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Proline Iminopeptidase from the Outer Cell Envelope of the Human Oral Spirochete *Treponema denticola* ATCC 35405

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Certain periodontopathic organisms have been shown to exhibit high activity of proline iminopeptidase (PIPase). The human oral spirochete *Treponema denticola* ATCC 35405 was found to contain an easily extractable, novel PIPase (EC 3.4.11.5), which was purified to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis-pure form by means of fast protein liquid chromatographic procedures. The range of the minimum monomeric molecular mass (280 amino acid residues) of the PIPase, based on amino acid analysis, was 30.35 to 30.39 kDa, but the likely in vivo form of the enzyme is a tetramer (minimum mass, 120.2 to 120.4 kDa). The molecular masses based on laser desorption mass spectrometry were 36.058 kDa for the monomer and 72.596 kDa for a dimer. The PIPase cleaves specifically the Pro-Y bond in dipeptides where Y is preferably Arg or Lys. Pro-Gln, Pro-Asn, and Pro-Ala were also good substrates, while Pro-Glu was hydrolyzed slowly and Pro-Asp was not hydrolyzed at all. Tripeptides were poor substrates or were not hydrolyzed (an exception was Pro-Gly-Gly, which cleaved at a moderate rate). Larger molecules, such as poly-L-Pro, were not hydrolyzed. The *T. denticola* enzyme can be regarded as a true PIPase, since replacing Pro in Pro-Y with other amino acid residues resulted in no hydrolysis. The activity of the PIPase may depend on an active carboxyl group and on an active seryl residue but not on metal cations. Diethylpyrocarbonate inactivated the enzyme in a reaction that was not reversible upon addition of NH_2OH. The enzyme contains a relatively large percentage (ca. 15%) of proline residues. The dominance of the PIPase activity among aminopeptidase activities present in *T. denticola* and the proposed location of the enzyme in the outer cell envelope suggest that it has a vital function in the propagation of the cells within their biological niche (inflamed human periodontal tissues). The biologic role of the PIPase may be envisaged as in the termination of the overall peptidolytic cascade (liberating free proline and other amino acids), whereby host tissue proteins and peptides are first processed and inactivated by other peptidases possibly present within the same confines as the PIPase.

The catabolism of proline may have particular importance in the metabolism of infectious microorganisms. Bacteria have alternative means of acquiring this amino acid that is critical to their viability. The reaction in which the N-terminal proline is liberated from small host tissue peptides offers one important mechanism for acquiring proline for the propagation of the pathogen. Such reactions can be catalyzed by proline iminopeptidases (PIPases), which are highly active in some organisms associated with periodontal disease (22, 23). Especially the cells of *Treponema denticola* were shown to be active producers of PIPases, and plaque samples of periodontitis patients, which were positive for *Eikenella corrodens*, showed significantly higher PIPase activity than the plaque of other patients (2, 3). The PIPase activity was termed dominant among all aminopeptidase activities present in the cells of *T. denticola* (23). Such a relatively high activity suggests that the PIPase plays a vital role in the propagation of *T. denticola*.

Since the first published description of PIPase activity (33, 34) in *Escherichia coli*, this enzyme group has remained an obscure and relatively scantily researched peptidase category, in spite of later investigations of a few bacteria and mammalian tissues (1–3, 5, 9, 14–17, 22, 23, 29, 37, 39–41). The initial reports (33, 34) established a PIPase concept still used by the Enzyme Commission (12) and in literature reviews (39). Currently, a PIPase (also called prolyl aminopeptidase or Pro-X-aminopeptidase; EC 3.4.11.5) is designated as a peptidase releasing the N-terminal proline from a peptide (12) or as an aminopeptidase that cleaves N-terminal proline from low- and high-molecular-weight peptides, including poly-L-Pro, salmine, Pro-Gly, Pro-Gly-Gly, and Pro-Leu-Gly-Lys (12). The enzyme was characterized as a Mn(II)-requiring peptidase present in the cytosol of mammalian and microbial cells. In contrast to the mammalian form, the bacterial PIPase was stated to hydrolyze both poly-L-proline and *N*-α-L-prolyl-*p*-nitroaniline (Pro-*p*NA) (12). The mammalian enzyme, which is not specific for prolyl bonds, is possibly identical to EC 3.4.11.1 (leucine aminopeptidase) (12). Recently, the occurrence of at least two types of PIPases was postulated (14): one group would be formed by the *Bacillus*, *Neisseria*, and *Lactobacillus* enzymes, while the other would be formed by enzymes such as the *Aeromonas* PIPase.

Our research on proline-specific peptidases (PSPases) (19, 21, 24–27, 35) of human pathogens demonstrated PIPase activity in the cells of all clinical strains of *T. denticola* investigated (22, 23). This paper reports new information on a PIPase isolated from a mild detergent extract of the outer cell envelope of *T. denticola* ATCC 35405. The proposed location of the enzyme in the cell structure, the strict specificity of the enzyme (exhibiting virtually infallible activity on certain Pro-Y dipeptides), its total lack of activity on poly-L-proline, the independence of its activity on metal ions, and other characteristics distance the *T. denticola* PIPase from PIPases included in the Enzyme Commission's list (12). Consequently, the present study attempts to elucidate general characteristics of a particular PSPase, i.e., the PIPase, present in a human pathogen.

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These microbial PSPases may contribute to the breakdown of human proline-containing bioactive peptides (21, 25, 26), collagen or collagen fragments (24, 35), or perhaps salivary proline-rich peptides. The role of the PIPase in this peptidolytic process would be to hydrolyze Pro-Y peptides liberated from larger molecules by other enzymes. Therefore, the present PIPase and other PSPases may contribute to the inflammatory status of human periodontal tissues.

MATERIALS AND METHODS

Source and cultivation of the organism and treatment of the cells. The cells of *T. denticola* ATCC 35405 were grown anaerobically for 48 h in a tryptone-yeast extract-heart infusion broth containing 10% heat-inactivated rabbit serum (30). For the purpose of the purification of the PIPase, 1.2-liter aliquots of the growth medium in 1.5-liter screw-capped flasks were inoculated with 100-ml aliquots of cultures (21) and incubated anaerobically for 4 days at 37°C. The optical density at 660 nm (A_{660}) was used to determine the number of cells; an extinction of 0.2 at A_{660} corresponded to 5×10^8 cells per ml (10). The cells were harvested by centrifugation for 10 min at 16,300 × *g*. This procedure and all subsequent steps of enzyme purification were carried out at 0 to 4°C, except for fast protein liquid chromatography (FPLC) separations, which were carried out at 22°C. For the purification of the enzyme, about 50 g of cells (wet weight) was washed thoroughly with water. The final washed cell pellet was resuspended in buffer (21). Small volumes of 10% Triton X-100 (Pierce) were added to a final concentration of 0.05%. Sixty minutes later, the suspension was centrifuged for 15 min at 27,000 × *g*. The supernatant fluid was passed through a Gelman 0.2-μm-pore-size membrane. The filtrate was subjected to FPLC as shown below.

Chemicals. Unless specifically mentioned, the chemicals were obtained from Sigma. The water used in this study was prepared with a Millipore Milli-Q system and had a resistivity of 18 MΩ cm^{-1}.

Purification of PIPase. The harvesting of the cells for enzyme purification was carried out after 4 days of growth (25). The following procedure was developed on the basis of a large number of tests and was found to be repeatable on 25- to 50-ml volumes of cell extract.

(i) Hydroxyapatite FPLC. About 46 ml of the filtered enzyme extract was passed through a Calbiochem high-resolution hydroxyapatite column with a phosphate gradient (Fig. 1A). Only one peak showing enzyme activity on Pro-*p*NA was detected.

(ii) Hydrophobic-interaction FPLC. The enzyme (26 ml) from the previous step was made 1.7 mol/liter with regard to ammonium sulfate. The solution was filtered through a Gelman 0.2-μm-pore-size membrane and passed through a Phenyl-Superose column with a descending ammonium sulfate gradient (Fig. 1B).

(iii) Anion-exchange FPLC. The enzyme (12 ml) from the previous step was dialyzed for 18 h against 100 volumes of 20 mmol of Tris (pH 8.0) plus 0.1 mmol of EDTA (each per liter). The dialysate was passed through a Mono Q HR5/5 anion-exchange column (data not shown) by use of a two-step ascending NaCl gradient from 0 to 0.3 mol/liter (from 40 to 100 min) and from 0.3 to 1.0 mol/liter (from 100 to 110 min) in 20 mmol of Tris (pH 8.0) plus 1.0 mmol of EDTA (each per liter). The flow rate was 0.5 ml/min, and the fraction size was 0.5 ml. The active PIPase fractions from each Mono Q FPLC were combined, and the enzyme was concentrated on a Centricon 30 filter (Amicon) to 0.4 ml.

(iv) Molecular permeation FPLC. Aliquots of ca. 0.35 ml of the enzyme from the previous step were passed through a Superose 12 column (Fig. 1C). The active fractions were combined, and the enzyme was concentrated to 0.4 ml as described above and rechromatographed on the same column (Fig. 1D). The purification is summarized in Table 1. The enzyme (stored in the final elution buffer (20 mmol of phosphate [pH 8.0], 0.1 mmol of EDTA, 0.25 mol of NaCl [each per liter]) was active for several months when stored at +4°C. Freezing reduced the activity.

Enzyme determinations. The activity of the PIPase was routinely (in enzyme purification and several other experiments; referred to as standard conditions) determined for 1.0-ml reaction mixtures containing 50 mmol of buffer (normally Bis-Tris propane [pH 7.8] plus 0.15 mol of NaCl per liter) per liter, 0.25 to 1.0 mmol of Pro-*p*NA (dissolved in water) per liter, and 1 to 10 μl of enzyme at 30°C. After enzyme addition, the increase in A_{405} was monitored with a Shimadzu UV 160U recording spectrophotometer and a thermostated cuvette holder. The normal reaction time was 20 to 60 s. The value of 8,800 M^{-1} cm^{-1} was used for ε_{405}. The determination of enzyme activity with 2-naphthylamine derivatives of amino acids as substrates was carried out by azo coupling (18). The modified ninhydrin procedure of Doi et al. (8) was used to study the hydrolysis of true peptides.

Protein determinations. The Pierce micro-bicinchoninic acid procedure was used to determine the protein concentrations, except in FPLC, where the A_{280} or A_{214} was monitored.

Amino acid sequencing. The enzyme was dialyzed against 1000 volumes of water with a Centricon 30 membrane (Amicon) and subjected to reversed-phase high-performance liquid chromatography (HPLC), amino acid analysis, and N-terminal sequencing. The reversed-phase HPLC was carried out on a Vydac (Hesperia, Calif.) analytical C_{18} column (no. 218TP52; 0.21 by 25 cm; particle size, 5 μm), with an acetonitrile gradient (20 to 70% in B eluent) containing 0.1% trifluoroacetic acid. The amino acid analyses were carried out with an Applied Biosystems (Foster City, Calif.) model 420H amino acid analyzer and by standard procedures (Table 2) (36). The tryptophan content was estimated after hydrolysis in the presence of dodecanethiol (7). The N-terminal sequencing was performed on Applied Biosystems models 420H and 473A protein sequencers, which use automated Edman degradation and phenylhydantoin-amino acid analysis (4, 11). In a typical process, 300 pmol of the PIPase (30 μl) was loaded. The yields at each cycle were 87 to 95%.

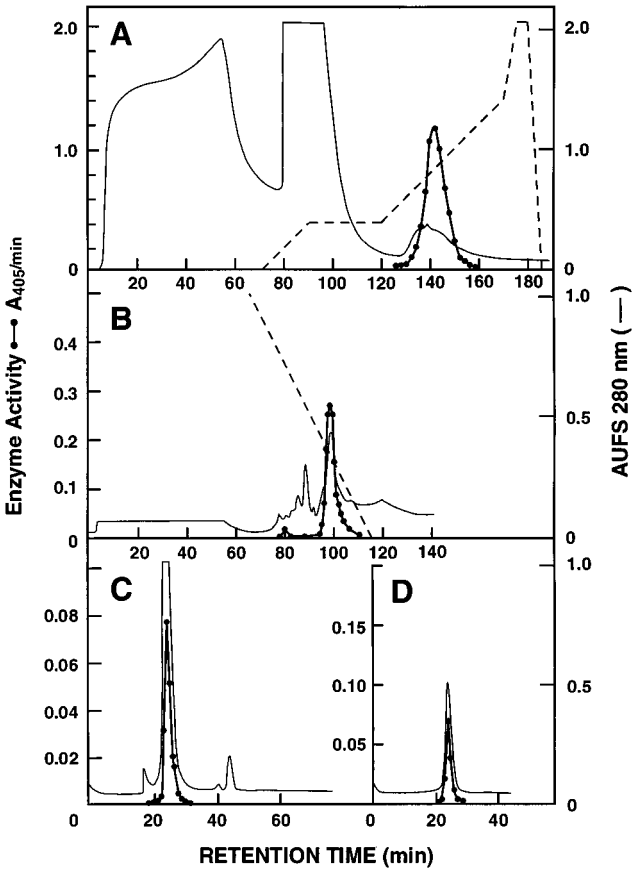

FIG. 1. Purification of the PIPase. (A) FPLC of the enzyme on a Calbiochem high-resolution hydroxyapatite gel, packed in a Pharmacia 10/10 column. The elution was carried out with 20 mmol of phosphate buffer (pH 8.0; 0.01 mmol of EDTA per liter) per liter (initial buffer), with a phosphate gradient from 0.02 to 0.2 mmol/liter (from 70 to 90 min), from 0.2 to 0.7 mmol/liter (from 90 to 170 min), and from 0.7 to 1.0 mmol/liter (from 170 to 175 min). The gradient was applied after the application of the 46-ml sample was complete. The fraction volume was 2.0 ml, and the flow rate was 1.0 ml/min. (B) Separation of the enzyme from the previous step by means of hydrophobic interaction FPLC on a phenyl-Superose column (Pharmacia). The elution was carried out with 20 mmol of phosphate buffer (pH 8.0; 0.1 mmol of EDTA per liter) per liter, with a descending ammonium sulfate gradient from 1.7 mol/liter to 0 (from 65 to 115 min). The gradient was applied after the application of the 26-ml sample (made in 1.7 mol of salt per liter) was complete. The fraction volume was 0.5 ml, and the flow rate was 0.5 ml/min. (C and D) Final separation of the enzyme by means of two consecutive FPLC steps on Superose 12 gel packed in a Pharmacia 10/30 column. The elution was carried out with 20 mmol of phosphate buffer (pH 8.0; 0.1 mmol of EDTA plus 0.25 mol of NaCl [each per liter]) per liter. The sample was obtained after step 3 (ion-exchange FPLC on a Mono Q column [Pharmacia]; see text and Table 1). The fraction volume was 0.5 ml, and the flow rate was 0.5 ml/min. The protein (solid lines) is shown as A_{280} (the scale on the left concerns both enzyme activity and protein; the AUFS [absorption units, full scale] used in protein monitoring is indicated). The gradients are represented by dashed lines.

Laser desorption mass spectrometry. The mass of the PIPase was studied by means of matrix-assisted laser desorption ionization time-of-flight mass analysis. The analyses were performed on a Vestec-2000 (Houston, Tex.) Lasertec Research laser desorption linear time-of-flight mass spectrometer, equipped with a

TABLE 1. Purification of the PIPase from *T. denticola* ATCC 35405

Step	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Sp act[a] (μmol/min/mg)	Total activity (μmol/min)
1. 0.05% Triton extract	46.0	2.35	107.98	0.40	42.76
2. After hydroxyapatite FPLC	26.5	0.077	2.05	10.42	21.41
3. After $NH_4(SO_4)_2$ precipitation and phenyl-Superose FPLC	12.3	0.038	0.47	19.46	9.15
4. After Mono Q and first Superose 12 FPLC	0.4	0.365	0.146	27.62	4.03
5. After second Superose 12 FPLC	1.5	0.046	0.070	21.1	1.47

[a] Determined with Pro-*p*NA under standard conditions described in Materials and Methods (with a 50-mmol/liter concentration of Bis-Tris propane buffer [pH 7.8]).

337-nm VSL-337ND nitrogen laser (Laser Science, Inc.) with a 3-ns pulse width and a 1.2-m flight tube, operated at 23-kV ion accelerating voltage and 3-kV multiplier voltage. The matrix solutions were saturated sinapinic acid in 1:1 acetonitrile and 0.1% aqueous trifluoroacetic acid; 0.5 pmol of myoglobin was loaded as an internal standard. The sample mixtures consisted of 1 μl of matrix, 1 μl of analyte, and 1 μl of internal standard. A 1-μl aliquot was loaded onto the probe.

Chemical modification of PIPase. The experiments to chemically modify PIPase were deliberately carried out to elucidate the possible role of seryl-, carboxyl-, and diethyl pyrocarbonate (DEP)-reactive residues in PIPase activity. Modification of seryl residues was performed mainly with diisopropyl fluorophosphate (DFP). The reactions of DEP with the enzyme were studied by the method of Miles (28). Treatment of the enzyme with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to study the involvement of carboxyl groups in enzyme activity was performed at 25°C (31, 32). Unless otherwise indicated, all modifications were carried out in an ice water bath as described previously (19, 21, 25). A larger number of other chemical reagents were used to study the inhibition characteristics of the enzyme. Such experiments were normally carried out in 1.0-ml reaction mixtures containing 0.8 ml of a 62.5-mmol/liter concentration of Bis-Tris-propane buffer (pH 7.8; 0.19 mol of NaCl plus 1.25 mmol of Pro-*p*NA [each per liter]) and 0.2 ml of water. Small aliquots of the effector were added to the reaction mixture (or the 0.2-ml volume of water was replaced with an aqueous solution of the substance to be tested). The reactions were performed in a thermostated cuvette (protected from room lighting) in the spectrophotometer and were normally initiated by the addition of a small (normally 10-μl) volume of enzyme. The reaction time was 60 s. Because several modifiers and inhibitors were dissolved in organic solvents (vide infra), appropriate controls were included.

Affinity and specificity constants. The values of K_m and V_{max} were studied at 30°C by use of the Enzpack 3 program (Biosoft, Ferguson, Mo.). The values of k_{cat} (catalytic constant) and k_{cat}/K_m were calculated on the basis of data computed with the same program.

TABLE 2. Amino acid composition of the PIPase from *T. denticola* ATCC 35405

Amino acid	Residues/molecule of enzyme[a]	
	Monomer	Tetramer[b]
Asparagine or aspartic acid	26	102
Serine	16	65
Histidine	7	27
Threonine	7	29
Proline	41	164
Valine	10	42
1/2 Cystine	ND[c]	ND
Leucine	23	90
Lysine	22	85
Glutamine or glutamic acid	27	106
Glycine	27	105
Arginine	9	37
Alanine	22	88
Tyrosine	10	38
Methionine	4	16
Isoleucine	14	55
Phenylalanine	15	61
Tryptophan[d]	0	0
Unknown	0	0
Total	280	1,110

[a] The hydrolysis of the enzyme (20 pmol) was carried out for 1 h at 200°C in 6 mol of HC1 per liter. The values were not corrected for possible loss of amino acids during hydrolysis.
[b] Based on direct chemical analysis of the tetramer.
[c] ND, not determined.
[d] Estimated after hydrolysis in the presence of dodecanethiol (7).

RESULTS

Production of enzyme during growth of cells. The growth of the cells of *T. denticola* ATCC 35405 and the production of the PIPase by the cells were monitored for up to 7 days. The maximum production of the enzyme when tested in the mild (0.05%) detergent extract occurred after 4 days of growth, declining thereafter. No extracellular PIPase activity was detected in the spent medium at any stage of growth. The enzyme was readily and at least by 90% extracted with 0.05% detergent (compared with the total activity obtained by ultrasonically [21] disintegrating washed cells). The PIPase is most likely located on the outer surface of the cells and is relatively loosely bound, being also able to react with substrates in its bound state (or the substrates, which were found to be of small size, may cross the cell membrane when incubated with intact cells). Thoroughly washed whole cells hydrolyzed Pro-*p*NA at a high rate. Microscopic examinations showed that the cells remained intact after incubation with Pro-*p*NA and other peptidase substrates.

Purification of the PIPase. The development of the specific activity of the PIPase during purification is shown in Table 1. The use of hydroxyapatite FPLC as the first separation step in the purification of treponemal peptidases from mild detergent extracts has been useful (20) and was found to be decisive also in the present study; the PIPase elutes relatively late from the column (Fig. 1A), facilitating the separation of the enzyme from other proteins. The purification factor shown in Table 1 should be viewed with regards to the relatively low number of proteins present in the mild detergent extract. Although ion-exchange FPLC on the Mono Q column was necessary for obtaining the PIPase in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-pure form, this step considerably reduced the enzyme activity and the yield. Exclusion of this step maintained the yield at 16% but resulted in the cofractionation of impurities.

Purity of the enzyme. The enzyme was found to be homogeneous in native PAGE tests (carried out with PhastGel gradient 8-25 and PhastGel native buffer strips [Pharmacia Biotechnology, Inc.]), and in SDS-PAGE (with PhastGel SDS buffer strips and PhastGel gradient 8-25) (Fig. 2). The proteins were stained by the silver staining procedure. The purity of the PIPase was verified independently at the University of Michigan Medical School Protein Structure and Sequencing Facility by means of microbore-HPLC on a Vydac C_{18} reversed-phase column (no. 218TP52; 0.21 by 25 cm).

Molecular mass, amino acid composition, and N-terminal sequence. The PIPase appeared in FPLC on a Superose 12 gel

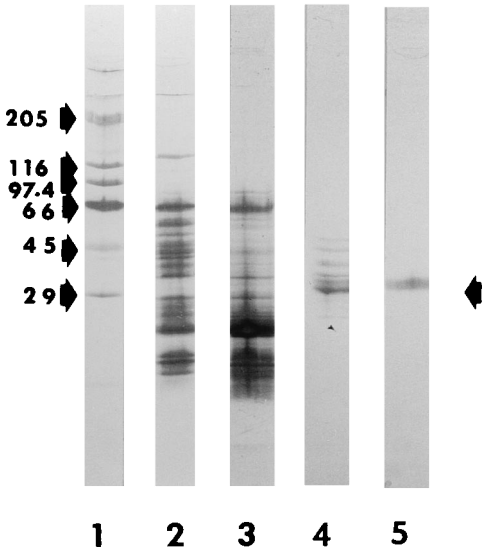

FIG. 2. SDS-PAGE of the PIPase after various purification steps. PhastGel gradient 8-25, PhastGel SDS buffer strips, and silver staining were used. Lanes: 1, molecular weight markers (Sigma SDS 6H); 2, 0.05% Triton X-100 extract of washed cells; 3, after hydroxyapatite-FPLC; (Table 1, step 2); 4, after phenyl-Superose FPLC (Table 1, step 3); 5, after second gel permeation FPLC (Table 1, step 6). The arrow on the right corresponds to a mass of about 30 kDa.

filtration column as a structure with a mass of 120 kDa. Upon treatment with SDS under conditions normally used in SDS-PAGE, the enzyme showed only one silver-stained band that corresponded to a mass of 30 kDa (Fig. 2). Amino acid analysis of the 30- and the 120-kDa forms gave 280 and 1,110 residues, respectively, as the minimum estimated lengths of the peptides. Consequently, amino acid analysis of the 120-kDa form gave a value which was about 10 amino acid residues smaller than that obtained in the analysis of four 30-kDa subunits (1,110 versus 4×280, or 1,120). From the behavior of these forms in FPLC and other tests, it was concluded that the 30-kDa form represents a monomer and that the 120-kDa form represents a tetramer of the PIPase. The estimated minimum molecular masses of these forms, based on amino acid analyses, were 30.37 kDa (range, 30.350 to 30.392 kDa) and 120.27 kDa (range, 120.191 to 120.357 kDa), respectively. Attempts to determine the N-terminal sequence of the 30-kDa form failed to reveal the N-terminal residue, while it was possible to obtain the sequence for residues 2 to 21, namely, KILYEKTPLFD EGFLKUSDI. The amino acid composition of the PIPase was characterized by a relatively high (14.6%) content of proline residues (Table 2). Treatment of the enzyme with cold SDS resulted in the formation of both a monomeric and a dimeric form of the enzyme. Studies by means of laser desorption mass spectrometry gave M_r values of 36.058 and 72.596 for the monomer and the dimer, respectively.

Substrate specificity. Among the *N*-aminoacyl 2-naphthylamines and *p*NAs, only Pro-2-naphthylamine and Pro-*p*NA were hydrolyzed (studied under standard conditions with up to 60-min reaction times). The Pro-dipeptides (Pro-Y) that were hydrolyzed at the highest rate had a positively charged residue (Lys, Arg) in position Y (Table 3). Accordingly, Pro-Asn and Pro-Gln were also good substrates, while a second carboxyl group in Y (as in Glu and Asp) lowered significantly the rate of hydrolysis. The arrangement of a basic versus an acidic side chain in Y was not the only decisive hydrolysis-determining factor, because Pro-Ala was also a good substrate. Table 4 gives the values of the affinity constant and specificity constant for several Pro-Y peptides. The affinities of the eight substrates shown were remarkably similar for the PIPase. Peptides longer than Pro-Y were not good substrates. However, Pro-Gly-Gly was hydrolyzed at a high rate as a result of the relatively small size of the Gly-Gly moiety. No hydrolysis was observed with poly-L-Pro and with several oligopeptides with an N-terminal Pro residue, such as des-Arg^1-bradykinin.

TABLE 3. Hydrolysis of peptides by the *T. denticola* PIPase[a]

Substrate	Relative rate of hydrolysis
Pro-*p*NA	100
Pro-Arg	153
Pro-Lys	147
Pro-Ala	115
Pro-Gly-Gly	98
Pro-Asn	98
Pro-Gln	96
Pro-Met	95
Pro-Trp	90
Pro-Leu	77
Pro-Phe-NH_2	75
Pro-Val	68
Pro-Phe	67
Pro-Tyr	63
Pro-Gly	42
Pro-Hyp	36
Pro-Pro	33
Pro-Ile	32
Pro-Glu	11
Pro-Pro-Pro	7
Pro-Asp	0
Pro-Leu-Gly-NH_2	0
Pro-Phe-Gly-Lys	0
Poly-L-Pro	0

[a] The reactions were carried out at 30°C in 50 mmol of Bis-Tris-propane buffer per liter (pH 7.8; containing 0.15 mol of NaCl per liter), with initial peptide concentrations of 1.0 mmol/liter. All amino acids were in the L-form. The rate of the hydrolysis of Pro-*p*NA under these conditions was 26.7 μmol min^{-1} mg^{-1} and was given the value of 100. Poly-L-Pro (Sigma; molecular size range, 1.0 to 10 kDa) was used at a concentration of 1.0 mg/ml.

Effect of temperature. The initial rate of hydrolysis of Pro-*p*NA relative to a temperature curve showed a sharp maximum at 37°C (data not shown). When the enzyme was first incubated for 5 min at various test temperatures and then tested by a standard assay of activity at 30°C, the enzyme activity started to decline sharply if the enzyme had been pretreated at temperatures higher than 30°C. It is not known if the PIPase exhibits this type of temperature effect in vivo.

TABLE 4. Kinetic constants of the hydrolysis of substrates of the PIPase from *T. denticola*

Substrate[a]	K_m (M [10^3])	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1} [10^6])	V_{max} (nmol min^{-1})
Pro-*p*NA	0.96	1,000	1.04	2.7
Pro-2NA	0.96	1,370	1.43	3.7
Pro-Lys	0.99	3,185	3.22	8.6
Pro-Arg	0.99	1,889	1.91	5.1
Pro-Asn	0.95	2,518	2.65	6.8
Pro-Gln	0.90	2,000	2.22	5.4
Pro-Ala	0.98	2,444	2.49	6.6
Pro-Hyp	0.99	1,296	1.31	3.5

[a] The reactions were performed in 50 mmol of Bis-Tris propane per liter (pH 7.5; 0.15 mol of NaCl per liter) at 30°C. All amino acids were in the L-form. 2NA, 2-naphthylamine.

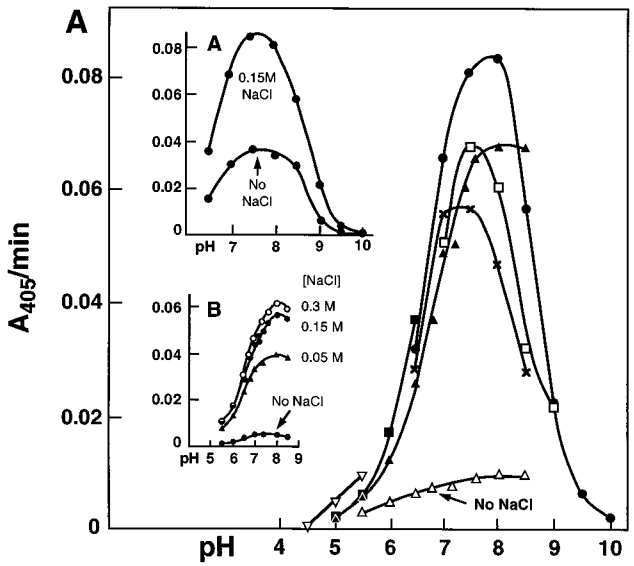

FIG. 3. Relationship between PIPase activity and NaCl concentration. The rate of the hydrolysis of Pro-*p*NA was measured in various 50-mmol/liter buffer systems with an initial substrate concentration of 0.25 mmol/liter. Symbols for main panel: ●, Bis-Tris propane; □, Trizma; ▲, phosphate; X, Tris; ■, morpholineethanesulfonic acid; ▽, acetate; △, phosphate buffer without added salt. All buffer systems except the last contained 0.15 mol of NaCl per liter. (Inset A) Effect of NaCl in Bis-Tris propane; (inset B) effect of NaCl in phosphate buffer.

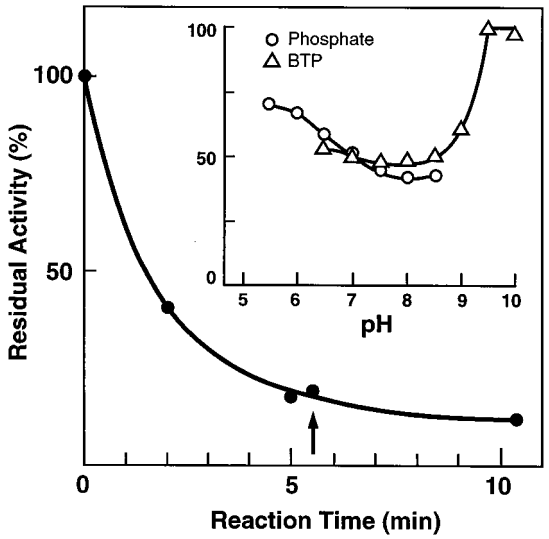

FIG. 4. Modification of the PIPase with DEP at 30°C. The enzyme was treated with 0.9 mmol of DEP per liter in 20 mmol of phosphate buffer (pH 6.8; plus 0.1 mmol of EDTA per liter), and the activity was monitored on small (10-μl) aliquots withdrawn from the reaction mixture at the times shown. After 5.5 min of reaction time (arrow), the mixture was made 0.1 mol/liter with regard to NH_2OH (pH of the 2.0-mol/liter stock solution was adjusted to pH 6.8). The residual activity values were calculated by considering the slight inhibitory effect of the ethanol used to dissolve DEP. Inset, Effect of pH on the DEP-associated inactivation of PIPase. The enzyme was treated for 3 min with 0.5 mmol of DEP per liter at the pH values shown (with 20-mmol/liter concentrations of phosphate and Bis-Tris propane [BTP] buffers).

Effect of pH and NaCl. NaCl was shown to exert selective effects on treponemal peptidases (29). The effect of pH on the rate of the hydrolysis of Pro-*p*NA was studied with several buffer systems (Fig. 3). The overall rate versus pH curve pattern was bell shaped, with a broader maximum between pH 7 and 8. NaCl was a potent activator of the hydrolysis, with the physiologic salt concentration (0.9% or 0.154 mmol/liter) occasioning an almost optimum activation; higher NaCl levels were not remarkably more effective. The salt increased the rate of hydrolysis to the same degree at all pH values tested (Fig. 3). Preincubation of the enzyme for 10 min at 30°C over a wide pH range [with 50 mmol of acetate, phosphate, Bis-Tris propane, tris(hydroxymethyl)aminomethane (Trizma), or 4-morpholineethanesulfonic acid buffer per liter] showed that the enzyme activity was stable after treatment within the pH range of 6 to 8.5, declining sharply after treatment at lower and higher pH values.

Chemical modification of the enzyme. Treatment with DEP inactivated the enzyme in a time-, concentration-, and pH-dependent manner. Figure 4 shows that the enzyme lost almost 90% of its activity in 5.5 min upon treatment with DEP (initial concentration, 0.9 mmol/liter). Addition of hydroxylamine (final concentration, 0.1 mmol/liter) had no effect on this inactivation (also, NH_2OH was slightly inhibitory). No observable inactivation by DEP took place at pH 5.5 and 6.0 (Fig. 4, inset). However, the inactivation progressed rapidly at pH values of 7.5 to 8.5, while the rate was greatly reduced and the inactivating effect of DEP was eventually eliminated at more-alkaline pH values. These results suggest that the activity of the PIPase is not dependent on histidyl residues. An active tyrosyl residue may be involved, however.

DFP (dissolved in isopropanol) (6) also caused a time- and concentration-dependent inactivation of the enzyme, but this reagent, especially when used at low millimolar levels, was not a potent inactivator: concentrations of DFP of 0.1, 1.0, and 10.0 mmol/liter caused, in 60 s, 1.8, 30, and 98% inactivations, respectively. Phenylmethylsulfonyl fluoride (at 1.0 mmol/liter, freshly prepared in methanol) inactivated the enzyme by only 2.5% in 60 s. EEDQ, tested at 25°C, inactivated the enzyme in a time-, concentration-, and pH-dependent manner, i.e., displaying inactivation characteristics previously interpreted as an indication of the involvement of a carboxyl group in enzyme activity (31, 32). Thus, EEDQ at 0.1 mmol/liter, tested in 20-mmol/liter phosphate buffers (pH range, 5.4 to 6.6; containing 0.1 mmol of EDTA per liter), inactivated the enzyme in 20 min by 90% at pH 5.4 and by 35% at pH 6.0; the rate of inactivation generally increased with decreasing pH. No inactivation was observed at pH 6.6 (data not shown). At a higher concentration (0.5 mmol/liter), EEDQ was more potent, causing a 90% inactivation in 5 min at pH 5.4.

None of the sulfhydryl compounds (L-cysteine, 2-mercaptoethanol, and dithiothreitol [DTT]) had any remarkable effect on enzyme activity, even when used at a concentration of 10 mmol/liter (at this level, 2-mercaptoethanol was slightly inhibitory). 5,5′-Dithio-bis(2-nitrobenzoic acid), a sensitive SH reagent, inhibited enzyme activity by 45% at concentrations of 0.1 to 0.4 mmol/liter. Freshly prepared solutions of iodoacetamide (0.1 to 1.0 mmol/liter) and *trans*-epoxy-succinyl-L-leucylamido(4-guanidino)butane (E-64) (6) (11.0 mmol/liter) were without effect. However, *p*-hydroxymercuribenzoic acid (*p*HMB) was a potent inhibitor; even 0.01 mmol of *p*HMB per liter caused a 95% inhibition. This inhibition was partly reversed by the addition of DTT, i.e., the activity of the 95% inactivated enzyme was restored to 68% of the original level by the addition of 10 mmol of DTT per liter, in 10 min. *p*HMB failed to inhibit if DTT was added first to the reaction mixture. Metal-chelating agents (1,10-phenanthroline and 8-hydroxyquinoline 5-sulfonic acid) had no effect on enzyme activity.

Of other reagents tested, bestatin (0.1 mmol/liter) and bacitracin (0.5 mmol/liter), both of which inhibit certain peptido-

lytic enzymes, had no effect on PIPase. Chlorhexidine gluconate (a membrane-active antibacterial agent) at a 0.2% level caused an inactivation of 70%, while 0.02 and 0.002% concentrations inhibited by 35 and 5%, respectively. Among the metal cations tested, Hg^{2+} and Zn^{2+} were potent inhibitors: 1.0 μmol of Hg^{2+} per liter and 100 μmol of Zn^{2+} per liter caused an almost 100% enzyme inhibition.

DISCUSSION

The outer cell envelope of *T. denticola* contains several proteases and oligopeptidases whose collective activity contributes to the breakdown of tissue proteins and oligopeptides to form gradually smaller peptides and free amino acids. The role of PIPase in this peptidolytic cascade seems to be the termination of catabolism of Pro-Y peptides for the propagation of the pathogen. The PIPase enzyme can be regarded as a means by which free proline is made available for the growth requirements of the pathogen. Suitable Pro-Y peptides can readily be formed from collagen and other host tissue proteins, but also from several bioactive peptides (26, 27), through the action of proteases and peptidases present within the same cell envelope confines as the present PIPase. Because the enzyme prefers peptides with arginine, lysine, asparagine, glutamine, and alanine in position Y, it is possible that the liberation of these amino acids from Pro-Y peptides also constitutes an important role of the PIPase. Several Pro-Y peptides derived from collagen and human bioactive peptides by means of other peptidolytic processes indeed contain one or more of the amino acid sequences indicated in Table 3, making such peptides potential in vivo substrates of the present PIPase. Future research should clarify whether salivary proline-rich peptides would also liberate substrates of PIPase after first being hydrolyzed by other enzymes. No cell fractionation studies were carried out to confirm the localization of the PIPase. Although the present experiments do suggest that the enzyme is located in the outer cell envelope, further studies must be performed to validate this suggestion.

The present PIPase may be classified as a true proline iminopeptidase (EC 3.4.11.5). However, the knowledge concerning PIPases in general has been defective since it appears that this enzyme group may include several PIPases that differ from each other with regard to amino acid homology and active site structure. Kitazono et al. (14) suggested that there may be at least two types of PIPases, the *Bacillus-Neisseria-Lactobacillus* group and the *Aeromonas* group. The latter (represented by *Aeromonas sobria*) PIPase type was suggested to be a tetramer with a molecular mass of 205 kDa. The tetrameric nature of the spirochete PIPase may link it with the *Aeromonas* group of PIPases. It is also possible that the *T. denticola* PIPase represents a distinct class of the PIPases because of the enzyme's different specificity profile and inhibition characteristics. More research should be carried out to substantiate this proposal and, in general, to determine the number of different types of microbial PIPases. An important characteristic of the present PIPase seems to be its relatively high proline content. Proline-rich regions are common in several diverse proteins (38), such as *E. coli* protein OmpA and bovine amelogenin (which have repetitive short proline-rich sequences), and in human saliva proline-rich proteins, human mucins, and *Plasmodium berghei* circumporozoite protein (which have tandemly repeated sequences).

The previously assumed noninvolvement of a serine residue in the activity of PIPases should be reevaluated on the basis of reports that at least some bacterial PIPases may not be sulfydryl enzymes but, instead, serine peptidases (13, 16). The present results obtained with DFP, but not those obtained with phenylmethylsulfonyl fluoride, could be regarded as supporting the idea of an active serine residue being located in the *Treponema* enzyme as well, although the DFP-occasioned inactivation was not pronounced. Phenylmethylsulfonyl fluoride is a much weaker serine reagent than DFP (6) and did not appreciably inhibit the chymotrypsin-like enzyme, a serine protease, from *T. denticola* (27). Also, the failure of E-64 and other compounds to inactivate the enzyme may indicate that an active SH group is not involved (6) and that the present enzyme may be a serine peptidase. In such a case, the *p*HMB-caused strong inhibition of the enzyme should be explained by a reaction other than that with an SH group. It is possible that the failure of any of the thiols tested to activate the present PIPase under standard conditions could also be explained as an absence of an active SH group in the enzyme. No thiols were needed to maintain full activity during enzyme purification.

The cellular location of the PIPase present in other bacteria is not well known because of the procedures used to treat the cells for enzyme purification. For example, the cells of *Bacillus coagulans* and *A. sobria* were disrupted with glass beads in a Dyno-Mill (14, 15). The Enzyme Commission refers to the PIPases as cytosol enzymes (12). The PIPase of *Lactobacillus delbrueckii* (5) was suggested to be located in the cell envelope. Our studies suggest that the molecules of the various peptidases of *T. denticola* are present in the cell envelope or associated with it at highly different quantities. These differences in the relative occurrence of the peptidases are readily reflected in the success of the purification procedures of these peptidases and, especially, in the yields achieved. For example, the oligopeptidase B of *T. denticola* (formerly known as trypsin-like enzyme, BANA-peptidase, or BAPNA-peptidase, where BANA and BAPNA stand for *N*α-benzoyl-L-arginyl-2-naphthylamine and *N*α-benzoyl-L-arginyl-*p*-nitroaniline, respectively) (20) is present at quite high concentrations in the outer cell envelope, accounting for even more than 1% of all proteins present in a mild Triton X-100 extract. The PIPase is present in significantly smaller quantities (i.e., in amounts less than 10% of the former). The reason why the cells of *T. denticola* would require a large number of oligopeptidase B molecules and a much smaller number of PIPase molecules within the same confines of the cell envelope is not known. An explanation of this occurrence pattern would perhaps also solve the problem of the exact in vivo role of those peptidases. Even if the molar concentration of the PIPase per cell is smaller, the high specific activity of the PIPase should nevertheless be emphasized: among various aminopeptidase activities present in the cells of *T. denticola*, the activity of PIPase was dominating (23).

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REFERENCES

1. **Albertson, N. H., and M. Koomey.** 1993. Molecular cloning and characterization of a proline iminopeptidase gene from *Neisseria gonorrhoeae*. Mol. Microbiol. **9:**1203–1211.
2. **Allaker, R. P., K. A. Young, and J. M. Hardie.** 1994. Production of hydrolytic enzymes by oral isolates of *Eikenella corrodens*. FEMS Microbiol. Lett. **123:**69–74.
3. **Allaker, R. P., K. A. Young, and J. M. Hardie.** 1994. Rapid detection of proline iminopeptidase as an indicator of *Eikenella corrodens* in periodontal infection. Lett. Appl. Microbiol. **19:**325–327.
4. **Andrews, P. C.** 1994. Neuropeptide structure determination. Methods Neurosci. **5:**5–15.
5. **Atlan, D., C. Gilbert, B. Blanc, and R. Portalier.** 1994. Cloning, sequencing

and characterization of the *pepIP* gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397. Microbiology **140:**527–535.
6. **Beynon, R. J., and G. Salvesen.** 1989. Commercially available protease inhibitors, p. 241–249. *In* R. J. Beynon and J. S. Bond (ed.), Proteolytic enzymes: a practical approach. IRL Press, Oxford.
7. **Bozzini, M., R. Bello, N. Cagle, D. Yamane, and D. Dupont.** 1991. Tryptophan recovery from autohydrolyzed samples using dodecanethiol. Appl. Biosystems Res. News **February:**1–4.
8. **Doi, E., D. Shibata, and T. Matoba.** 1981. Modified colorimetric ninhydrin methods for peptidase assays. Anal. Biochem. **72:**248–254.
9. **Gilbert, C., D. Atlan, B. Blanc, and R. Portalier.** 1994. Proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: purification and characterization. Microbiology **140:**537–542.
10. **Haapasalo, M., V. Singh, B. C. McBride, and V.-J. Uitto.** 1991. Sulfydryl-dependent attachment of *Treponema denticola* to laminin and other proteins. Infect. Immun. **59:**4230–4237.
11. **Hunkapiller, M. W., R. M. Hewick, W. J. Dreyer, and L. E. Hood.** 1983. High-sequencing with a gas-phase sequenator. Methods Enzymol. **91:**339–413.
12. **International Union of Biochemistry and Molecular Biology.** 1992. Enzyme Nomenclature 1992. Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes, p. 375. Academic Press, Inc., San Diego, Calif.
13. **Kitazono, A., K. Ito, and T. Yoshimoto.** 1994. Prolyl aminopeptidase is not a sulphydryl enzyme: identification of the active serine residue by site-directed mutagenesis. J. Biochem. **116:**943–945.
14. **Kitazono, A., A. Kitano, D. Tsuru, and T. Yoshimoto.** 1994. Isolation and characterization of the prolyl aminopeptidase gene (pap) from *Aeromonas sobria*: comparison with the *Bacillus coagulans* enzyme. J. Biochem. **116:**818–825.
15. **Kitazono, A., T. Yoshimoto, and D. Tsuru.** 1992. Cloning, sequencing, and high expression of the proline iminopeptidase gene from *Bacillus coagulans*. J. Bacteriol. **174:**7919–7925.
16. **Klein, J. R., U. Schmidt, and R. Plapp.** 1994. Cloning, heterologous expression, and sequencing of a novel proline iminopeptidase gene, *pepI*, from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290. Microbiology **140:**1133–1139.
17. **Mäkinen, K. K.** 1969. The proline iminopeptidases of the human oral cavity. Partial purification and characterization. Acta Chem. Scand. **23:**1409–1438.
18. **Mäkinen, K. K., and P.-L. Mäkinen.** 1978. Purification and characterization of two human erythrocyte arylamidases preferentially hydrolysing N-terminal arginine or lysine residues. Biochem. J. **175:**1051–1067.
19. **Mäkinen, K. K., and P.-L. Mäkinen.** 1987. Purification and properties of an extracellular collagenolytic protease produced by the human oral bacterium *Bacillus cereus* (strain Soc 67). J. Biol. Chem. **262:**12488–12495.
20. **Mäkinen, K. K., P.-L. Mäkinen, W. J. Loesche, and S. A. Syed.** 1995. Purification and general properties of an oligopeptidase from *Treponema denticola* ATCC 35405—a human oral spirochete. Arch. Biochem. Biophys. **316:**689–698.
21. **Mäkinen, K. K., P.-L. Mäkinen, and S. A. Syed.** 1992. Purification and substrate specificity of and endopeptidase from the human oral spirochete *Treponema denticola* ATCC 35404, active on furylacryloyl-Leu-Gly-Pro-Ala and bradykinin. J. Biol. Chem. **267:**14285–14293.
22. **Mäkinen, K. K., S. A. Syed, P.-L. Mäkinen, and W. J. Loesche.** 1986. Benzoylarginine peptidase and iminopeptidase profiles of *Treponema denticola* strains isolated from the human periodontal pocket. Curr. Microbiol. **14:**85–89.
23. **Mäkinen, K. K., S. A. Syed, P.-L. Mäkinen, and W. J. Loesche.** 1987. Dominance of iminopeptidase activity in the human oral bacterium *Treponema denticola* ATCC 35405. Curr. Microbiol. **14:**341–346.
24. **Mäkinen, K. K., S. A. Syed, S. L. Salvadore, and P.-L. Mäkinen.** 1990. Hydrolysis of the Leu-Gly bond of phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (a substrate of microbial collagenases) by treponemes isolated from the subgingival plaque of periodontitis patients. Curr. Microbiol. **20:**69–74.
25. **Mäkinen, P.-L., D. Clewell, F. An, and K. K. Mäkinen.** 1989. Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from *Streptococcus faecalis* (strain OG1-10). J. Biol. Chem. **264:**3325–3334.
26. **Mäkinen, P.-L., K. K. Mäkinen, and S. A. Syed.** 1994. An endo-acting proline-specific oligopeptidase from *Treponema denticola* ATCC 35405: evidence of hydrolysis of human bioactive peptides. Infect. Immun. **62:**4938–4947.
27. **Mäkinen, P.-L., K. K. Mäkinen, and S. A. Syed.** 1995. Role of the chymotrypsin-like membrane-associated proteinase from *Treponema denticola* ATCC 35405 in inactivation of bioactive peptides. Infect. Immun. **63:**3567–3575.
28. **Miles, E. W.** 1977. Modification of histidyl residues in proteins by diethypyrocarbonate. Methods Enzymol. **47:**431–442.
29. **Nordwig, A., and H. Mayer.** 1973. The cleavage of prolyl peptides by kidney peptidases. Detection of a new peptidase capable of removing N-terminal proline. Hoppe-Seyler's Z. Physiol. Chem. **354:**380–383.
30. **Ohta, K., K. K. Mäkinen, and W. J. Loesche.** 1986. Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. Infect. Immun. **53:**213–220.
31. **Pougeois, R., M. Satre, and P. V. Vignais.** 1978. *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, a new inhibitor of the mitochondrial F_1-ATPase. Biochemistry **17:**3018–3023.
32. **Saccomani, G., M. L. Barcellona, and G. Sachs.** 1981. Reactivity of gastric (H^+ and K^+)-ATPase to *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. J. Biol. Chem. **256:**12405–12410.
33. **Sarid, S., A. Berger, and E. Katchalski.** 1959. Proline iminopeptidase. J. Biol. Chem. **234:**1740–1744.
34. **Sarid, S., A. Berger, and E. Katchalski.** 1962. Proline iminopeptidase. II. Purification and comparison with iminodipeptidase (prolinase). J. Biol. Chem. **237:**2207–2212.
35. **Söderling, E., S. A. Syed, P.-L. Mäkinen, and K. K. Mäkinen.** 1994. Proteolytic activity of treponemes from the subgingival plaque of periodontitis patients. Microb. Ecol. Health Dis. **7:**71–81.
36. **Tarr, G. E.** 1986. Manual Edman sequencing system, p. 155–194. *In* J. E. Shively (ed.), Methods of protein microcharacterization. Humana Press, Clifton, N.J.
37. **Turzynski, A., and R. Mentlein.** 1990. Prolyl aminopeptidase from rat brain and kidney: action on peptides and identification as leucyl aminopeptidase. Eur. J. Biochem. **190:**509–515.
38. **Williamson, M. P.** 1994. The structure and function of proline-rich regions in proteins. Biochem. J. **297:**249–260.
39. **Yaron, A., and F. Neider.** 1993. Proline-dependent structural and biological properties of peptides and proteins. Crit. Rev. Biochem. Mol. Biol. **28:**31–81.
40. **Yoshimoto, T., T. Saeki, and D. Tsuru.** 1985. Proline iminopeptidase from *Bacillus megaterium*: purification and characterization. J. Biochem. **93:**469–477.
41. **Yoshimoto, T., and D. Tsuru.** 1985. Proline iminopeptidase from *Bacillus coagulans*. J. Biochem. **97:**1477–1485.

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