Kadowaki et al. (12). The quantity of protein was determined by the DC protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Purification of the protease. Cells of *T. denticola* ATCC 35405 grown in 4 liters of the medium were harvested by centrifugation at 8,000 × *g* for 20 min at 4°C and washed twice with 20 mM Tris-HCl buffer (pH 8.0). The cells were then suspended in 20 mM Tris-HCl buffer (pH 8.0) containing 1% of the zwitterionic detergent 3[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS) (Dojindo, Kumamoto, Japan). The cells were disrupted with a sonicator (Branson, Danbury, Conn.) at 100 W for 5 min on ice. Insoluble material was removed by ultracentrifugation at 105,000 × *g* for 1 h. The supernatant was absorbed to a Q-Sepharose fast-flow column (2.6 by 10 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1% (wt/vol) CHAPS. After the column was washed with the buffer, the enzyme was eluted with a 0 to 0.3 M NaCl gradient. The enzymatically active fractions were pooled, diluted with distilled water containing 1% CHAPS, and concentrated by ultrafiltration through a 50,000-molecular-weight-cutoff mem-

TABLE 1. Purification of the chymotrypsin-like protease from *T. denticola* ATCC 35405

Fraction	Vol (ml)	Total activity (U)[a]	Sp act (U/mg)	Purification (fold)
Sonicate	220	2,531.6	1.46	1
Q-Sepharose	220	1,054.2	4.94	3.4
Rotofor	9	345.4	5.05	3.5
G3000SWXL	2.23	26.0	11.16	7.6
DEAE-5PW	0.33	10.2	61.7	42.3

[a] The enzyme activity was determined at pH 8.0 with the synthetic substrate SAAPNA. The units are micromoles per minute at 37°C.

brane (Centriprep 50; Amicon, Beverly, Mass.). The fraction was brought to 50 ml with 1 mM Tris-HCl buffer (pH 8.0) containing 10 mM NaCl, 1% (wt/vol) CHAPS, and 2% (wt/vol) ampholytes (pH 5 to 7) (Bio-lyte; Bio-Rad Laboratories). This preparation was applied to a Rotofor isoelectric focusing cell (Bio-Rad Laboratories). The electrolytes in the anode and cathode chambers were 100 mM H_3PO_4 and 100 mM NaOH, respectively. Isoelectric focusing in the Rotofor cell was accomplished at 12 W of constant power at an initial voltage of 500 V at 4°C for 4 h. The focusing was continued until the voltage had been stabilized (1,200 V) for 30 min. Active enzyme fractions were concentrated and applied to a G3000SWXL gel filtration column (7.5 mm by 30 cm; TOSO, Tokyo, Japan) equilibrated with phosphate-buffered saline (pH 7.2) (PBS) containing 1% CHAPS. The column was washed at a rate of 0.8 ml/min, and fractions of 0.5 ml were collected. The fraction possessing protease activity was dialyzed against 10 mM Tris-HCl buffer (pH 8.5) containing 0.8% *n*-octyl-β-D-glucopyranoside (Dojindo) (octylglucoside) and concentrated with a Centriprep membrane. This fraction was then applied to an anion-exchange column containing DEAE-5PW (7.5 mm by 7.5 cm; TOSO) equilibrated with the same buffer with 0.8% octylglucoside and then eluted with 110 mM NaCl. The enzyme preparation from the gel filtration column was analyzed by high-performance liquid chromatography (HPLC) (Shimazu, Kyoto, Japan). A reverse-phase chromatography phenyl-5PW-RP column (4.6 mm by 7.5 cm; TOSO) was used, with the following HPLC parameters: solvent A was 0.02% trifluoroacetic acid in H_2O, solvent B was acetonitrile-H_2O-trifluoroacetic acid (80:20:0.02, vol/vol/vol), and the flow rate was 1 ml/min with a linear gradient of 5 to 80% acetonitrile over 40 min. Protein was detected at 220 nm.

Inhibition studies. The effect of various protease inhibitors on the purified enzyme was examined with SAAPNA as a substrate as described above. Five microliters of enzyme was incubated with 50 μl of inhibitor at various concentrations in 100 mM Tris-HCl buffer (pH 8.0) at 37°C for 10 min. The reaction was started by addition of 100 μl of 1.5 mM SAAPNA. The enzymatic activity was determined as described above.

Hydrolysis of natural substrates. Samples containing 10 μg of protein substrate were incubated with 2 μg of the enzyme for 6 h at 37°C. Human plasma fibronectin, human plasma α1-antitrypsin, human IgG, and human placenta type IV collagen (all obtained from Sigma) were used as substrates. Reaction mixtures were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. After electrophoresis, protein bands were stained with Coomassie brilliant blue. For the zymogram, the enzyme preparation was incubated with SDS sample buffer at 4°C overnight, and mixtures were separated on 6% SDS-PAGE gels containing 200 μg of gelatin per ml. After electrophoresis, the gels were incubated in 100 mM Tris-HCl buffer (pH 8.0) for 1 h and stained with Coomassie brilliant blue.

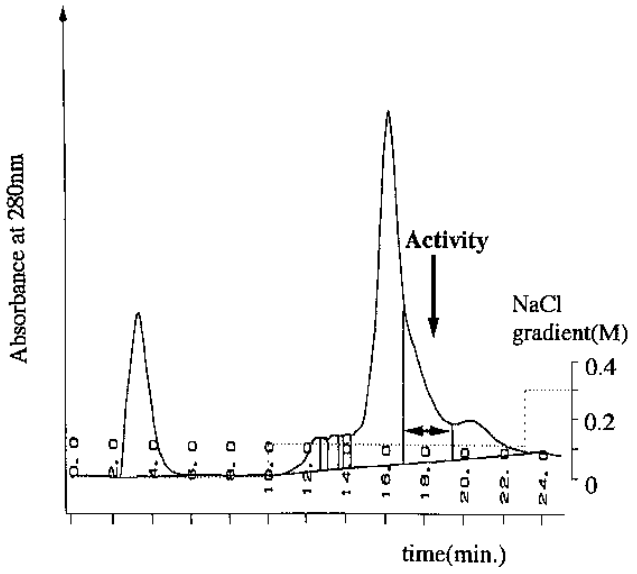

FIG. 1. Anion-exchange chromatography on a DEAE-5PW column. ·····, NaCl gradient; ——, A_{280}.

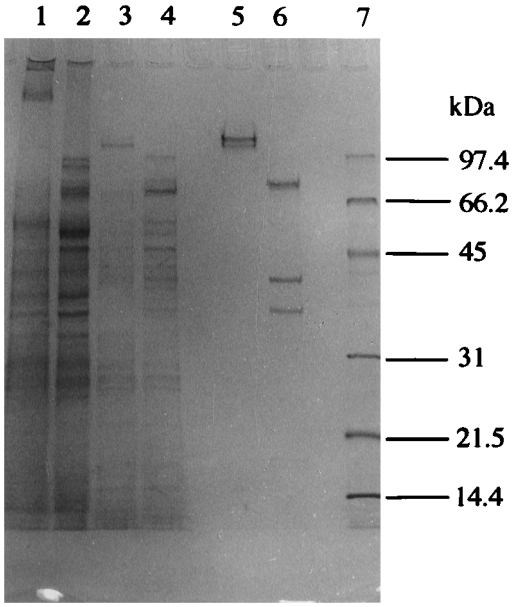

FIG. 2. SDS-PAGE of the enzyme after various purification steps. Lane 1, CHAPS extract of cells (without boiling); lane 2, CHAPS extract of cells (boiled); lane 3, Q-Sepharose fast-flow chromatography (without boiling); lane 4, Q-Sepharose fast flow chromatography (boiled); lane 5, DEAE-5PW chromatography (without boiling); lane 6, DEAE-5PW chromatography (boiled); lane 7, molecular size marker. After electrophoresis, the gel was stained with Coomassie brilliant blue.

Determination of N-terminal amino acid sequences. The purified samples (samples which were resolved with phenyl-5PW-RP columns or separated by SDS-PAGE under reducing conditions) were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, Mass.) and stained with Coomassie blue R-250 (Sigma). The stained bands were excised, and the absorbed proteins were sequenced with an automatic peptide sequencer (model 476A; Applied Biosystems, Foster City, Calif.).

Oligonucleotides and DNA amplification by PCR. For amplification of DNA fragments encoding the N-terminal amino acid sequences from the purified protease, four synthetic oligonucleotide primers were designed on the basis of the amino acid sequences of the N-terminal regions of the purified enzyme (positions 1 to 20 for the 72-kDa protein and 1 to 17 for the 43-kDa protein). The primers PR72D (5′-GGNYTNACNGAYGGNAAYTAY-3′), PR72U (5′-NA RNCCRTARTCNGCRTTRTT-3′), PR43D (5′-GCNYTNAAYCCNATHGA RGGN-3′), and PR43U (5′-RAANACRAANGCNGCNACRTA-3′) (N denotes complete degeneracy), which corresponded to the N- and C-terminal regions of the 72- and 43-kDa sequences, respectively, were synthesized on an Applied Biosystems model 391 DNA synthesizer.

Amplification of the DNA fragments was carried out with the two pairs of degenerate oligonucleotide primers in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–200 mM dATP–200 mM dCTP–200 mM dGTP–200 mM dTTP–2.5 U of AmpliTaq (Perkin-Elmer Cetus, Foster City, Calif.). The following PCR thermal cycle, repeated 25 times, was used: step 1, 94°C for 30 s; step 2, 55°C for 2 min; and step 3, 72°C for 2 min. Ten microliters of PCR products was blunt ended with 2.5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). The blunted fragments were purified by $CHCl_3$-phenol treatment and cloned into the plasmid vector pCR-Script (Stratagene). Sequences from the resulting clones were determined to encode the anticipated N-terminal amino acid sequences. According to these sequences, oligonucleotide probes (72P [5′-GGTAACTAT TTGGATGACCCTGAGGCAAACAACGC-3′] for the 72-kDa protein and 43P [5′-TTCTTCATAACGTTGCAGCCTT-3′] for the 43-kDa protein) were synthesized. These oligonucleotides were used as probes for further screening.

Construction of genomic library and screening. Genomic DNA from *T. denticola* was isolated as described previously (9). Standard procedures for recom-

TABLE 2. Effects of chemical agents on the chymotrypsin-like protease activity

Chemical agent[a]	Concn[b]	Relative activity[c] (%)
PMSF	1	6.1
DFP	1	3.2
Benzamidine	1	109
	5	108
Soybean trypsin inhibitor	10	51
Antichymotrypsin	10	93
PMBS	1	2.2
Iodoacetic acid	1	101
	5	94
N-Ethylmaleimide	5	90
TLCK	1	85
Leupeptin	1	98
	5	84
DTT	1	103
	10	109
Cysteine HCl	10	102
EDTA	5	59
$CaCl_2$	1	106
	10	115
$MgCl_2$	1	91
	10	99
$ZnCl_2$	1	54

[a] PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; TLCK: *p*-toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride; DTT, dithiothreitol.

[b] The concentrations of soybean trypsin inhibitor and antichymotrypsin are in micrograms per milliliter; all other concentrations are in millimolar.

[c] The enzyme activity was determined at pH 8.0 with SAAPNA as a substrate and with the sample after DEAE-5PW chromatography.

binant DNA manipulations were carried out as described by Sambrook et al. (29). For construction of the genomic bank, chromosomal DNA from *T. denticola* ATCC 35405 was partially digested with *Sau*3AI to yield fragments of 2 to 10 kbp, and the fragments were ligated with equimolar amounts of the phage vector Zap Express (Stratagene), which was linearized and treated with alkaline phosphatase. In vitro packaging was performed with the Gigapack II Gold packaging extract kit (Stratagene). The resulting lysate was used to transduce *E. coli* XL1-Blue MRF, and the infected cells were used to identify positive clones from the Zap Express library as follows. The library was plated out, and plaques were transferred to charged nylon membranes (Hybond-N+; Amersham, Arlington Heights, Ill.). The oligonucleotide probes (72P and 43P) were labeled with digoxigenin (DIG) by using a DIG oligonucleotide tailing kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Hybridization was carried out for 18 h in 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–1% blocking buffer (Boehringer Mannheim)–0.1% sarcosine–0.01% SDS–0.1 mg of poly(A) (Boehringer Mannheim) per ml at 54°C with probe 72P or 43P. Positive clones were detected with the DIG DNA detection kit (Boehringer Mannheim) and subjected to subcloning.

Southern blot analysis. Southern hybridizations were performed by standard procedures (29). Chromosomal DNA from *T. denticola* was digested with *Hin*dIII. Restricted chromosomal DNA was electrophoresed through 1.0% agarose gels, denatured, and transferred to Hybond-N+ by capillary transfer (35). A DNA probe (571-bp *Cla*I-*Pst*I fragment [see Fig. 5]) was labeled with DIG-dUTP by using a DIG DNA labeling system (Boehringer Mannheim) according to the manufacturer's protocol. Hybridization was performed at 42°C in aqueous buffer containing 50% formamide, 5× SSC, 1% blocking buffer (Boehringer Mannheim), 0.1% sarcosine, and 0.01% SDS for 18 h. Hybridized membranes were washed at high stringency as specified by the supplier, and hybridizing probes were detected on Hybond-N+ by using a DIG DNA detection kit.

DNA sequencing. The phage vector Zap Express, which contained the protease gene, was excised with the ExAssist helper phage (Stratagene) and recircularized to generate subclones in the pBK-CMV phagemid according to the manufacturer's instructions. The cloned DNA fragments from chimeric plasmids pKAZ16 and pMCH14 were subcloned into pBluescript SK+ in *E. coli* XL1-Blue MRF. Template DNA was prepared with the Wizard miniprep system (Promega, Madison, Wis.). DNA was sequenced with double-stranded plasmid DNA as a template by the dideoxynucleotide chain termination method (30) with a dye primer sequencing kit (Applied Biosystems) or a dye terminator sequencing kit (Applied Biosystems) together with a synthetic oligonucleotide primer. The sequence was determined with an Applied Biosystems model 373A automated DNA sequencer. Both DNA strands were sequenced, with all fragments containing overlaps of the adjacent sequence. Nucleotide sequences were assembled and analyzed with the DNASIS software package (Hitachi, Tokyo, Japan). Amino acid homology searches and comparisons were done with the FASTA and BLAST network services of DDBJ.

Purification of the 72-kDa protein expressed in *E. coli* and preparation of antisera. Amplification of the protease gene-encoding fragments and cloning of amplified fragments were carried out as described above with Expand High Fidelity (Boehringer Mannheim) and synthesized primers (*prtP* head primer, 5′-CGGTCTGACAGACGGTAATTATTTGG-3′; *prtP* tail primer, 5′-ACGGATCCCCTGTAAACCGTAACTC-3′). After amplification, blunted fragments were digested with *Bam*HI, purified by $CHCl_3$-phenol treatment, and cloned into the *Nru*I-*Bam*HI site of the expression plasmid Xa3 (Promega). The resulting plasmid was designated Tag3 and was transformed into *E. coli* HB101. After incubation of HB101/Tag3 cells for 18 h, the cultures were incubated for 4 h with IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were harvested and washed with 10 mM Tris-HCl buffer (pH 8.0). The cells were next resuspended with 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 0.5% CHAPS and were disrupted with a sonicator (Branson) at 100 W for 5 min on ice. Unbroken cells were removed by centrifugation (8,000 × *g*, 15 min). The crude extract was applied to 5 ml of Softlink Resin (Promega) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 0.5% CHAPS. The resin was washed with 30 ml of the same buffer, and the absorbed fraction was eluted with the buffer containing 5 mM biotin (Sigma). Eluted material was collected and characterized by SDS-PAGE analysis. The 72-kDa protein (20 μg) purified from Tag3 was injected intramuscularly into a New Zealand White rabbit with incomplete Freund's adjuvant. Subsequent intramuscular injections, with the adjuvant, were carried out at days 7 and 14. The rabbit was then injected intravenously without adjuvant on day 21. The rabbit was finally bled via the marginal vein on day 30. The specificity of the resulting serum was monitored by immunoblotting (38) with a Transblot cell (Bio-Rad) with the serum as the first antibody and goat anti-rabbit IgG (Bio-Rad) as the second antibody.

Immunogold labeling. Immunogold labeling was performed according to the method of Grenier et al. (6). Briefly, 2-day cultures of *T. denticola* cells were harvested, washed twice with PBS (pH 7.2), and adjusted to an A_{600} of 1.0. The treponemal cell suspension (200 μl) was incubated with an equal volume of anti-72-kDa serum or normal rabbit serum at 4°C for 1 h. Cells were washed twice with PBS and suspended in 200 μl of PBS. The cells were then mixed with 25 μl of gold particles (15 nm) conjugated with goat anti-rabbit IgG fraction (BioCell, Golden Gate, England) at 4°C overnight. The unbound secondary antibody was removed by centrifugation, and the cells were washed twice with PBS and resuspended in PBS. The cell suspensions were negatively stained with 1% phosphotungstic acid. Bacteria coated with nonimmune serum were included as a control. Observations were made with an H7100 electron microscope (Hitachi).

Nucleotide sequence accession numbers. The nucleotide sequences of KAZ16 and MCH14 have been assigned DDBJ accession number D83264.

RESULTS

Purification of the prolyl-phenylalanine-specific protease. Table 1 shows a representative scheme for the purification of the chymotrypsin-like protease from *T. denticola* cells. The

TABLE 3. Substrate specificity of the chymotrypsin-like protease

Substrate[a]	Enzyme activity (U/mg)[b]
Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide	61.7
Alanyl-alanyl-phenylalanine *p*-nitroanilide	0
N-Succinyl-glycyl-glycyl-phenylalanine *p*-nitroanilide	0
Glycyl-phenylalanine *p*-nitroanilide	0
L-Phenylalanine *p*-nitroanilide	0
Glutaryl-L-phenylalanine *p*-nitroanilide	0
N-Benzoyl-L-tyrosine *p*-nitroanilide	0
N-α-Benzoyl-L-arginine *p*-nitroanilide	0
Succinyl-L-alanyl-L-alanyl-L-prolyl-L-leucine *p*-nitroanilide	5.8
Succinyl-L-alanyl-L-alanyl-L-alanyl *p*-nitroanilide	0
N-Succinyl-L-leucyl-L-leucyl-L-valyl-tyrosine 7-AMC	0
Glycyl-proline *p*-nitroanilide	0
Lysine *p*-nitroanilide	0

[a] All substrates were purchased from Sigma. AMC, amido-4-methylcoumarin.

[b] The enzyme activity was determined at pH 8.0 with the sample after DEAE-5PW chromatography.

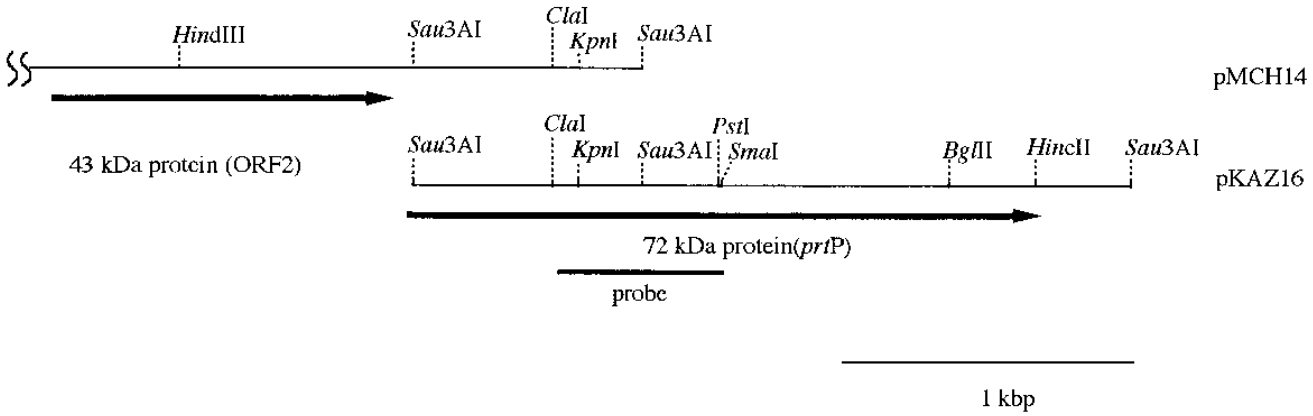

FIG. 3. Restriction map of inserts of pKAZ16 and pMCH14.

protease was extracted from the *T. denticola* cells with 10 mM Tris-HCl buffer (pH 8.0) containing 1% CHAPS by sonication. The solubilized protease was absorbed to Q-Sepharose fast-flow columns and eluted at approximately 200 mM NaCl from the resin. The crude preparation from Q-Sepharose was next separated with the Rotofor apparatus. The fraction at pH 4.95 exhibited the greatest SAAPNA-hydrolyzing activity, and no activity was found in other fractions. Following gel filtration chromatography on G3000 SWXL (data not shown), the active fractions were further purified on DEAE-5PW anion-exchange columns (Fig. 1). By this step, a 42.3-fold purification from the original sample was accomplished (Table 1). As shown in Fig. 2, SDS-PAGE with 10 to 20% gradient gels without boiling of the sample yielded two bands at 100 and 98 kDa. When the sample was boiled before electrophoresis, three bands were observed, at 72, 43, and 38 kDa. However, several other modes of chromatography, including hydrophobic chromatography on phenyl-5PW (TOSO) and hydroxyapatite chromatography on HW 1000 (TOSO), were unsuccessful in resolving the 100- and 98-kDa bands. These results suggested that the SAAPNA-hydrolyzing protease dissolved as an oligomeric form when solubilized with CHAPS. A proteolytic band at around 100 kDa was observed in a zymogram with a 6% SDS-PAGE gel containing gelatin (data not shown).

Enzymatic activity of the purified protease. The optimal pH of this enzyme was found to be approximately 8.0. The activity was drastically affected by the serine protease inhibitors phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate and by the cysteine protease inhibitor *p*-mercuribenzenesulfonic acid (PMBS) (Table 2). However, other cysteine protease inhibitors, such as *N*-ethylmaleimide and iodoacetic acid, had no significant effects. The activity was also reduced by EDTA, $ZnCl_2$, and soybean trypsin inhibitor. The naturally occurring protease inhibitor α1-antichymotrypsin did not affect the activity. The purified protease hydrolyzed SAAPNA strongly and *n*-succinyl-alanyl-alanyl-prolyl-leucine *p*-nitroanilide weakly but did not hydrolyze other substrates containing phenylalanine or tyrosine in the P1 position (Table 3). This result indicated that the enzyme activity required phenylalanine in the P1 position and proline in the P2 position in order to maximally hydrolyze the peptide bond. Therefore, the enzyme appears to be a prolyl-phenylalanine-specific protease. To determine the natural substrates of the enzyme, several proteins were examined. The enzyme strongly hydrolyzed fibronectin and α1-antitrypsin, while human IgG and type IV collagen were weakly digested (data not shown).

Determination of the amino-terminal amino acid sequences of the purified protease. The N-terminal amino acid sequences of the proteins resolved from the purified enzyme (Fig. 2) were directly determined by Edman degradation. Both the 100- and 98-kDa protein bands consisted of mixtures of the 72- and 43-kDa proteins which were resolved following boiling of the enzyme in SDS-PAGE sample buffer. The N-terminal amino acid sequences of the 72- and 43-kDa proteins were Gly-Leu-Thr-Asp-Gly-Asn-Tyr-Leu-Asp-Asp-Pro-Glu-Ala-Asn-Asn-Ala-Asp-Tyr-Gly-Leu and Ala-Leu-Asn-Pro-Ile-Glu-Gly-His-Asn-Ser-Ser-Tyr-Val-Ala-Ala-Phe-Val-Phe, respectively. The 38-kDa polypeptide could not be sequenced, since its amino terminus appeared to be blocked.

Isolation of the protease gene. In order to characterize the protease gene, the genes expressing the 72- and 43-kDa proteins were cloned. On the basis of the sequences of the N termini of both proteins, oligonucleotide primers were synthesized and the DNA fragments which encode the amino termini of the 72- and 43-kDa proteins were amplified by PCR. The amplified fragments were cloned into the plasmid vector pCR-Script. Ten clones were sequenced to check the validity of the sequences. The clones for the 72- and 43-kDa proteins included 60- and 54-bp fragments, respectively. Following nucleotide sequencing of the inserts, the deduced amino acid sequences from the cloned fragments were determined to be identical to the N-terminal amino acid sequences of the 72- and 43-kDa proteins. The central regions of the nucleotide sequences were then utilized for synthesizing oligonucleotide primers to screen a *T. denticola* genomic library amplified in Lambda Zap for the genes encoding both proteins. One positive clone which reacted with the 72P primer and two positive clones which reacted with the 43P primer were independently obtained from a total of 6,000 plaques by plaque blot hybridization. Southern blot analyses indicated that the latter clones contained identical fragment inserts (data not shown). The clones which hybridized with the probes for the 72- and 43-kDa proteins were designated KAZ16 and MCH14, respectively. These *T. denticola* inserts from the phage DNA were excised from the phage DNA with helper phages and were circularized as plasmids, which were designated pKAZ16 and pMCH14, respectively.

The results of restriction mapping as well as Southern blot analyses indicated that pKAZ16 and pMCH14 contained overlapping DNA fragments (Fig. 3). To confirm that the insert fragments in plasmids pKAZ16 and pMCH14 were indeed derived from *T. denticola*, Southern blot analysis was carried

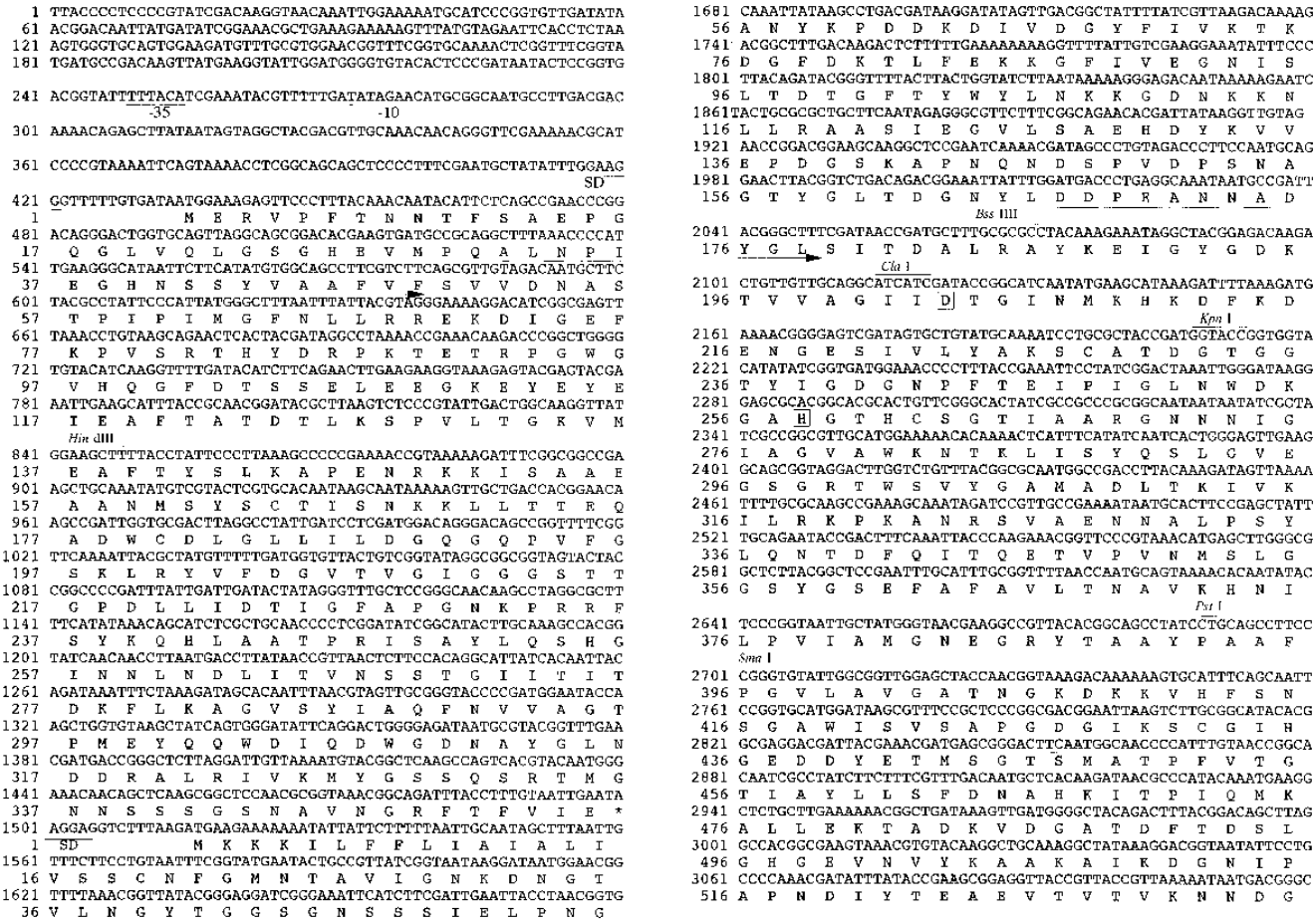

FIG. 4. Nucleotide sequences of the *prtP* and 43-kDa protein genes from *T. denticola* and the deduced amino acid sequences. The locations of the endonuclease restriction sites are indicated above the sequence. The predicted catalytic triad is indicated by boxes. N-terminal amino acid sequences determined from the purified enzyme are indicated by arrows. Possible Shine-Dalgarno (SD) sequences are double underlined. Potential promoter sequences are underlined.

out. The DIG-dUTP-labeled *Cla*I-*Pst*I fragment (Fig. 3) reacted with all *T. denticola* strains tested but not with other oral treponemes, such as *T. vincentii*, which also expresses proteolytic activity (data not shown). Interestingly, restriction polymorphism was observed among the *T. denticola* strains. A 6.2-kbp band was detected in *T. denticola* ATCC 33520, and a 2.8-kbp band was observed with *T. denticola* ATCC 35404 and ATCC 35405 (data not shown). This result confirmed that the insert DNAs of the plasmids pKAZ16 and pMCH14 were derived from *T. denticola.*

Sequence analysis of the protease gene. In order to identify and characterize the protease gene, both insert DNA strands of plasmids pKAZ16 and pMCH14 were sequenced. The sequence data indicated the presence of two tandemly located open reading frames (ORFs) on the inserts contained in the two plasmids (Fig. 3). In addition, both the 43- and 72-kDa N-terminal sequences were identified in each of the ORFs (Fig. 4). The downstream ORF codes for the 72-kDa protein and was designated the *prtP* (protease with phenylalanine specificity) gene. The presumed initiation codon of this gene was preceded by a sequence which is highly homologous to the *E. coli* Shine-Dalgarno consensus sequence. No potential stem-loop structure or ORFs were identified 400 bp downstream from the *prtP* gene. The *prtP* gene, which contains 2,169 bp, would code for a putative protein of 722 amino acids with a calculated molecular mass of 77,471 Da and an estimated pI of 5.42. The G+C content of the gene (44.4%) is somewhat higher than that estimated for the chromosomal DNA from *T. denticola* strains (37 to 38%) (33). The first 26 amino acid residues appeared to have the characteristics of a signal sequence with a basic amino acid terminus followed by a hydrophobic sequence. Furthermore, this region exhibits a potential

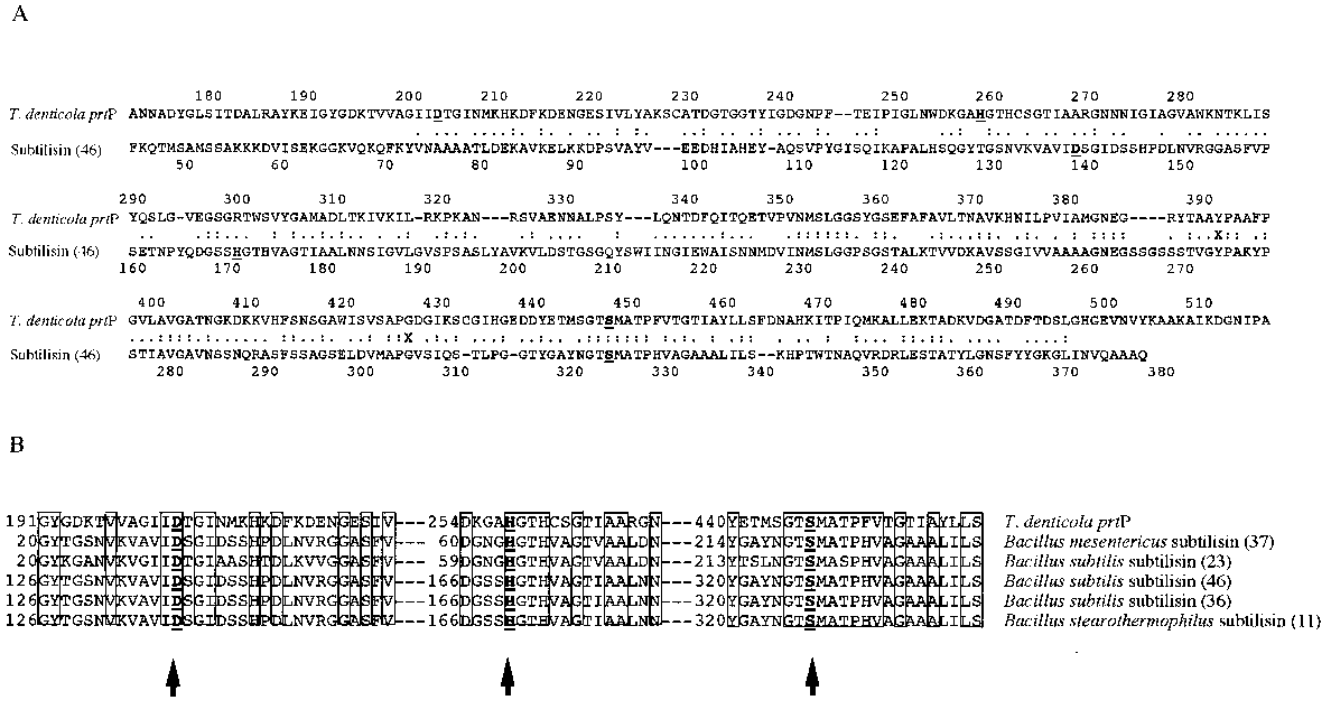

FIG. 5. Comparison of the deduced amino acid sequences of the prolyl-phenylalanine-specific protease (*T. denticola prtP* protease) and subtilisins. (A) Overall similarity. The active-site residues are in boldface and underlined. (B) Similarities around catalytic residues. The active-site residues are indicated by arrows, and identical residues are indicated by boxes. Numbers in parentheses are reference numbers.

signal sequence cleavage site (between Ala-26 and Val-27), as judged by the rules proposed by von Heijne (42). The residues from amino acids 27 to 158 are likely a prosequence, since this sequence precedes the N-terminal amino acid of the purified 72-kDa protein. The calculated molecular mass and predicted isoelectric point of the mature enzyme would be 60,389 Da and 5.36, respectively. This pI value is approximately the same as that deduced from isoelectrofocusing fractionation with the Rotofor. The codon bias, such as TTT for Phe, CTT for Leu, and TAT for Tyr, was similar to that of other cloned genes from *T. denticola* (8, 9). The ORF which codes for the 43-kDa protein (ORF 2) contains 1,068 bp and would code for a putative protein of 355 amino acids with a calculated molecular mass of 38,854 Da and an estimated pI of 5.42.

A comparison of the amino acid sequence encoded by the *prtP* gene with those of other proteins in the DDBJ database with the FASTA program revealed 25.9% identity with the subtilisin type serine proteases in a 309-amino-acid overlap (Fig. 5). According to the BLAST program, the amino acid sequences of the putative active-site residues of the subtilisins, containing the catalytic triad (Asp, His, and Ser), were conserved in the corresponding region of the *prtP* protein (Fig. 5).

To confirm the expression of the *prtP* protease in *T. denticola*, the expression of a fusion protein containing the biotin-binding protein transcarboxylase and mature *prtP* protein was evaluated by using the plasmid vector Xa3. The plasmid expressing the fusion protein was designated Tag3. The product expressed in *E. coli* did not yield detectable protease activity. We obtained rabbit antiserum by immunization with the recombinant 72-kDa protein. The result of immunoblotting showed that the rabbit antiserum reacted with the sonicate of *T. denticola* ATCC 35405 at 72 kDa (data not shown). Electron microscopic observation of *T. denticola* that had been incubated with the antiserum against the 72-kDa protein and gold particles conjugated with goat anti-rabbit IgG revealed that the gold particles were concentrated on the cell surface (Fig. 6). This result further suggested that the *prtP* protease is expressed on the cell surface of *T. denticola.*

DISCUSSION

Several enzymes with SAAPNA-hydrolyzing activity have been reported for *T. denticola* (1, 20, 39). The profiles of natural substrates hydrolyzed by the *prtP* protease indicate that the enzyme differs from the *prtA* and *prtB* proteases, whose genes were previously isolated (1, 26). The protease described here shares a number of enzymatic properties, such as susceptibility to phenylmethylsulfonyl fluoride and PMBS and substrate specificity, with the chymotrypsin-like protease previously purified from *T. denticola* (20, 39). However, the optimal pH and the effects of sulfhydryl reagents and EDTA differ somewhat. These discrepancies may derive from differences in the methods of enzyme purification utilized in the three laboratories. Therefore, it is possible that the enzymes previously characterized (20, 39) are identical to the *prtP* prolyl-phenylalanine-specific serine protease characterized in the present investigation. The observation that the expression of the *prtP* protein in *E. coli* did not result in enzymatic activity may explain why previous attempts to isolate the chymotrypsin-like protease in *E. coli* by using activity screening have not succeeded and resulted in the isolation of two other genes, *prtA* and *prtB* (1, 26), which are clearly distinct from that encoding the *prtP* protein.

In the present study, the purified protease appeared to be a serine protease on the basis of the results of the inhibition studies and the deduced amino acid sequence of the *prtP* gene, which encodes a 72-kDa protein with similarity to the serine protease subtilisin. These results suggested that the 72-kDa

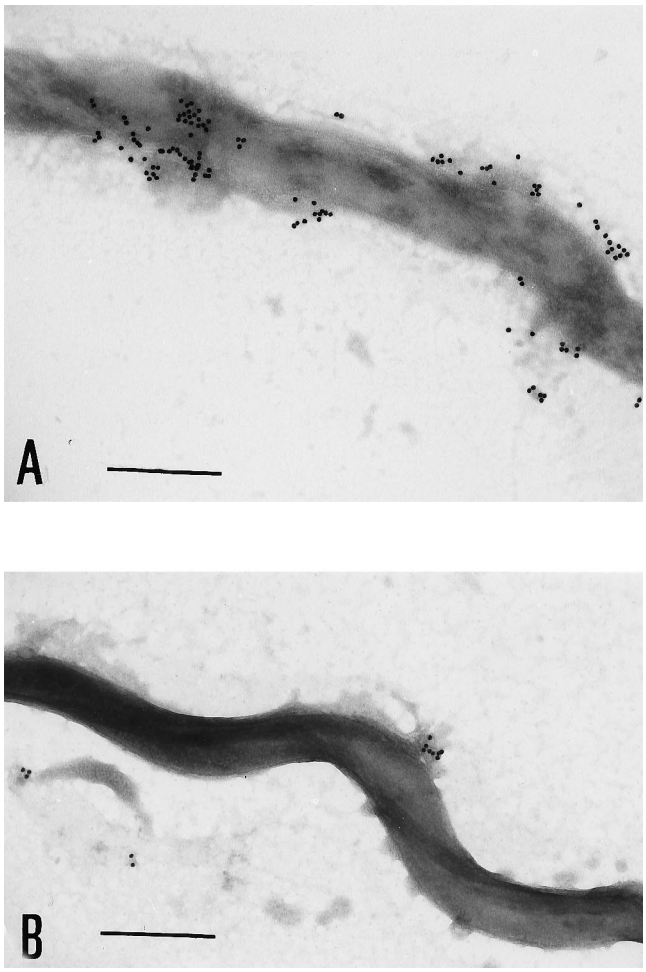

FIG. 6. Immunoelectron microscopy of *T. denticola* ATCC 35405 cells reacted with the anti-72-kDa antibody. Many gold particles can be seen on the surface of *T. denticola* ATCC 35405 incubated with anti-72-kDa serum (A) but not on the cell incubated with preimmune serum (B). Bars, 0.33 μm.

protein is the prolyl-phenylalanine-specific protease of *T. denticola*. Interestingly, the chymotrypsin-like proteases previously characterized (20, 39) formed a single band on SDS-PAGE with unboiled samples; however, they also consisted of three proteins. The protease purified by Uitto et al. (39) was composed of 72-, 27-, and 23-kDa proteins, and that purified by Mäkinen et al. (20) was composed of 76-, 39-, and 32-kDa proteins. The protease in this study contains 72-, 43-, and 38-kDa proteins. Two possible explanations for these differences could be that the active protease (72 kDa) associates with smaller proteins or that the protease (72 kDa) is autodegraded into smaller proteins. The observation that the sequence of the 43-kDa protein was identified upstream of the *prtP* sequence makes the latter possibility unlikely. In the previous reports and the present study, the sizes of the protein bands are similar, especially those of the 72-kDa proteins. The present results indicated that the 100- and 98-kDa proteins are readily separated into three proteins with organic solvents (data not shown). The sequence results indicated that the 72- and 43-kDa proteins contain cysteine residues. However, no difference was found in the structure of the 100-kDa protein complex in the presence or absence of 2-mercaptoethanol. These results indicated that the 100-kDa protein does not appear to be stabilized by disulfide bonds. There are several reports concerning high-molecular-mass oligomeric polypeptides in spirochetes (3, 5, 13, 43, 45). Weinberg and Holt (45) reported that some proteins of *T. denticola* were detected at between 100 and 200 kDa in SDS-PAGE without boiling. However, these proteins were detected as smaller proteins following boiling. Several attempts using reagents such as urea or dithiothreitol at different temperatures also did not resolve these proteins. The association of the 72- and 43-kDa or the 72- and 38-kDa proteins may generate bands of higher molecular mass on SDS-PAGE with unboiled samples (Fig. 3). In addition, the 43-kDa protein exhibits no sequence similarity with any protease and may not contain intrinsic protease activity. It is likely that detergents such as SDS or CHAPS dissolve the protease as an oligomeric form. This oligomeric protein may consist of protease and other membrane-associated proteins. Further analysis is required to define the interactions of the protease constituting the 100-kDa oligomer.

The molecular mass of the putative protease in the purified enzyme is 72 kDa on SDS-PAGE gels. An analysis of the deduced amino acid sequence of the *prtP* gene suggested that the protease consists of three domains: a 26-amino-acid signal sequence, a 132-amino-acid prosequence, and then the mature protein domain. According to the BLAST program, residues 440 to 462, 254 to 272, 191 to 233, 271 to 280, and 117 to 158 showed 56, 57, 32, 70, and 23% identity, respectively, with subtilisin (37). In addition, the *prtP* protease exhibited similarity with other subtilisins (11, 23, 36, 46). The amino acid sequences of the putative active-site residues of the subtilisins were conserved in the corresponding region of the *prtP* protein (Fig. 5). These observations strongly suggest that this mature protein contains a protease domain. The molecular mass of the deduced amino acid sequence of the mature protein is 60,389 Da. This molecular mass agrees well with that of the expressed fusion protein with transcarboxylase (72 kDa) in *E. coli*. However, the size is smaller than that of the purified enzyme from *T. denticola*. This suggests the possibility that the mature protein may migrate anomalously in SDS-PAGE gels.

The purified enzyme was inhibited by only one of the cysteine protease inhibitors examined, PMBS. Some proteases belonging to the subtilisin family require thiol activation. This type of enzyme has cysteine residues near the active-site histidine residue (27). The deduced amino acid sequence of the mature *prtP* protease contains six cysteine residues. Specifically, Cys-262 is located near His-258, which is a component of the proposed catalytic site. It is possible that this Cys residue interacts with PMBS and alters the enzymatic activity. The distance between the putative active-site residues is not the same for subtilisins and the chymotrypsin-like enzyme. These differences may reflect the difference in substrate specificity between these two enzymes. The *prtP* enzyme has been previously characterized as a chymotrypsin-like enzyme on the basis of its substrate specificity. This enzyme appears to require phenylalanine in the P1 position and proline in the P2 position in order to maximally hydrolyze the peptide bond. This is distinct from the case for chymotrypsin, which requires tyrosine, phenylalanine, or tryptophan in the P1 position and does not require proline in the P2 position. The position of the active-site triad of chymotrypsin is His-Asp-Ser, while that for the triad of the proline-phenylalanine-specific protease appears to be Asp-His-Ser, which is the same as that for subtilisins (27). However, the substrate specificity of the enzyme differs from that of subtilisins, which possess broad substrate specificity. Therefore, this enzyme should be classified as a

member of the subtilisin, rather than the chymotrypsin, family. Accordingly, the name dentilisin is proposed for the *prtP* enzyme.

Protease activity was not detected for the mature *prtP* protein expressed in *E. coli*. Similar results were obtained for the cloned cysteine proteases from another anaerobic periodontopathic organism, *Porphyromonas gingivalis* (25). Expression of active enzyme in *T. denticola* may require processing of the prosequence as well as interaction with other proteins, such as the 43-kDa protein. Further characterization of the expression of the *prtP* protease activity and the 43-kDa protein is currently in progress in our laboratories. Recently, pathogenic effects of the *T. denticola* chymotrypsin-like protease on host cells were reported (41). The fibronectin- and IgG-hydrolyzing activities of this enzyme may play an important role in such toxic effects. The recent development of a gene inactivation system for *T. denticola* (14) will now allow for the assessment of the role of the dentilisin enzyme in such toxicity.

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