Prolyl Tripeptidyl Peptidase from *Porphyromonas gingivalis*

A NOVEL ENZYME WITH POSSIBLE PATHOLOGICAL IMPLICATIONS FOR THE DEVELOPMENT OF PERIODONTITIS*

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Agnieszka Banbula‡, Pawel Mak‡, Marcin Bugno‡, Jerzy Silberring§, Adam Dubin‡, Daniel Nelson¶, James Travis¶, and Jan Potempa¶‡

From the ¶Institute of Molecular Biology and §Faculty of Chemistry and Regional Laboratory, Jagiellonian University, 31-120 Kraków, Poland and the ¶Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602

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Porphyromonas gingivalis possesses a complex proteolytic system, which is essential for both its growth and evasion of host defense mechanisms. In this report we characterized, both at a protein and genomic level, a novel peptidase of this system with prolyl tripeptidyl peptidase activity. The enzyme was purified to homogeneity, and its enzymatic activity and biochemical properties were investigated. The amino acid sequence at the amino terminus and of internal peptide fragments enabled identification of the gene encoding this enzyme, which we refer to as *PtpA* for prolyl tripeptidyl peptidase A. The gene encodes an 82-kDa protein, which contains a GWSYGG motif, characteristic for members of the S9 prolyl oligopeptidase family of serine proteases. However, it does not share any structural similarity to other tripeptidyl peptidases, which belong to the subtilisin family. The production of prolyl tripeptidyl peptidase may contribute to the pathogenesis of periodontal tissue destruction through the mutual interaction of this enzyme, host and bacterial collagenases, and dipeptidyl peptidases in the degradation of collagen during the course of infection.

Porphyromonas gingivalis, a Gram-negative, asaccharolytic, anaerobic bacterium, is one of the predominant members of the human periodontal flora and has been strongly implicated as a causative agent of adult type periodontitis (1, 2). At least part of its pathogenic potential appears to be attributable to the production of a variety of proteolytic enzymes that facilitate, directly or indirectly, both local tissue damage and evasion of host defense mechanisms (for review, see Ref. 3). Recent studies have indicated that this periodontopathogen produces at least seven different enzymes belonging to the cysteine and serine catalytic classes of peptidases, among which three cysteine proteases (gingipains) are predominant (for review, see Ref. 4). Because their structure, function, enzymatic properties, and pathological significance are known, these endopeptidases are the best characterized group of *P. gingivalis* enzymes. From *in vitro* studies it is apparent that two gingipain Rs (HRgpA and RgpB), enzymes specific for cleavage at Arg-Xaa peptide bonds, have a significant potential to contribute to the development and/or maintenance of a pathological inflammatory state in infected periodontal pockets through (i) activation of the kallikrein-kinin cascade (5, 6), (ii) the release of neutrophil chemotactic activity from native and oxidized C5 of the complement pathway (7), and (iii) activation of both factor X (8) and prothrombin. ¹ This latter effect leads to the uncontrolled generation of thrombin, an enzyme with multiple functions, including strong proinflammatory activity (9). In addition, gingipain K, an enzyme that cleaves Lys-Xaa peptide bonds, degrades fibrinogen, and this may add to a bleeding on probing tendency associated with periodontitis (10). Finally, the presence of a hemagglutinin adhesion domain in the noncovalent multiprotein complexes of HRgpA and gingipain K (11) suggests participation of these enzymes in the binding of *P. gingivalis* to extracellular matrix proteins, which may facilitate tissue invasion by this pathogen.

In comparison with the gingipains, relatively little is known about other cysteine proteinases produced by *P. gingivalis*. Two genes, referred to as *tpr* (12) and *prtT* (13), have been cloned and sequenced, and although they encode putative papain- and streptopain-like cysteine proteinases, respectively, neither has been purified and characterized at the protein level. However, a gene homologous to *prtT* has now been identified in the partially sequenced *P. gingivalis* genome, and the proteinase encoded by this gene, referred to as periodontain, has been purified from culture media and characterized.²

Despite the fact that the presence of serine proteinase activity in cultures of *P. gingivalis* has been known for several years (14), only limited information is available about such enzymes. Indeed, a serine endopeptidase has been isolated from culture media, although it was only superficially characterized (15). On the other hand, an enzyme referred to as glycylprolyl peptidase (dipeptidyl peptidase IV) was found to be associated with bacterial surfaces (16), and two molecular mass forms of this peptidase have been described (17, 18). This enzyme(s) has also been shown to possess the ability to hydrolyze partially degraded type I collagen, releasing the Gly-Pro dipeptide, and it was suggested that, in collaboration with collagenase, dipeptidyl peptidase IV may contribute to the degradation of the periodontal ligament (19). In addition to this potential pathological function, glycylprolyl peptidase may also play a vital role in providing *P. gingivalis* with dipeptides, which can be transported inside the cell and serve as a source of carbon, nitrogen, nitrogen,
and energy for this asaccharolytic organism. Recently, a gene encoding glycolylproline peptidase has been cloned and sequenced, and it is now apparent that this enzyme is homologous to dipeptidyl peptidase IV from other organisms (20). In this report we describe the purification and characterization of another cell surface-associated serine proteinase of P. gingivalis with a novel prolyl tripeptidyl peptidase activity but with a surprising primary structure related to dipeptidyl peptidases. This enzyme liberates tripeptides from the free amino terminus and possesses the absolute requirement for the proline residue in the P1 position.

EXPERIMENTAL PROCEDURES

Materials

Disopropylfluorophosphate (DFP)3, leupeptin, and 3,4-dichloroisocoumarin were purchased from Calbiochem. Antipain, iodoacetamide, substance P, bradykinin, and bradykinin-related peptides were obtained from Sigma. Other peptides used in this study were synthesized at the Molecular Genetic Instrumental Facility using an FMoc (N-(9-fluorenyl)methoxycarbonyl) protocol with an advanced ChemTech MPS350 automated synthesizer. H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, and H-Pro-pNA were obtained from Bachem. Pronase was kindly provided by Dr. James Powers (Georgia Institute of Technology, Atlanta, GA), and cystatin C was provided by Dr. Magnus Abrahamsson (University of Lund, Lund, Sweden).

Methods

Source and Cultivation of Bacteria—P. gingivalis HG66 was a gift of Dr. Roland Arnold (University of North Carolina, Chapel Hill, NC), and the strains W50 and ATCC 33277 were obtained from the American Type Culture Collection. All cells were grown as described previously (21).

Enzyme Activity Assays—Routinely, the tripeptidyl peptidase amidoacytic activity was measured with H-Ala-His-Pro-pNA (1 mM) in 0.2 mM HEPES, pH 7.5, at 37 °C. The assay was performed in a total volume of 0.2 ml on microplates, and the initial turnover rate was recorded at 405 nm using a microplate reader (Spectramax, Molecular Devices). In inhibition studies, the enzyme was first preincubated with inhibitor for 15 min at 37 °C, substrate was added, and residual activity was recorded. H-Gly-Pro-pNA, Z-Ala-Pro-pNA, Z-Gly-Pro-pNA, and H-Pro-pNA (1 mM) were finally assayed in the same manner.

Protein Determination—Protein concentration was determined using the BCA reagent kit (Sigma), using bovine serum albumin as a standard.

Localization of Tripeptidyl Peptidase Activity— Cultures of P. gingivalis HG66, W50, and ATCC 33277, at different phases of growth, were subjected to the following fractionation procedure. The cells were removed by centrifugation (10,000 × g, 30 min), washed twice with 10 mM Tris, 150 mM NaCl, pH 7.4, resuspended in 50 mM Tris, pH 7.6, and disintegrated by ultrasonication in an ice bath at 1500 Hz for 5 cycles (5 min of sonication and 5 min of brake). Unbroken cells and large debris were removed by centrifugation (10,000 × g, 30 min), and the opalescent supernatant was subjected to ultracentrifugation (150,000 × g, 120 min), yielding a pellet containing bacterial membranes and a supernatant that was considered membrane-free extract. All fractions, as well as the full culture, culture medium, and full culture after sonication, were assayed for amidoacytic activity against H-Ala- Pro-Phe-pNA.

Enzyme Purification—All purification steps were performed at 4 °C except for FPLC separations, which were carried out at room temperature. Cells were harvested by centrifugation (6000 × g, 30 min), washed with 50 mM potassium phosphate buffer, pH 7.4, and resuspended in the same buffer (150 ml/50 g of cells, wet weight). Triton X-100 (10% v/v in H2O) was added slowly to the bacterial cell suspension to a final concentration of 0.05%. After 120 min of gentle stirring, unbroken cells were removed by centrifugation (28,000 × g, 60 min). Proteins in the supernatant were precipitated with cold acetone (−20 °C) added to a final concentration of 60%, collected by centrifugation, redissolved in 50 mM potassium phosphate buffer, pH 7.0, and extensively dialyzed against 20 mM potassium phosphate, pH 7.0, containing 0.02% sodium azide. The dialyzed fraction was clarified by centrifugation (28,000 × g, 30 min) and applied to a hydroxyapatite column (Bio-Rad) equilibrated with 20 mM potassium phosphate, pH 7.0, a flow rate of 20 ml/h, after which the column was washed until the A280 fell to zero. Bound proteins were eluted with a gradient from 20 to 300 mM potassium phosphate, and fractions (7 ml) were analyzed for dipeptidyl- and tripeptidyl peptidase activity using H-Gly-Pro-pNA and H-Ala-Phe-Pro-pNA, respectively. The activity against the latter substrate was pooled, saturated with 1 mM ammonium sulfate, clarified by centrifugation, and directly loaded onto a phenyl-Sepharose (Amersham Pharmacia Biotech) column equilibrated with 50 mM potassium phosphate, pH 7.0, containing 1 mM ammonium sulfate. The column was washed with two volumes of equilibration buffer, followed by buffer containing 0.5 mM ammonium sulfate, and developed with a descending gradient of ammonium sulfate from 0.5 to 0 M. Active fractions were pooled, dialyzed against 25 mM Bis-Tris, pH 6.3, and subjected to chromatofocusing on a MonoP FPLC column equilibrated with Bis-Tris buffer, using a pH gradient developed with 50 ml of 0–20 mM Bis-Tris Polybuffer 74 (Amersham Pharmacia Biotech) adjusted to a pH of 4.0.

Electrophoretic Techniques—The SDS-PAGE system of Schagger and von Jagow (22) was used to monitor enzyme purification and estimate the enzyme molecular mass. For amino-terminal sequence analysis, proteins resolved in SDS-PAGE were electroblotted to polyvinylidene difluoride membranes using 10 mM 3-(cyclohexylamino)propanesulfonic acid, pH 11, 10% methanol (23). The membrane was washed thoroughly with water and stained with Coomassie Blue G250. The blot was air dried, and protein bands were cut out and subjected to amino-terminal sequence analysis with an Applied Biosystems 491 protein sequencer using the program designed by the manufacturers.

Enzyme Fragmentation—The purified prolyl tripeptidyl peptidase (PTP-A) was partially denatured by incubation in 6 M urea in 0.02 mM Tris, pH 7.6, for 60 min. Low molecular mass gingipain B (RgpB) from P. gingivalis was then added to make an enzyme:substrate molar ratio of 1:100; the reaction mixture was made in 1 mM cysteine and the sample was incubated overnight at 37 °C. Generated peptides were separated by reverse phase HPLC using a μBondapak C-18 column (3.9 × 300 mm; Waters, Milford, MA). Peptides were eluted with 0.1% trifluoroacetic acid and acetonitrile containing 0.05% trifluoroacetic acid, using a gradient from 0 to 80% acetonitrile over 60 min. The column was monitored at 220 nm and collected manually.

For determination of the active site serine residue and to confirm that the purified enzyme was, indeed, a serine proteinase, 100 μg of purified PTP-A was first incubated with 170 μg of (1,3’-DHPDF (Amersham Pharmacia Biotech) for 30 min at 25 °C in 20 mM HEPES, pH 7.0, after which the reaction was quenched by addition of 0.1% Triton X-100 to a final concentration of 10 mM. The radiolabeled material was analyzed by SDS-PAGE, followed by autoradiographic analysis. The gel was dehydrated, soaked in 2,5-diphenyloxazole solution for 2 h, and dried, and the DFP-binding proteins were detected by fluorography after an exposure time of 96 h on x-ray film (XAR; Eastman Kodak). The bulk of the radiolabeled protein was subjected to proteolytic fragmentation with RgpB, and peptides obtained were separated by reverse phase HPLC as described above. Radioactivity in each peptide fraction was measured using a β liquid scintillation counter, and the labeled peptide as well as other selected peptides were subjected to sequence analysis.

Identification of the PTP-A Gene—The data base containing the unfinished P. gingivalis W83 genome, available from The Institute for Genomic Research, was searched for the presence of nucleotide sequences corresponding to the amino-terminal and the internal PTP-A amino acid sequences using the TBLASTN algorithm (24). An identified clone, gnfTIGR/P. gingivalis_126, was retrieved from The Institute for Genomic Research (TIGR) data base. The position of the PTP-A gene was localized using the National Center for Biotechnology Information open reading frame (ORF) finder, and the amino acid sequence, obtained by conceptual translation of the entire ORF, was then used for homology screening by use of the NCBI BLAST search tool.

Enzyme Specificity—Peptides were incubated with 1 μg of PTP-A at an enzyme:substrate molar ratio of 1:100 for 3 or 24 h in 50 μl of 200 mM HEPES, pH 7.5, at 37 °C, and the reaction was stopped by acidification with trifluoroacetic acid. The samples were then subjected to reverse phase high pressure liquid chromatography using a μBondapak C-18
column (3.9 × 300 mm) (Waters, Milford, MA) and an acetonitrile gradient (0–80% in 0.075% trifluoroacetic acid in 50 min). Each peak, detected at 220 nm, was collected, lyophilized, redissolved in 50% (v/v) methanol, 0.1% acetic acid, and subjected to analysis by mass spectrometry.

Mass Spectrometry—A Finnigan MAT 95S sector mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an electrospray source (ESI) was operated essentially as described previously (25). Peptides were identified by fitting of the obtained spectra to specific sequences using an Internet application program MsFit available at http://falcon.ludwig.ucl.ac.uk/msfit.html.

RESULTS

Enzyme Localization, Purification, and Initial Characterization—Analysis of amidolytic activity against H-Ala-Phe-Pro-pNA in several fractions of \textit{P. gingivalis}, HG66, W50, and ATCC 33277, clearly indicated that an enzyme(s) with prolyl tripeptidyl peptidase activity is localized on the cell surface in all strains tested, with 5% of the total activity being found in the medium regardless of the growth phase of the bacterial culture. Cell-associated enzyme was easily detached from the bacterial surface by treatment with a low concentration (0.05%) of Triton X-100. This procedure released 85–90% of activity in a soluble form. Subsequent acetone precipitation of proteins in the Triton X-100 fraction successfully separated the activity from pigment that remained in solution. The redissolved protein fraction, after dialysis, was applied to hydroxyapatite chromatography, and at this step substantial separation of the

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Step & Volume & Protein & Total activity & Specific activity & Purification fold & Yield \\
& ml & mg & & units/mg & & % \\
\hline
Triton X-100 extract after centrifugation & 200 & 1200 & 757,673 & 642 & 1 & 100 \\
Acetone precipitate & 50 & 600 & 537,622 & 896 & 1.4 & 71 \\
Hydroxyapatite chromatography & 50 & 22 & 400,039 & 18,183 & 28 & 53 \\
Phenyl-Sepharose & 48 & 10 & 312,505 & 31,250 & 48 & 41 \\
MonoQ & 3 & 1.5 & 244,828 & 163,218 & 254 & 32 \\
MonoP & 4 & 0.7 & 188,400 & 269,142 & 420 & 25 \\
\hline
\end{tabular}
\caption{Purification of the PTP-A from \textit{P. gingivalis}}
\end{table}

* Based on the enzymatic activity using H-Ala-Phe-Pro-pNA, where 1 unit = mOD/min/1 μl.
PTP-A activity from both the dipeptidyl peptidase IV and bulk protein was achieved (Fig. 1A). Further purification performed by subsequent chromatography steps, including phenyl-Sepharose (Fig. 1B), MonoQ (Fig. 1C), and MonoP columns (Fig. 1D), resulted in the isolation of purified enzyme. Significantly, the chromatography step on MonoP yielded an $A_{280}$ profile much sharper than the activity peak. Although this imperfect overlap of protein and activity profiles may suggest that the protein component does not represent active enzyme, the rest of data argue with such a contention. This apparent contradiction may be likely explained by the enzyme inhibition by the reaction product of H-Ala-Phe-Pro-pNA hydrolysis, but this possibility has not been been explored. The yield of protein and activity recovery in a typical purification procedure is summarized in Table I.

SDS-PAGE analysis of the purified enzyme revealed the presence of two protein bands with apparent molecular masses of 81.8 and 75.8 kDa, respectively (Fig. 2, lane f). Autoradiograph of the enzyme sample radiolabeled with [1,3-$^3$H]DFP (Fig. 2, lane g) clearly indicated that the bands represented either two distinct serine proteinases or different molecular mass forms of the same enzyme. In an attempt to distinguish between these two options, the electrophoretically resolved proteins were subjected to amino-terminal sequence analysis, but, unfortunately, it was found that the 81.8-kDa form of PTP-A had a blocked amino terminus. In contrast, the sequence NH$_2$-SAQTRTRFSADNLALMP- was found at the amino terminus of the lower molecular mass form of the enzyme. This result led us to the possibility that the 75.8-kDa form of PTP-A was derived from the 81.8-kDa form through proteolytic cleavage of a 6-kDa amino-terminal peptide. To confirm this hypothesis and, in addition, to localize the active site residue within $P. gingivalis$ PTP-A, the mixture containing both radiolabeled enzymes was proteolytically fragmented, and peptides were resolved by reverse phase HPLC. This procedure yielded only one major radioactive peptide peak (data not shown), and the purified peptide was found to have a single amino acid sequence: IGVH-GWYGGFMTTNL, where X apparently represents the DFP-modified serine residue. These data convincingly indicate that the two protein bands of purified PTP-A represents different forms of the same enzyme.

**pH Optimum, Stability, and Inhibition Profile**—Using the

### TABLE II

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diisopropyl fluorophosphate</td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>Phenylmethanesulfonyl fluoride</td>
<td>10 mM</td>
<td>96</td>
</tr>
<tr>
<td>Pefabloc SC</td>
<td>1 mg/ml</td>
<td>20</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>5 mM</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
<td>98</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>0.1 mM</td>
<td>100</td>
</tr>
<tr>
<td>Antipain</td>
<td>0.1 mM</td>
<td>100</td>
</tr>
<tr>
<td>Prolinal</td>
<td>0.1 mM</td>
<td>100</td>
</tr>
</tbody>
</table>

### TABLE III

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cleavage site</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td>H-Arg-Pro-Pro-</td>
<td>*-Gly-Phe-Ser-Pro-Phe-Arg</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>H-Arg-Pro-Pro-</td>
<td>-Gly-Phe</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
<td></td>
</tr>
<tr>
<td>Peptide 4</td>
<td>H-Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
<td></td>
</tr>
<tr>
<td>Peptide 5</td>
<td>H-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg</td>
<td></td>
</tr>
<tr>
<td>Peptide 6</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met-NH$_2$</td>
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<tr>
<td>Peptide 7</td>
<td>H-Val-Pro-Pro-</td>
<td>Gly-Glu-Asp-Ser-Lys-Glu-Glu-Val-Ala-Ala-Pro-His-Gln</td>
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<tr>
<td>Peptide 8</td>
<td>H-Val-Pro-Pro-</td>
<td>-Gly-Glu-Asp-Ser-Lys</td>
</tr>
<tr>
<td>Peptide 9</td>
<td>Ac-Val-Pro-Pro-Gly-Glu-Asp-Ser-Lys</td>
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<tr>
<td>Peptide 10</td>
<td>H-Val-Glu-Pro-</td>
<td>-Ile-Pro-Tyr</td>
</tr>
<tr>
<td>Peptide 11</td>
<td>H-Arg-Gly-Pro-</td>
<td>-Phe-Pro-Ile</td>
</tr>
<tr>
<td>Peptide 12</td>
<td>H-Ala-Arg-Pro-</td>
<td>-Ala-D-Lys-amide</td>
</tr>
<tr>
<td>Peptide 13</td>
<td>H-Pro-Asn-Pro-</td>
<td>-Asn-Glu-Gly-Asp-Phe-Ile</td>
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<tr>
<td>Peptide 14</td>
<td>H-Arg-His-Pro-</td>
<td>-Lys-Tyr-Lys-Thr-Glu-Leu</td>
</tr>
<tr>
<td>Peptide 15</td>
<td>H-Gly-Val-Pro-</td>
<td>-Lys-Thr-His-Leu-Glu-Leu</td>
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<td>Peptide 16</td>
<td>H-Lys-Gly-Pro-Pro-Ala-Leu-Thr-Leu</td>
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<td>Peptide 17</td>
<td>H-Gly-Lys-Gln-Met-Ser-Asp-Arg-Arg-Glu-Asp-Met-Ser-Pro-Ser-Asn-Asn-Val-Val-Pro-Ile-His-Pro-Ile-Pro-Thr-Glu-Asn-Lys-Pro-Lys-Val-Gln</td>
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<tr>
<td>Peptide 18</td>
<td>H-Phe-Leu-Arg-Glu-Pro-Val-Ile-Phe-Leu</td>
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<tr>
<td>Peptide 19</td>
<td>H-Gly-Ile-Arg-Pro-Tyr-Glu-Ile-Leu-Ala</td>
<td></td>
</tr>
<tr>
<td>Peptide 20</td>
<td>H-Leu-Pro-Asp-Leu-Asp-Ser-Ser-Leu-Ala-Ser-Asp-Glu-Leu-Leu-Ser-Pro-Glu-Pro-Arg-Pro-Glu-Ala</td>
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</tr>
<tr>
<td>Peptide 21</td>
<td>H-Cys-Leu-Ser-Ser-Gly-Thr-Leu-Glu-Pro-Gly-Glu-Asp-Asp-Asp-Ser-Arg-Glu-Leu-Glu-Ser</td>
<td></td>
</tr>
<tr>
<td>Peptide 22</td>
<td>H-Lys-Ile-Ala-Gly-Tyr-His-Leu-Glu-Leu</td>
<td></td>
</tr>
</tbody>
</table>

* $\downarrow$, cleavage site.
amidolytic activity assay with H-Ala-Phe-Pro-pNA, it was found that the enzyme has a broad pH optimum from pH 6.0 to 8.0, and in 0.2 M HEPES, pH 7.6 was stable for at least 12 h at 25 or 37 °C.

PTP-A activity was not affected by class-specific synthetic inhibitors of cysteine or metalloproteinases (Table II). In contrast, preincubation of the enzyme with DFP or Pefablock resulted in total loss of activity, supporting its classification as a serine proteinase. Surprisingly, however, 3,4-dichloroisocumarin was only a poor inhibitor, and phenylmethylsulfonyl fluoride, leupeptin, antipain, and prolinol had no effect at all. Interestingly, preincubation of PTP-A with iodoacetamide, but

![Image of multiple sequence alignment](http://www.jbc.org/)

**Fig. 3.** Multiple sequence alignment of *P. gingivalis* PTP-A (Pg-PTP-A) and its bacterial and eukaryotic homologues. Sequences of dipeptidyl peptidase from *P. gingivalis* (Pg-DPP; Ref. 20; note that the Pg-DPP amino-terminal sequence was corrected according to the data from the *P. gingivalis* W83 genome), dipeptidyl peptidase from *Flavobacterium meningosepticum* (Fn-DPP), human dipeptidyl peptidase IV (Hs-DPP), and mouse fibroblast activation protein (Mm-FAP) were aligned using the ClustalW multiple sequence alignment tool. Peptide sequences obtained from PTP-A analysis are indicated with arrows (note that the sequence of the peptide 81–97 corresponds to the amino terminus of the lower molecular weight form of PTP-A); catalytic triad is marked with asterisks; and the proposed PTP-A membrane-anchoring amino-terminal α-helix is underlined. Homologous regions are highlighted.
not with N-ethylmaleimide, stimulated enzyme amidolytic activity --2-fold. Human plasma inhibitors, such as α1-proteinase inhibitor, α1-antichymotrypsin, and α2-macroglobulin, did not affect the enzyme activity, nor were they cleaved by PTP-A (data not shown).

Substrate Specificity—Among several chromogenic substrates tested, including H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, Z-Gly-Pro-pNA, Z-Ala-Pro-pNA, and H-Pro-pNA, only H-Ala-Phe-Pro-pNA was hydrolyzed by PTP-A, indicating a prolyl-specific tripeptidyl peptidase activity. To further confirm this specificity, several synthetic peptides composed of 5–34 amino acid residues and containing at least 1 proline residue were tested as substrates for PTP-A. Of 22 peptides tested only those with a proline residue in the third position from the amino-terminal end were cleaved (Table III), with the significant exception of peptides with adjacent proline residues (peptides 3, 4, and 16). In addition, a free α-amino group was absolutely required for cleavage after the third proline residue, as exemplified by resistance to enzymatic hydrolysis of peptide 9, which differs from peptide 8 only in acylation of the α-amino group of the amino-terminal valine residue. Except for these two limitations, the peptide bond -Pro - Yaa- was cleaved at the same rate in all peptides with the general formula NH₂-Xaa-Xaa-Pro-Yaa-(Xaa)ₙ, where Xaa represents any amino acid residue, whereas Yaa could be any residue except proline, regardless of the chemical nature of the amino acids and the length of the peptide. In all cases the reaction was completed within 3 h, and prolonged incubation for 24 h did not affect the pattern of cleavage, confirming the absolute requirement for a proline residue at the third position from the unblocked amino terminus. In addition, these data indicate that the preparation of PTP-A was free of any contamination with aminopeptidase, dipeptidyl peptidase, or endopeptidase activities.

The lack of cleavage after internal proline residues in the synthetic peptides corresponds well with the absence of any proteolytic activity on several protein substrates, including IgA, IgG, albumin, azocasein, carboxymethylated ribonuclease, and gelatin. However, the size of the substrate, which is a limiting factor in the activity of oligopeptidases (26), is not restricting in the case of PTP-A, because the enzyme is able to cleave a tripeptide (NH₂-Xaa-Xaa-Pro) from the amino terminus of both human cystatin C and interleukin 6 (data not shown).

PTP-A Sequence Analysis—Partial PTP-A amino acid sequence data allowed us to identify the P. gingivalis genomic clone gnl/TIGR_P. gingivalis_126 in the Unfinished Microbial Genomes data base, TIGR. An ORF corresponding to the PTP-A amino acid sequence was found, as indicated by the fact that all sequences of the PTP-A-derived peptides obtained by the enzyme polypeptide fragmentation with RgpB were present in the protein primary structure inferred from the nucleotide sequence of the ORF. The 732-amino acid polypeptide with a calculated mass of 82,266 Da was encoded in this ORF. The homology search performed using the NCBI TBLASTN tool against GenBank, EMBL, DDBJ, and PDB data bases, and subsequent multiple sequence alignments (Fig. 3) indicated that PTP-A is a new member of the prolyl oligopeptidase family of serine proteinases (27). Within this large and diverse family of evolutionary and functionally related enzymes both from prokaryotes and eukaryotes, PTP-A was most closely related to bacterial dipeptidyl peptidase IV from Flavobacterium meningosepticum, Xantomonas maltophilus, and P. gingivalis, sharing 31.6, 30.4, and 28.5% amino acid sequence identity, respectively. Remarkably, the carboxyl-terminal region of the PTP-A molecule (residues 502–732) shows a significant similarity to the eukaryotic proline oligopeptidases, with 34 and 33% identity to human dipeptidyl peptidase IV and mouse fibroblast activation protein (FAP), respectively (Fig. 3). This part of the molecule contains the amino acid residues that encompass the catalytic triad in all characterized prolyl oligopeptidases, and from the multiple alignments with dipeptidyl peptidase IV of confirmed active site residues (28) it is apparent that Ser-603, Asp-768, and His-710 represent the catalytic triad of PTP-A (Fig. 3). Such an inference is further supported by the direct labeling of Ser-603 by DFP. In addition, the computer-assisted search for sequential motifs characteristic for transmembrane domains revealed the presence of such a putative region within the amino-terminal sequences of PTP-A, with residues 5–25 most likely folded into a hydrophobic α-helix responsible for membrane anchoring of this enzyme.

DISCUSSION

Because of their cyclic aliphatic character, proline residues bestow unique conformational constraints on polypeptide chain structure, significantly affecting the susceptibility of proximal peptide bonds to proteolytic cleavage (29). It is assumed, therefore, that those proline residues that often appear near the amino termini of many biologically active peptides protect them against proteolytic degradation by proteinases with general specificity. For the cleavage of peptide bonds adjacent to proline residues a specialized group of proteolytic enzymes has evolved, and their activity in vivo may have important physiological significance, because it may lead to inactivation of many biologically active peptides and/or transformation of the activity of others (30). In addition, hydrolysis of prolyl-X bonds in conjunction with general catabolic pathways should allow the complete reutilization of amino acids by living organisms, including bacteria. However, prolyl peptidebidas from bacterial pathogens, if released into the host environment, may interfere with the physiological functions of biologically active peptides and proteins and, therefore, contribute to the pathogenicity of infectious diseases.

In this report we have described the purification, properties, and amino acid sequence of a novel prolyl-specific tripeptidyl peptidase from P. gingivalis. PTP-A releases tripeptides from small proteins such as interleukin 6 and cystatin C as well as from peptides. It works as an exopeptidase because those peptide bonds with a blocked α-amino group are not processed. In addition, the enzyme has an absolute requirement for Pro in the P1 position because the substitution of proline by any other amino acid, including hydroxyproline, is not tolerated.
PTP-A was classified as a serine protease based on its inhibition by DFP and its resistance to sulphydryl blocking and chelating agents, and this was also confirmed by sequencing of the peptide containing the DFP-labeled active site residue. The sequence GWSYG is the signature for a recently identified group of serine proteinases, the prolyl oligopeptidase (S9) family, and indeed, sequence alignment clearly indicates that PTP-A is a new member of this family, most closely related to bacterial and eukaryotic dipeptidyl peptidases IV. In parallel with other members of the S9 family, P. gingivalis PTP-A contains the typical proline oligopeptidase catalytic triad topology of Ser-603-Asp-711 located at the carboxyl-terminal end of the amino acid sequence in a region now designated the protease domain.

Despite structural similarities to proteinases from the prolyl oligopeptidase family, the tripeptidyl peptide activity of PTP-A is unusual for this group of enzymes, and no other known similar activity has so far been attributed to any other member of the S9 family. In fact, all strict tripeptidyl peptidases belong only to the subtilisin family (S8) and the S33 family of serine proteinases; however, they neither share a structural relationship with PTP-A nor have activity limited to cleavage after proline residues (31). In this respect, the P. gingivalis tripeptidyl peptidease is a unique enzyme, and it will be interesting to learn the structural basis for its narrow specificity.

In P. gingivalis PTP-A, as well as in dipeptidyl peptidease IV (19), all activities are cell surface associated, and it is conceivable that the enzymes are membrane anchored through putative signal sequences, which are not cleaved but remain as a membrane-spanning domain similar to other members of the prolyl oligopeptidase family (32). However, a significant portion of the purified PTP-A has a truncated amino terminus, apparently attributable to cleavage by Lys-specific proteinase and likely to be an artifact that has occurred during the purification procedure. Nevertheless, the proteolytic shedding of membrane-bound PTP-A and dipeptidyl peptidease IV also occurs during cultivation of the bacteria, as indicated by variable amount of soluble activities found in cell-free culture media. The cell surface localization of dipeptidyl and tripeptidyl peptidases also supports a putative physiological function in providing nutrients for growing bacterial cells. Here, the inability of asaccharolytic P. gingivalis to utilize free amino acids (34) makes the bacterium entirely dependent on an external peptide supply. In this regard, dipeptidyl peptidease IV and PTP-A activities are probably very important, if not indispensable, for bacterial growth. This suggestion is strongly corroborated by the fact that the P. gingivalis genome contains three additional genes encoding proteinases homologous with dipeptidyl peptidease IV and PTP-A and one related to aminopeptidase B. If expressed, each gene product would probably have enzymatic activity, because each has a well preserved catalytic triad (Fig. 4). In addition, all of these genes encode a putative signal peptide, which may act in providing membrane anchorage motifs. Whether each of these enzymes is constitutively expressed or forms a backup system to provide nutrients in a specific environment is presently unknown, but our preliminary data indicate that at least one aminopeptidase B homologue, also having tripeptidyl peptidease activity, is produced during P. gingivalis growth.

Although there is a little doubt that P. gingivalis homologues of prolyl proteinases can be considered important housekeeping enzymes, their external localization and uncontrolled activity may contribute significantly to runaway inflammation in the human host and the pathological degradation of connective tissue during periodontitis. In working in concert, the bacterial PTP-A and dipeptidyl peptidease IV have the ability to completely degrade collagen fragments locally generated by endogenous or bacterial collagenases. Because type I collagen is the major component of periodontal ligament, its enhanced degradation by dipeptidyl peptidease IV and PTP-A, as well as by functionally related enzymes released by other periodontopathogens (33), may contribute to tooth attachment loss and periodontal pocket formation; thus these enzymes must be considered important pathogenic factors of these bacteria.

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Prolyl Tripeptidyl Peptidase from *Porphyromonas gingivalis*: A NOVEL ENZYME WITH POSSIBLE PATHOLOGICAL IMPLICATIONS FOR THE DEVELOPMENT OF PERIODONTITIS

Agnieszka Banbula, Pawel Mak, Marcin Bugno, Jerzy Silberring, Adam Dubin, Daniel Nelson, James Travis and Jan Potempa

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