Porphyromonas gingivalis DPP-7 Represents a Novel Type of Dipeptidylpeptidase

Received for publication, September 26, 2000, and in revised form, November 28, 2000.
Published, JBC Papers in Press, November 28, 2000, DOI 10.1074/jbc.M008789200

Agnieszka Banbula, Jane Yen, Aneta Olekszky, Paweł Mak, Marcin Bugno, James Travis, and Jan Potempa

From the Department of Biochemistry and Molecular Biology, University of Athens, Athens, Georgia 30602 and the Institute of Molecular Biology, Jagiellonian University, 34-120 Krakow, Poland

A novel dipeptidylpeptidase (DPP-7) was purified from the membrane fraction of Porphyromonas gingivalis. This enzyme, with an apparent molecular mass of 76 kDa, has the specificity for both aliphatic and aromatic residues in the P1 position. Although it belongs to the serine class of peptidases, it does not resemble other known dipeptidylpeptidases. Interestingly, the amino acid sequence around the putative active site serine residue shows significant similarity to the C-terminal region of the Staphylococcus aureus V-8 endopeptidase. The genes encoding homologues of DPP-7 were found in genomes of Xylella fastidiosa, Shewanella putrefaciens, and P. gingivalis. It is likely that at least in P. gingivalis, DPP-7 and its homologue, in concert with other di- and tripeptidases, serve nutritional functions by providing dipeptides to this asaccharolytic bacterium.

Porphyromonas gingivalis, an oral anaerobic bacterium, has been implicated as a causative agent of adult type periodontitis. As an asaccharolytic organism, P. gingivalis is totally dependent on external sources of peptides, which are necessary for its growth and proliferation. To fulfill such a fastidious nutritional requirement, this bacterium evolved a complex system of proteolytic enzymes, which are now recognized as important virulence factors in the development of periodontal disease (1). The best known and well-characterized enzymes of this system are gingipains R and K, arginine- and lysine-specific cysteine proteinases (2). Working in concert with the proteinases periodontain (3), collagenases/gelatinases (4–6), prIT (7), and Tpr (8) as well as host proteinases, this array of enzymes has the potential to degrade proteins from both the periodontal ligamentum and surrounding tissues. Their concerted action leads to the formation of a large pool of oligopeptides, which can be further utilized by P. gingivalis and other oral bacteria. However, P. gingivalis cannot transport poly- and oligopeptides into the cell, although it has the ability to thrive on dipeptides as a sole source of carbon. For this reason, we have focused our attention on a specialized group of P. gingivalis peptidases capable of hydrolyzing oligopeptides to di- and tripeptides, which can be subsequently metabolized by this periodontopathogen. In our previous report (9), we presented the purification, characterization, and cloning of prolyl tripeptidylpeptidase A, an enzyme that liberates tripeptides from the N-terminal regions of substrates containing proline residues in the third position. DPP-IV, an enzyme with similar specificity but only dipeptidylpeptidase activity, has also been cloned (10), purified, and characterized (11, 12). Together with a recently described angiotensinogen-converting enzyme analogue (13), all of these proteases can hydrolyze peptide bonds containing proline residues. In addition, the P. gingivalis genome contains three further putative genes encoding proteinases homologous with dipeptidyl peptidase IV, although their activities have not yet been identified (9).

In the present study, we show purification, biochemical characterization, and the gene sequence of a new cell surface-associated serine protease with dipeptidylpeptidase activity. This enzyme liberates dipeptides from the free amino terminus and has a broad specificity for both aliphatic and aromatic residues in the penultimate position.

EXPERIMENTAL PROCEDURES

Source and Cultivation of Bacteria—P. gingivalis DPP-7 was purified from strain H6G6, a kind gift of Dr. Roland Arnold (University of North Carolina, Chapel Hill, NC). The cells were grown as described previously (14).

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

Localization of Dipeptidylpeptidase Activity—The localization of active enzyme was checked in bacterial cells that had been subjected to a previously described fractionation procedure (12). All fractions, as well as the full culture, culture medium, and full culture after sonication, were assayed for amidolytic activity against H-Ala-Phe-pNA.

Enzyme Purification—All purification steps were performed at 4°C except for FPLC separations, which were carried out at room temperature. The cells were collected by centrifugation (6000g, 30 min) and resuspended in 50 mM potassium phosphate buffer, pH 7.4. The outer membrane proteins were solubilized with 0.05% Triton X-100. After 2 h of gentle stirring, unbroken cells were removed by centrifugation (28,000g, 60 min). Proteins from the supernatant were precipitated with cold acetone (60% final concentration), collected by centrifugation, and redissolved in 50 mM potassium phosphate buffer, pH 7.0. After extensive dialysis against the same buffer, the sample was loaded onto a hydroxyapatite column (Bio-Rad) previously equilibrated with 20 mM potassium phosphate, pH 7.0, at a flow rate of 20 ml/h. The column was then washed until the A280 fell to 0. Bound proteins were eluted with a potassium phosphate gradient (20–300 mM), and fractions (7 ml) were assayed for amidolytic activity against H-Ala-Phe-pNA. The active fractions were saturated with 1 mM ammonium sulfate and loaded onto a phenyl-Sepharose HP column (Amersham Pharmacia Biotech) equilibrated with 50 mM potassium phosphate, pH 7.0, containing 1 mM ammonium sulfate. The column was washed with two volumes of the equilibration buffer, followed by a wash with buffer containing 0.4 M

* This work was supported by National Institutes of Health Grant DE09761 and by Committee of Scientific Research (KBN, Poland) Grant 6PO4A 047 17. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 706-542-1713; Fax: 706-542-3719; E-mail: potempa@arches.uga.edu.

1 The abbreviations used are: DPP, dipeptidylpeptidase; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; pNA, p-nitroanilide; Suc-, succinyl-; Z -, benzoyloxycarbonyl; contig, group of overlapping clones; ORF, open reading frame.
ammonium sulfate and developed with a descending gradient of ammonium sulfate from 0.4 to 0 M. Active fractions were pooled, extensively dialyzed against 20 mM MES, pH 6.6, and applied onto a MonoS HR 5/5 FPLC (Amersham Pharmacia Biotech) column equilibrated with the same buffer. Bound proteins were eluted with a 0–300 mM NaCl gradient. This allowed us to obtain a homogenous preparation of active proteinase.

Electrophoretic Techniques—The SDS-PAGE system of Schagger and von Jagow (15) was used to monitor enzyme purification and estimate the enzyme molecular mass. For amino-terminal sequence analysis, proteins resolved in SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes using 10 mM CAPS, pH 11, 10% methanol (16). After staining 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.

Kinetic Analysis—Routinely, the dipeptidylpeptidase amidolytic activity was measured with H-Ala-Phe-pNA (1 mM) in 0.2 M HEPES, pH 7.8, at 37 °C. The reaction was followed for specific time intervals in a thermostated enzyme-linked immunosorbent assay reader (SpectraMax, Applied Biosystem), and the release of p-nitroaniline was monitored at 405 nm. Other p-nitroanilide substrates were used in the same manner. For inhibition studies, the enzyme was first preincubated with an inhibitor for 15 min at 37 °C, substrate was added, and residual activity was recorded. The initial steady-state velocity (v₀) was determined by continuous assay for the range of substrate concentrations (100 nM to 1 mM). Kᵣ and Vₑₚₐₓ were determined by hyperbolic regression of the kinetic data using the software package Hyper Version 1.02 obtained from Dr. J. S. Easterby (University of Liverpool, United Kingdom).

Enzyme Fragmentation—The purified dipeptidylpeptidase was subjected to in-gel tryptic digestion (17). Peptides were extracted and separated by microbore reverse-phase high pressure liquid chromatography. Fractions absorbing at 210 nm were manually collected, and their masses were determined by reflectron matrix-assisted laser desorption ionization-time-of-flight mass spectrometry using a Bruker Daltonics ProFlex instrument as described previously (18). Selected peptides were subjected to Edman degradation in a model Procise-cLS sequencer (PE Biosystems).

Identification of the DPP-7 Gene—An unfinished P. gingivalis W83 sequence data for DPP-7 was obtained from the Institute for Genomic Research site on the World Wide Web.
buffers including HEPES ( ), PIPES ( ), Tris ( ), potassium phosphate ( ), and MES ( ).

Enzyme Specificity—The determination of substrate specificity was based on the separation of the products of peptide hydrolysis by reverse-phase chromatography. Peptides were first incubated with 1 μg of DPP-7 at an enzyme/substrate molar ratio of 1:100 for 3 or 24 h in 50 μl of 20 mM HEPES, 100 mM NaCl, pH 8.0, 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 Supelco LC 18 column (Supelco) with an acetonitrile gradient of 0–60% in 0.075% trifluoroacetic acid in 50 min.

Each peak, detected at 210 nm, was collected, lyophilized, redissolved in 50% (v/v) methanol plus 0.1% acetic acid, and subjected to analysis by mass spectrometry.

RESULTS

A 76-kDa dipeptidylpeptidase associated with P. gingivalis membranes was solubilized by mild detergent treatment. This procedure released more than 90% of the amidolytic activity against H-Ala-Phe-pNA into the medium. After acetone precipitation and subsequent chromatography steps including the use of hydroxyapatite, phenyl-Septarose, and MonoS columns (Fig. 1), a pure enzyme preparation was obtained. The homogeneity of the preparation and molecular mass of the protein were checked both by SDS-PAGE (Fig. 2) and gel filtration on a TSK G3000 SW column (data not shown).

Inhibition Profile—Based on the inhibition studies (Table I) DPP-7 was classified as a serine protease, being inactivated by disopropylfluorophosphate, Pefablock, and 3,4-dichlorosocoumarin but not by typical cysteine class inhibitors such as E-64 or iodooacetic acid. Metal chelators including EDTA and 1,10-orthophenanthroline, as well as reducing agents did not influence its activity. The enzyme was not sensitive to inactivation by either detergents (0.5% SDS, 1% Triton X-100) or heavy metal ions including Zn2+, Co2+, and Ni2+. Human plasma inhibitors, such as α1-proteinase inhibitor, α1-antichymotrypsin, and α2-macroglobulin, did not affect enzyme activity, nor were they cleaved by DPP-7 (data not shown).

pH Optimum and Stability—Purified DPP-7 was active against H-Ala-Phe-pNA over a broad pH range, from neutral to basic pH (6.5–9.0) (Fig. 3). This activity also changed with the ionic strength of the buffer, reaching 200% at 0.5 M NaCl concentration in 100 mM HEPES, pH 8.0. DPP-7 was stable in 0.2 M HEPES, pH 8.0, for 1 week at 4 °C. The protease showed no appreciable loss of activity when kept frozen at −80 °C for 1 month. After a 3-h incubation at either room temperature or 37 °C, activity was reduced to 62 and 20%, respectively. The optimum temperature for the hydrolysis of H-Ala-Phe-pNA was determined to be 43 °C.

Substrate Specificity—Among the several chromogenic substrates tested, only those with aliphatic or aromatic side chains residues in the second, penultimate position were rapidly hydrolyzed by DPP-7 (Table II). To further confirm specificity, several synthetic peptides were also tested as substrates for this enzyme. Again, only those with an aliphatic or aromatic residue in the second position from the amino-terminal end were cleaved (Table III), with glycine, proline, or charged amino acids being not acceptable in the P1 position. The protease did not show any endopeptidase activity on gelatin, insulin β-chain, carboxymethylated lysozyme, and azocasein or type I collagen (data not shown). Purified DPP-7 was devoid of any aminopeptidase activity and did not cleave model substrates with blocked amino termini.

DPP-7 Sequence Analysis—Purified DPP-7 was resolved on SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. It had an amino-terminal sequence ADKGM-.

**TABLE II**

| Substrate                   | $K_m$ (μM) | $V_{max}$ (pmol/mg/min) |
|-----------------------------|-----------|-------------------------|
| H-Ala-Ala-pNA               | 0.313     | 129.65                  |
| H-Ala-Phe-pNA               | 0.441     | 170.06                  |
| H-Gly-Phe-pNA               | 0.256     | 54.54                   |

**TABLE III**

| Peptides cleaved         | Peptides not cleaved                  |
|--------------------------|---------------------------------------|
| Trp-Ala-γ-Arg-Asp-Ala-Ser-Gly-Glu | Trp-His-Trp-Leu-Glu-Leu-Lys-Pro-Gly-Glu-Pro-Met-Tyr |
| Ile-Ala-γ-Arg-Arg-His-Pro-Tyr-Phe-Leu | Ser-Pro-Tyr-Ser-Ser-Glu-Thr-Thr |
| Lys-Ile-γ-Ala-Gly-Tyr-His-Leu-Glu-Leu | Ala-Pro-Val-Arg-Ser-Leu |
| Phe-Leu-γ-Arg-Glu-Pro-Val-Ile-Phe-Leu | Gln-Lys-Gln-Met-Ser-Asp-Arg-Glu |
FIG. 4. ORF for the gene coding for the gene coding for P. gingivalis DPP-7. Underlined are sequences obtained from the Edman degradation of the trypsin fragmented DPP-7 polypeptide chain. The putative active site serine residue is marked by the black background.

FIG. 5. Comparison of the C-terminal regions of the P. gingivalis DPP-7 (residues 664–695) and S. aureus V8 endopeptidase (residues 704–863).
FIG. 6. Multiple sequence alignment of *P. gingivalis* DPP-7 and its putative homologues. Sequences of DPP-7-related proteinases were obtained from the conceptual translation of the following ORFs retrieved from unfinished and finished genome databases: S1, *S. putrefaciens* gnl [vert]TIGR_24[vert]sputre 6401; S2, *S. putrefaciens* gnl [vert]TIGR_24[vert]sputre 6410; X, *X. fastidiosa* gb[vert]AE004008.1[vert]; P1, *P. gingivalis* gnl [vert]TIGR[vert]P. gingivalis_CP.G_con; P2, *P. gingivalis* DPP-7 gnl [vert]TIGR[vert]P. gingivalis_CP.G_con. The sequences were subsequently aligned using the ClustalW multiple sequence alignment tool.
tained, including DNKPYK, EMTRYL, FAQFAN, VLPAML, SVVPY, and LFPGAGL. All of this sequence data allowed us to identify the P. gingivalis genomic contig gnl[vert]TIGR[vert]P. gingivalis in the Unfinished Microbial Genomes data base, the Institute of Genomic Research. An ORF corresponding to the DPP-7 amino acid sequence was found, as indicated by the fact, that all sequences of the DPP-7-derived peptides obtained by the enzyme polypeptide fragmentation by trypsin were present in the protein primary structure inferred from the nucleotide sequence of the ORF as shown in Fig. 4. The entire ORF corresponds to a 675-amino acid polypeptide with a calculated mass of 76247.4 Da. Interestingly, the DPP-7 ORF contains the consensus sequence for the active-site serine residue of serine type proteases, TGGSNSGPVF. As indicated in Fig. 5, the DPP-7 carboxyl terminus exhibits a high degree of identity to that of the V8 serine protease, particularly around the putative active site serine residue. This is surprising, since the P. gingivalis DPP-7 is a dipeptidyl peptidase specific for small aliphatic and aromatic residues, whereas Staphylococcus aureus V8 endopeptidase is specific toward substrates containing glutamic or aspartic acid residues in the P1 position. The similarity search performed using the NCBI TBLASTN tool against GenBank™, EMBL, DDBJ, and PDB data bases showed no significant similarity of DPP-7 to any other known dipeptidyl peptidases, indicating that this enzyme could be regarded as a member of a new family of proteases. Additional searches against data bases containing unfinished and finished microbial genomes allowed us to identify more genes coding for similar proteases with consensus active site sequence TGGSNSGPVF (Fig. 6). A gene of related protein has been found in similar proteases with consensus active site sequence TG-. DPP-7 displays the consen....
DPP-7 from *P. gingivalis*

20. Barrett, A. J., Rawlings, N. D., and Woessner, J. (1998) *Handbook of Proteolytic Enzymes*, Academic Press, London

21. McGuire, M. J., Lipsky, P. E., and Thiele, D. L. (1992) *Arch. Biochem. Biophys.* 295, 280–288

22. Vacheron, M. J., Guinand, M., Francon, A., and Michel, G. (1979) *Eur. J. Biochem.* 100, 189–196

23. Beauvais, A., Monod, M., Debeauquis, J. P., Diaquin, M., Kobayashi, H., and Latge, J. P. (1997) *J. Biol. Chem.* 272, 6238–6244

24. Ellis, S., and Nueske, J. M. (1967) *J. Biol. Chem.* 242, 4623–4629

25. Ogasawara, W., Kobayashi, G., Okada, H., and Morikawa, Y. (1996) *J. Bacteriol.* 178, 6288–6295

26. Carmona, C., and Gray, G. L. (1987) *Nucleic Acids Res.* 15, 6757
Porphyromonas gingivalis DPP-7 Represents a Novel Type of Dipeptidylpeptidase
Agnieszka Banbula, Jane Yen, Aneta Oleksy, Pawel Mak, Marcin Bugno, James Travis
and Jan Potempa

J. Biol. Chem. 2001, 276:6299-6305.
doi: 10.1074/jbc.M008789200 originally published online November 28, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008789200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 25 references, 13 of which can be accessed free at
http://www.jbc.org/content/276/9/6299.full.html#ref-list-1