ASP- AND GLU-SPECIFIC NOVEL DIPEPTIDYL PEPTIDASE 11 OF *Porphyromonas gingivalis* THAT ENSURES UTILIZATION OF PROTEINEOUS ENERGY SOURCES*

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Running head: DPP11, a novel dipeptidylpeptidase specific for Asp and Glu

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***Porphyromonas gingivalis* and *Porphyromonas endodontalis*, asaccharolytic black-pigmented anaerobes, are predominant pathogens of human chronic and periapical periodontitis, respectively. They incorporate di- and tri-peptides from the environment as carbon and energy sources. In the present study, we cloned a novel dipeptidyl peptidase (DPP) gene of *P. endodontalis* ATCC 35406, designated as DPP11. The DPP11 gene encoded 717 amino acids with a molecular mass of 81,090, and was present as a 75-kDa form with an N-terminus of Asp_{22}. A homology search revealed the presence of a *P. gingivalis* orthologue, *PGN0607*, which has been categorized as an isoform of authentic DPP7. *P. gingivalis* DPP11 was exclusively cell-associated as a truncated 60-kDa form and the gene ablation retarded cell growth. DPP11 specifically removed dipeptides from oligopeptides with the penultimate N-terminal Asp and Glu, and has a P2-position preference to hydrophobic residues. Optimum pH was 7.0 and the k_{cat}/K_m value was higher for Asp than Glu. Those activities were lost by substitution of Ser_{652} in *P. endodontalis* and Ser_{655} in *P. gingivalis* DPP11 to Ala, and they were consistently decreased with increasing NaCl concentration. Arg_{670} is a unique amino acid completely conserved in all DPP11 members distributed in the genera *Porphyromonas*, *Bacteroides*, and *Parabacteroides*, whilst this residue is converted to Gly in all authentic DPP7 members. Substitution analysis suggested that Arg_{670} interacts with an acidic residue of the substrate. Considered to preferentially utilize acidic amino acids, DPP11 ensures efficient degradation of oligopeptide substrates in these gram-negative anaerobic rods.**

Porphyromonas gingivalis, a gram-negative black-pigmented anaerobe, is a major causative organism of aggressive forms of chronic periodontitis (1,2), which leads to loss of permanent teeth (3-5). Recently, much attention has been paid to this bacterium because of its close relationship with systemic diseases, such as cardiovascular diseases (6), decreased kidney function (7), and rheumatoid arthritis (8). An important relative of the bacterium is *Porphyromonas endodontalis*, which is predominantly isolated from periapical periodontitis sites, i.e., infected root canals with acute symptoms such as pain, swelling, and drainage (9-11).

Both *Porphyromonas* species are asaccharolytic in principal and do not ferment glucose, cellobiose, lactose, or sucrose (12), and require proteinous substrates as carbon and energy sources. *P. gingivalis* possesses extracellular arginine aminopeptidase activity, which is mediated by Arg-gingipains (Rgps), isoforms A and B of Arg-X-specific cysteine proteinase, whereas other aminopeptidase activities are not present (13, 14).

Lys-specific gingipain (Kgp), another potent cysteine proteinase, does not exhibit aminopeptidase activity (15). In accord with the lack of predominant aminopeptidase activities, it has been demonstrated that *P. gingivalis* mainly incorporates nutritional amino acids as forms of di- and tri-peptides, not as single amino acids, and produces metabolic end-products such as ammonia, acetate, propionate, and butyrate (16, 17), which are considered to be virulence factors of this bacterium, causing host tissue damage (18, 19). Accordingly, the cell-surface and extracellular peptidases of *P. gingivalis* that produce di- and tri-peptides are considered to play critical roles in cell growth as well as its pathogenicity.

Although entire genome sequencing has annotated 72 peptidase genes in *P. gingivalis* W83 (20) as well as ATCC 33277 (21), at present only the several peptidases that have been well characterized allow us to explain their proteinous substrate utilization. First, the predominant proteolytic activities of Kgp and Rgps (22-25) are believed to digest nutritional proteins into oligopeptides. Subsequently, oligopeptides are processed by dipeptidyl peptidase IV (EC 3.4.14.5, DPPIV) (26), DPP7 (27) and prolyl tripeptidyl peptidase-A (PTP-A) (28, 29). *P. gingivalis* DPPIV (PgDPPIV) preferentially cleaves NH_2-X-Pro-|-$(X)_n$ and less potently NH_2-X-Ala-|-$(X)_n$ (26, 30). DPP7 (PgDPP7) cleaves NH_2-X-Y-|-$(X)_n$, where Y is aliphatic or aromatic amino acid (27). When Pro is located at the third position from the N-terminus, neither DPPIV nor DPP7 cleaves the peptides, and instead, PTP-A cleaves an NH_2-X-X-Pro-|-$(X)_n$ bond (29, 31). Therefore, the integrated actions of DPPIV, DPP7, and PTP-A may be responsible for utilization of peptides from scarce resources in the oral cavity. On the other hand, though the growth of a *DPPIV*-, *DPP7*-, and *PTP-A*-triple knockout *P.* gingiva*lis* strain was demonstrated to be retarded, the mutant showed growth (32), which may indicate the presence of a complementary mechanism for supplying substances for the metabolic pathway. Moreover, Asp/Asn and Glu/Gln are the most intensively consumed nutritional peptides in tryptone-based medium (16). Therefore, the existence of *P. gingivalis* DPP that hydrolyzes Asp and Glu at the P1 position is reasonably surmised. In the case of *P. endodontalis*, which does not have the proteolytic activity equivalent to gingipains (10, 33), the mechanism on oligopeptide metabolism is further obscure.

In the present study, we started from the analysis of a DPP activity of *P. endodontalis*, an important pathogenic organism in periapical lesions, because substantial DPP activities were observed in the extracellular fraction obtained from a dialysis-membrane-culture on an agar plate, while DPPs seemed completely cell-associated in *P. gingivalis*. In addition, since *P. endodontalis* does not have marked gingipain-like activities, this microorganism has a great advantage over *P. gingivalis* in handling of the activities without care about degradation or modification by gingipain-like proteinases (33).

We isolated a gene of *P. endodontalis* using a PCR method with degenerated primers designed based on the sequence homology of DPP7-family genes, and subsequently found the existence of its *P. gingivalis* orthologue, *PGN0607*, in a homology search, which had been already proposed as an isoform of DPP7 (27). Recombinant proteins of the cloned gene and *PGN0607* expressed in *Escherichia coli* did not show DPP7-like activity, indicating their identity distinct from DPP7. Since Nos. I-VI and 6-10 have been already allocated to DPPs with various specificities and their subtypes, we designated this novel DPP as DPP11. The enzymatic and biochemical analyses on of DPP11 from *P. endodontalis* (PeDPP11) and *P. gingivalis* (PgDPP11) revealed that they possessed the activity to hydrolyze NH_2-X-Asp/Glu-|-$(X)_n$. We also found

Arg_{670} that is critical for the substrate specificity of DPP11. Furthermore, a MEROPS database (34) search indicated that DPP11 orthologues are widely distributed in anaerobic gram-negative species in the genera *Porphyromonas*, *Bacteroides*, and *Parabacteroides*.

Experimental Procedures

Materials- The expression and cloning vectors used were pTrcHisTOPO from Invitrogen, pQE60 from Qiagen, and pGEM-Teasy from Promega. DEAE-Sephacel, Sephacryl S200HR, CNBr-Sepharose, low-molecular-weight molecular markers and rainbow markers, were obtained from GE Healthcare. Restriction enzymes and DNA-modifying enzymes came from TAKARA BIO and New England Biolabs, respectively, while KOD Plus DNA polymerase came from Toyobo (Tokyo, Japan). MCA peptides and neuromedin B were obtained from the Peptide Institute Inc. (Osaka, Japan). Variants of neuromedin B with an amino acid substitution to Asp at the first, second, third, or fifth position were synthesized by BEX (Tokyo, Japan), and LD-, LE-, acetyl (ac)-LD-, and benzyloxycarbonyl (Z)-LLQ-MCA were synthesized by Thermo Fisher Scientific (Ulm, Germany) and TORAY (Tokyo, Japan). A Genome Walker Universal kit and Talon metal affinity resin came from Clontech Laboratories. Thermolysin from *Bacillus thermoproteolyticus rokko* and trifluoroacetic acid were obtained from Sigma-Aldrich, and polyvinylidene difluoride membranes and $ZipTip_{\mu\text{-}C18}$ came from Millipore. α-Cyano-4-hydroxycinnamic acid was purchased from Applied Biosystems, while oligonucleotide primers came from FASMAC (Atsugi, Japan) and alkaline-phosphatase-conjugated rabbit anti-mouse Ig(G+A+M), from Zymed Laboratories. Recombinant Glu-specific endopeptidase from *S. aureus* V8 strain (GluV8) was expressed using a method reported previously (35).

Bacterial strains and growth conditions- P. endodontalis ATCC 35406 and *P. gingivalis* ATCC 33277 were obtained from American Type Culture Collection. They were cultured in anaerobic bacteria culture media (ABCM) (Eiken Chemical, Tokyo, Japan) in the presence of 1 μg/ml of menadione (Sigma-Aldrich) at 35˚C under an anaerobic condition (90% N_2/5% H_2/5% CO_2). To separate the cellular and soluble extracellular fractions, bacterial cells grown to the early stationary phase were inoculated onto ABCM agar plates covered with a sterilized dialysis membrane (cut-off, 6 kDa) and cultured further for 2 days. The cells were then suspended with 10 ml of phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 10,000 x *g* for 20 min at 4˚C. The resultant supernatant was filtered with a 0.45-μm membrane and used as the extracellular fraction. The cell pellet was washed once with PBS, and the resultant pellet was re-suspended in 10 ml of PBS and used as the cellular fraction. CDC anaerobe blood agar plates (Nippon Becton Dickinson, Tokyo, Japan) were used to examine black pigmentation. *E. coli* XL-1 Blue was grown at 37˚C in Luria-Bertani broth and on agar plates supplemented with 75 μg/ml of ampicillin.

PCR cloning of gene encoding a DPP-family member from P. endodontalis- Genomic DNA from *P. endodontalis* and *P. gingivalis* (0.1 μg) was prepared as described previously (36), and used as templates. Degenerate 5'- and 3'-oligonucleotide primers (DPP7deg244-276 and DPP7deg676-643) (Table S1) were synthesized in consideration of the homology of DPP7-related peptidases from *P. gingivalis* (*PG0491*/Q7MWU6 and *PG1283*/ BAG33126), *X. fastidiosa* (ZP00652070), and *Shewanella putrefaciens* (YP001182286 and YP001182445) (27). Degenerate PCR was performed with 7 cycles at 94˚C for 3 sec and 72˚C for 3 min, then 25 cycles at 94˚C for 3 sec and 60˚C

for 3 min using a Genome Walker Universal kit. The resultant 448-bp DNA fragment was amplified, inserted into a pGEM-T Easy vector, and sequenced. Genomic DNA of *P. endodontalis* (2 μg) was digested with *Dra*I, *Eco*RV, *Pvu*II, or *Stu*I, then ligated with an adaptor nucleotide, and the obtained DNA fragments were used as a template for genome walking. PCR was performed using one of the gene specific primers (8Fcomo127-102 and 8Fcomp326-350, Fig. 1 and Table S1) in combination with the adaptor primer (5'-GTAATACGACTCACTATAGGGC-3'), resulting in amplification of 1- and 2-kbp DNA fragments from *Dra*I- and *Eco*RV-digested genome DNA, respectively. Consequently, a 2,303-bp nucleotide containing an open reading frame composed of 2,154 bp was identified. The gene was designated as *P. endodontalis* DPP11 (PeDPP11, registered as AB610284 in DNA Data Bank of Japan).

Construction of expression plasmids and in vitro *mutagenesis*- Since the N-terminus of the soluble form of PeDPP11 was Asp_{22} (see Fig. 3), a DNA fragment encoding Asp_{22}-Phe_{717} was amplified by PCR with genomic DNA and a set of primers (5PeDPP11D22Bgl, 3PeDPP11F717Bgl) containing *Bgl*II sites (Table S1), then the *Bgl*II-digested 2.2-kDa PCR fragment was cloned into the *Bam*HI site of pTrcHisTOPO (designated pTrcHis-PeDPP11). A DNA fragment encoding from Asp_{22} to the C-terminal Pro_{720} of *PGN0607*, the *P. gingivalis* gene (PgDPP11) most homologous to *PeDPP11*, was amplified by PCR with a set of primers (5PgDPP11D22Bam/3PgDPP11P720Bam, Table S1) and genomic DNA, then cloned into a *Bam*HI site of pQE60 to generate pQE-PgDPP11. *In vitro* mutagenesis was performed using a PCR technique with primers to introduce an amino acid substitution (Table S1). The substitutions of Ser652Ala and Ser655Ala were introduced into plasmids encoding pTrcHis-PeDPP11 and pQE-PgDPP11, respectively, while substitution of Arg_{670} to Gly, Lys, His, Gln, or Asp was introduced to pTrcHis-PeDPP11. All mutations were confirmed by nucleotide sequencing.

Expression of DPP in E. coli *and production of polyclonal antibodies*- Recombinant PeDPP11 and PgDPP11 were expressed in *E. coli* by induction with 0.2 mM isopropyl-β-thiogalactopyranoside at 30˚C for 4 h. Recombinant proteins were purified by Talon affinity chromatography as reported previously (35). For usage as antigens for developing antibodies, Talon affinity column-purified PeDPP11 and PgDPP11 were further separated by size-exclusion gel chromatography with a Sephacryl S200HR column (1 x 86 cm) equilibrated with 20 mM ammonium bicarbonate (pH 8.0). The peak fraction was lyophilized and used for immunization of rabbits.

Construction of P. gingivalis *strain with disrupted PGN0607*- A DNA fragment encoding Asp_{22}-Pro_{720} of *PGN0607* was amplified with a primer set (5PgDPP11D22Bam and 3PgDPP11-P720Bam), and cloned into a pGEM-T Easy vector (pGEM-T Easy-PGN0607). An erythromycin-resistant gene fragment from *Bacteroides fragilis* was amplified by PCR from pUC19-Em with 5ErmF-AM-Cla and 3ErmF-AM-Cla primers (37, 38), digested with *Cla*I, and inserted into a *Cla*I site of pGEM-T Easy-*PGN0607* (designated pGEM-T Easy-*PGN0607*-Em). Electro-transformation of *P. gingivalis* ATCC 33277 was carried out with *Sal*I-linearlized pGEM-T Easy-*PGN0607*-Em, and *PGN0607*-disrupted strains were selected on ABCM agar supplemented with 1 μg/ml of menadione and 10 μM erythromycin, according to a previously reported method (39).

Hydrolyzing activity toward MCA peptides- Generally, various fractions of native DPP11 at 10 μl or 2 ng of recombinant DPP11 were used for measurement of proteolytic activity in 200 μl of reaction solution composed of 50 mM sodium

phosphate (pH 7.0) and containing 5 mM EDTA. The reaction was started with an addition of 20 μM MCA-peptides and continued at 37˚C for 1 h. Fluorescence intensity was measured with excitation at 380 nm and emission at 460 nm with a Fluorescence Photometer F-4000 (Hitachi). In some experiments, appropriate buffers (50 mM) from pH 4 to 10.5 or NaCl concentrations from 0 to 1.6 M were used. Dipeptidyl-MCA substrates not commercially available were prepared from tri- or tetra-peptidyl-MCA (0.4 mM) through digestion with 0.3 μg of GluV8 in 50 mM Tris-HCl (pH 8.0) containing 5 mM EDTA (100 μl) or 0.3 μg of thermolysin in 10 mM sodium borate (pH 8.0) and 2 mM $CaSO_4$ containing 0.005% (v/v) Triton-X100 (100 μl) at 37˚C for 4 h (35). The resultant protease-pretreated MCA peptides were used as substrates for DPP11 at 20 μM, as described above.

Ion-exchange chromatography of P. endodontalis *extracellular fraction*- Chromatographic procedures were performed at 4˚C. Soluble extracellular fraction (100 ml) obtained from dialysis membrane-cultures of *P. endodontalis* was dialyzed against 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (Buffer A), and subjected to anion-exchange chromatography on a DEAE-Sephacel column (1.5 x 19 cm) at a flow rate of 2 ml/min. After washing with Buffer A, bound proteins were eluted with a linear gradient of 0-0.6 M NaCl in Buffer A (300 ml). Eighty-drop fractions (c.a. 4 ml) were collected, then the proteolytic activities toward ac-DNLD-, KA- and succinyl (suc)-AAA-MCA were determined, as described above.

Mass spectrometry- Neuromedin B and its derivatives (1 nmol) were separately incubated with recombinant PeDPP11 (50 nM) or ion-exchange-fractionated fractions (10 μl) in 50 μl of 50 mM Tris-HCl (pH 8.0) at 30˚C for 1 h. The reaction was stopped by addition of trifluoroacetic acid (final 0.1%), then hydrolysates were adsorbed to $ZipTip_{\mu\text{-}C18}$, washed with 0.1% trifluoroacetic acid, and eluted with 50% of acetonitrile containing 5 mg/ml of α-cyano-4-hydroxycinnamic under an isocratic condition. The molecular masses of the products were determined by mass spectrometry using a Voyager DE-PRO (Applied Biosystems).

SDS-PAGE, immunoblotting, and N-terminal sequencing- Proteins were separated by PAGE in the presence of 0.1% (w/v) of SDS with a polyacrylamide concentration of 11% (w/v), then stained with Coomassie Brilliant Blue (CBB) or transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using anti-PeDPP11 or anti-PgDPP11 serum (10^4- to 10^6-fold dilutions), and the blots were visualized with alkaline phosphatase-conjugated anti-rabbit Ig(G+A+M) using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega). For N-terminal sequencing, separated proteins were transferred to a Sequi-Blot membrane (Bio-Rad) and stained with CBB. The N-terminal sequences of stained bands were determined with a model Procise 49XcLC protein sequencer (Applied Biosystems), as previously described (40).

Immunoadsorption of PeDPP11- Recombinant PeDPP11-coupled Sepharose was prepared using a CNBr-Sepharose 4B (GE Healthcare), according to the manufacturer's protocol. A PeDPP11-specific antibody was purified from rabbit antiserum using PeDPP11-Sepharose affinity chromatography, then PeDPP11 antibody-conjugated Sepharose was prepared as above. For immunoadsorption assays, aliquots (100 μl) of DEAE-Sephacel-separated fractions were incubated with 15, 45, or 135 μl of PeDPP11-antibody resin (50% suspension). After rotation at 0˚C for 8 h, samples were centrifuged and the activity remaining in the supernatant was determined. To determine the N-terminal sequence, fractions (3 ml) obtained by DEAE-Sephacel chromatography were immunoadsorbed to anti-PeDPP11 antibody resin (0.4 ml as 50% suspension), then the resin was washed with 1 ml of

PBS 4 times. After centrifugation, materials bound to the resin were extracted with SDS-sample buffer (100 μl) at 94˚C for 10 min and aliquots were separated on SDS-PAGE.

RESULTS

Degenerate PCR cloning of potential DPP. We first attempted to purify DPP7-like activities from extracellular fraction of *P. endodontalis*, which exhibited higher activity than those of *P. gingivalis* (33), however, they were not purified to homogeneity and thus their N-terminal sequences were not determined. Then, gene cloning by degenerate PCR was undertaken with a set of primers (DPP7deg244-276 and DPP7deg676-643, Table S1) designed in consideration of the amino acid homology of DPP7s among *P. gingivalis*, *X. fastidiosa*, and *S. putrefaciens*. Using a combination of degenerate PCR and subsequent genome walking, we cloned a 2,303-bp nucleotide sequence carrying a gene of 2,154-bp ORF, which encoded a 717-amino acid protein with a deduced molecular mass of 81,090 (Fig. 1). This gene was designated as DPP11. The amino acid sequence identity of PeDPP11 to DPP7 from *P. gingivalis* ATCC 33277 (*PGN1479*) (21) was 38.3% (Table 1). The arrangement of potential catalytic triad for the serine proteases His_{84}, Asp_{197}, and Ser_{652} in PeDPP11 was identical to that in PgDPP7 (Fig. 2), and the amino acid sequence at around Ser_{652} shared significant homology with the Glu-specific serine protease GluV8 (Fig. 2). However, the arrangement of the catalytic triad was distinct from that of PgDPPIV (Ser_{593}, Asp_{668}, and His_{700}). Interestingly, a BLAST search identified *PGN0607* as having the highest homology (57.9%) to the PeDPP11 gene (Table 1) and the corresponding gene of *P. gingivalis* W83 (*PG1283*), which has only 4-amino acid substitutions with *PGN0607* and has been annotated to be an isoform of authentic DPP7 in *P. gingivalis*, whereas its enzymatic property was not directly examined (27).

When the PeDPP11 gene and *PGN0607* were expressed in *E. coli*, 85-kDa proteins were purified in accordance with their estimated molecular masses (Fig. S1). However, neither of those proteins showed hydrolyzing activity toward KA-MCA, a typical substrate for DPP7. Furthermore, none of 76 commercially available MCA substrates were cleaved by the recombinant proteins. These findings could be interpreted in the following 3 ways: they are pseudogenes, they are recombinant proteins that require maturation processing to achieve the activity, or they have novel substrate specificity that can not be detected with commercial MCA substrates. Although the possibility of pseudogenes can not be completely excluded, the maintenance of the ORF of 717 amino acids comparable to the 712 amino acid ORF of PgDPP7 and the similarity to *PG0607* suggest its integrity. To achieve *in vitro* maturation, recombinant proteins were incubated with trypsin, chymotrypsin, thermolysin, papain, or GluV8, as reported on recombinant GluV8 (35). However, they noted no activity for KA-MCA (data not shown). Hence, based on the third assumption, we attempted to reveal the peptidase activity of DPP11. To identify and purify native DPP11, antibodies against recombinant PeDPP11 and PgDPP11 were developed. Immunoblotting (Fig. S1*B*) and enzyme-linked immunosorbent assay (data not shown) demonstrated that these antibodies reacted with 7-8-fold higher affinities for the original antigens than the counterparts.

Peptidase activities in extracellular fraction of P. endodontalis. Initially, we arranged 76 commercially available mono- to octa-peptidyl MCA substrates with or without N-terminal modification, in which the P1-position was 14 amino acids other than Cys, His, Ile, Gln, Ser, and Thr, then determined the peptidase activities of

extracellular and cell-associated fractions from *P. endodontalis* as well as *P. gingivalis.* As a result, KA-MCA was predominantly hydrolyzed with the *P. endodontalis* extracellular fraction, whereas the Rgp and Kgp activities were negligible (Fig. S2, ref. 33). In addition, we detected activities hydrolyzing suc-AAA- and ac-DNLD-MCA in *P. endodontalis*, and the hydrolysis was significantly increased by prolonged incubation, suggesting a multiple step reaction. These activities were also present in the *P. endodontalis* cellular fraction (data not shown). Noticeably, the hydrolyzing activities toward KA-MCA, suc-AAA-MCA, and ac-DNLD were very low in the extracellular fraction of *P. gingivalis* (Fig. S2). Based on these observations, we assumed the presence of 2 kinds of DPPs in *P. endodontalis*, one that is responsible for the cleavage of KA-MCA and another that cleaves the NH_2-X-Asp/Asn-|-$(X)_n$ bonds. Considered the suc group as N-terminal amino acid, suc-AAA-MCA could be cleaved by the DPP responsible for the cleavage of KA-MCA, most likely to DPP7.

Separation of DPP11 activity from extracellular fraction of P. endodontalis *by anion-exchange gel chromatography and immunochemical techniques.* To determine whether the hydrolyzing activity toward the NH_2-X-Asp/Asn-|-$(X)_n$ bonds was related to DPP11, soluble extracellular proteins from *P. endodontalis* were separated by DEAE-Sephacel chromatography (Fig. 3). The DPP7-like activity hydrolyzing KA-MCA was split into 3 peaks at fractions 56, 64, and 74. Among them, the first major KA-MCA hydrolyzing activity was co-eluted with that for suc-AAA-MCA, further suggesting their identity. The activity for ac-DNLD-MCA was eluted as a major peak at around fraction 52, which was separate from the DPP7-like Ala-specific activity. In addition, the 75-kDa band immunoblotted with the PeDPP11 antibody was co-eluted with the peak of hydrolyzing activity toward ac-DNLD-MCA. Moderate staining was found at 80 kDa at around fraction 70 and weak staining at 75 kDa at fraction 64. These 75- and 80-kDa substances were purified by immunoadsorption, and their N-terminal amino acid sequences were determined. The N-terminal sequence of the 75-kDa molecule, DGGMXLMQQ, coincided with the deduced sequence of PeDPP11 (D_{22}GGMWLMQQ_{30}), with an estimated molecular mass of M_r=78,706 (Asp_{22}-Phe_{717}). Although the N-terminal sequence of the 80-kDa species was not determined because of impurity, it was speculated to be a full-length form (Met_1-Phe_{717}) with a calculated molecular mass of 81,095. Furthermore, the ac-DNLD-MCA-hydrolyzing activity of fraction 52 was decreased by incubation with PeDPP11-antibody-Sepharose in a dose-dependent manner (Fig. 3), whereas the DPP7-like activity of fraction 56 toward KA-MCA did not change. Thus, the ac-DNLD-MCA-hydrolyzing activity was tightly associated with the 75-kDa PeDPP11.

Identification of peptidase activity in recombinant DPP11. Despite the prominent activity of native PeDPP11, recombinant PeDPP11 did not exhibit activity for ac-DNLD-MCA. Apparently, since tetrapeptidyl-MCA is suboptimal to measure DPP activity, ac-DNLD-MCA was pre-treated by thermolysin, which was expected to produce LD-MCA. Consequently, PeDPP11 was shown for the first time to exhibit peptidase activity, whereas hydrolysis was not detected when ac-DNLD-MCA was preincubated with GluV8, which did not produce dipeptidyl-MCA (Fig. 4*A*). Furthermore, by use of ac-VEID-MCA pre-treated with thermolysin or GluV8, both of which could produce ID-MCA, the activity was evidently demonstrated. Similar results were obtained with PgDPP11 (Fig. 4*A*). The P1-positon specificity of DPP11 was further examined using synthesized dipeptidyl substrates, i.e., LD-, ac-LD- and LE-MCA, and additional dipeptidyl-MCA substrates, such as ID-,

LE-, AN-, LQ-MCA, which were enzymatically prepared from ac-VEID-, Z-LLE-, Z-AAN-, Z-LLQ-MCA, respectively, and commercially available suc-AE-, KA- and GP-MCA. We found that both PeDPP11 and PgDPP11 most efficiently hydrolyzed LD-MCA, while ID- and LE-MCA did so moderately, whereas they did not hydrolyze ac-LD-, suc-AE-, AN-, LQ-, KA-, or GP-MCA (Fig. 4*B*). These findings demonstrate that DPP11 cleaves peptides with penultimate N-terminal Asp and Glu, while does not cleave those either with Asn, Gln, Ala and Pro at the position or N-terminal modification.

The enzymatic activity of DPP11 was also studied using a bombesin-related human neuropeptide, neuromedin B, and its derivatives, in which Asp residue was located at the first, second, third, or fifth position (D_1-, D_2-, D_3- or D_5-neuromedin B, respectively) from the N-terminus. D_2-neuromedin B was solely cleaved to the two peptides, NH_2-GD-COOH and NH_2-LWATGHFM-NH_2 (Table 2, data not shown). Moreover, DPP11 did not cleave at NH_2-GN-|-L, NH_2-DG-|-L, or NH_2-GS-|-D/L, nor at NH_2-LW-|-A. Therefore, our results explicitly show that DPP11 has genuine Asp and Glu-specific DPP activity.

We also tested the activity of fraction 52 from ion-exchange chromatography (see Fig. 3), and found that D_2-neuromedin B was completely cleaved at NH_2-GD-|-L and neuromedin B was partially hydrolyzed at NH_2-GN-|-L (Table 2). These results suggested that the hydrolysis of ac-DNLD-MCA by fraction 52 as well as the culture supernatant was probably achieved through a 2-step reaction mediated by a co-existing Asn-targeting peptidase and DPP11.

Next, we examined the P2-position preference of DPP11. The dipeptidyl MCA substrates ID-, VD-, and TD-, and HD-MCA were prepared by pre-treatment with GluV8 from ac-VEID-, ac-DEVD-, ac-IETD-, and ac-LEHD-/ac-WEHD-MCA, respectively. After pre-treatment, these substrates were hydrolyzed by DPP11 with the efficiency in order of ID->VD->TD->HD-MCA (Fig. 4*C*). Taken the data of Fig. 4*A* together, the order of the substrates suitable for DPP11 was aligned to LD->ID->VD->TD->HD-MCA, suggesting a hydrophobic residue preference at the P2 position.

Biochemical properties of DPP11. The optimum pH of both PeDPP11 and PgDPP11 was 7.0 (Fig. S3*A*). This pH was slightly lower than those of the other members, i.e., pH 7.5 for DPPIV, pH 6.5-9.0 for DPP7, and pH 7.0-8.0 for PTP-A from *P. gingivalis* (26-28). Inhibitor analysis demonstrated that the activity of PeDPP11 was completely blocked by diisopropyl fluorophosphates (DFP), moderately by phenylmethylsulfonyl fluoride (PMSF) and 4-(2-methyl)benzenesulfonyl fluoride (AEBSF), and slightly by pepstatin (data not shown), while PgDPP11 showed a similar tendency. All of the potent inhibitors were serine protease inhibitors, except for pepstatin, an aspartyl protease inhibitor. The substitution of Ser_{652} in PeDPP11 and Ser_{655} in PgDPP11 to Ala completely abolished their hydrolyzing activities toward LD- and LE-MCA (Fig. S3*B*). Therefore, $Ser_{652/655}$ is likely the essential amino acid forming the catalytic triad of the chymotrypsin-superfamily serine proteases, as proposed in Figure 2, and the target of DFP, PMSF, and AEBSF.

The activity was maximal without NaCl and consistently declined in a concentration dependent manner (Fig. S3*C*), which was in contrast to PgDPP7, which had a maximal activity at 0.5 M NaCl in 100 mM HEPES buffer (pH 7.0) (27). To confirm the salt effect, the activities hydrolyzing GP-, KA-, and LD-MCA, specific for DPPIV, DPP7, and DPP11, respectively, were compared with the cellular fraction of *P. gingivalis* (Fig. S3*C*). Activities specific for native DPPIV and DPP11

were maximal without NaCl, and then decreased in a concentration-dependent manner. In contrast, the activity of DPP7 was maximal at 0.1 M NaCl, confirming that NaCl at appropriate concentrations enhances the activity of DPP7.

Although we attempted to determine their enzymatic parameters, it was impossible to directly measure K_m and V_{max} for these MCA substrates, because the K_m values seemed much higher than the maximal concentration (20 μM) that could be employed in the present study. Hence, k_{cat}/K_m toward LD- and LE-MCA was determined by non-linear regression analysis for fitting the Michaelis-Menten equation using GraphPad Prism software. k_{cat}/K_m ($S^{-1}M^{-1}$) values of PeDPP11 for LD- and LE-MCA were 126,300±4,600 and 55,000±4,400 (mean ± SD, n=4), respectively, while those of PgDPP11 were 36,100±1,100 and 33,900±300, respectively. Therefore, we concluded that PeDPP11 had k_{cat}/K_m values higher than PgDPP11 for the 2 substrates and that LD-MCA was a more preferable substrate as compared to LE-MCA.

Localization of DPP11 and effect of gene disruption on P. gingivalis. A PgDPP11 gene (*PGN0607*) disruptant was prepared with *P. gingivalis* ATCC 33277 by homologous recombination, whereas the same attempt with *P. endodontalis* was not successful due to feebleness of the bacterium. Potent activities corresponding to Kgp and Rgps were observed in both cell-associated and extracellular forms in the disruptant as in the wild type (Fig. 5*A*). The hydrolyzing activities toward KA-MCA of DPP7 and GP-MCA of DPPIV, which were exclusively cell-associated, were also demonstrated in both the wild-type and disruptant strains. Similarly, the hydrolyzing activities toward ac-DNLD- and LE-MCA were exclusively cell-associated, but were abolished in the disruptant, suggesting that PgDPP11 was solely responsible for the hydrolysis in *P. gingivalis*. Furthermore, these results demonstrated that the DPP11-gene defect did not affect other major proteolytic activities. In addition, absence of a soluble form of PgDPP11 in *P. gingivalis* was distinguishable from its presence in *P. endodontalis*, thus the lack of hydrolyzing activity toward ac-DNLD, as shown in Figure S2, could be explained by PgDPP11 localization on the cells.

Differential localization of DPP11 between *P. gingivalis* and *P. endodontalis* was reproduced by immunoblotting (Fig. 5*B*). A 75-kDa species was observed in the soluble and cell-associated fractions from *P. endodontalis*, and a truncated 55-60-kDa species was increased in the extracellular fraction. In contrast, a relatively low amount of a 60-kDa species was detected in the cell-associated fraction of the *P. gingivalis* wild-type strain. In the DPP11 gene disruptant, this species disappeared. Exclusive cellular localization was not specific for PgDPP11, as it was also seen in DPPIV and DPP7 (Fig. 5). Hence, the lack of soluble forms of these DPPs may be caused by degradation with gingipains predominantly produced in *P. gingivalis*.

The growth of the DPP11 gene disruptant was retarded in ABCM medium containing 10 μg/ml of erythromycin (Fig. 5*C*). These results suggest that, though not essential, DPP11 is involved in nutrition metabolism. Growth retardation was also reported in a DPPIV, DPP7, and PTP-A triple disruptant (29). Incomplete arrest in growth of the DPP11 disruptant may be explained by compensation with the remaining DPPs and *vice versa*. In addition, black pigmentation on sheep blood agar was observed with both the wild-type and mutant strains (data not shown).

Complete loss of hydrolysis for ac-DNLD- and LE-MCA by the disruptant suggested an absence of Asp- and Glu-cleaving peptidases other than DPP11. In fact, none of the 8 acetylated tetrapeptidyl MCAs carrying Asp at the P1 position

were cleaved by the disruptant (Fig. 5*D*). Moreover, suc-AE-, Z-LLE- and LE-MCA were scarcely hydrolyzed by the disruptant (Fig. 5*E*). Taken together, we concluded that DPP11 accounted for all Asp-specific and most Glu-specific cleavages of environmental peptide substrates in *P. gingivalis.*

Arg_{670} of DPP11 as an essential residue. Since Arg_{670} (PeDPP11 numbering) is a unique amino acid residue that is conserved in all DPP11 orthologues and thoroughly converted to Gly in all DPP7 orthologues (details described in DISCUSSION section), we examined whether Arg_{670} is indispensable for Asp- and Glu-specific DPP11 activities by its substitutions. The LD-MCA-hydrolyzing activity became negligible in the mutants with the substitution from Arg_{670} to Gly, Lys, His, Gln or Asp, indicating that Arg_{670} was truly important for DPP11 activity. Moreover, when a 250-fold excess of mutant protein was subjected to the assay, qualitative alterations in the remaining activities among the substituted amino acids emerged (Fig. 6*A*). Among the mutants, the hydrolysis of LD-MCA was maximal with substitution by His, followed by Lys. On the other hand, that of LE-MCA was maximal with the substitution by Gly, followed by Lys, His, and Gln. Again, no hydrolysis was observed in the mutant substituted to Asp. Importantly, in contrast to the wild type of PeDPP11, the hydrolysis of LE-MCA exerted by the mutations was consistently higher than those of LD-MCA, while the ratio of hydrolysis of LD-MCA to that of LE-MCA was correlated to the size of the amino acid residue (Fig. 6*B*). It seems important to note that the van der Waals volume of Asp (91 $Å^3$) is smaller than that of Glu (109 $Å^3$), which reasonably explains why the size reduction from Arg_{670} (148 $Å^3$) to other amino acids (48-135 $Å^3$) more significantly affected the activity for LD-MCA than for LE-MCA.

Next we investigated whether the basic charge of Arg_{670} is indispensable for the activity. For this purpose, we determined the pH profile of the Arg670His mutant. If the positive charge is indispensable for the activity, the pH optimum of the mutant was expected to shift to acidic side. Truly, the Arg670His mutant possessed the pH optimum one unit lower than that of the wild type (Fig. 6*C*). Taken together, it is proposed that the guanidium group of Arg_{670} directly interacts with the carboxyl group of Asp or Glu in a peptide substrate. Along this line, complete loss of the activity to LD- and LE-MCA observed in the Arg670Asp species can be explained by the repulsion between Asp_{670} and Asp/Glu.

Cooperative actions of DPP11 and other peptidases on protein metabolism. The efficiency of peptide utilization of proteinous nutrients in the wild type and DPP11 disruptant was calculated under the following hypothetical culture conditions containing human albumin (pI=4.7) and human hemoglobin $\alpha 1_2\beta_2$ (pI=7.2) as the most probable substrates for *P. gingivalis*: (i) Rgps and Kgp cleave all Arg-X and Lys-X bonds, respectively, of protein substrates; (ii) subsequently, DPPIV and DPP7 liberate dipeptides with any residues, except for Asp and Glu at the second position, and Pro at the third position; (iii) when Pro is located at the third position from the N-terminus, PTP-A liberates tripeptides; (iv) DPP11 liberates dipeptides with Asp and Glu at the second position, except that Pro is located at the third position, because its incapability is reasonably postulated by analogy to the properties of DPPIV and DPP7; (v) the resultant di- and tri-peptides are incorporated into the bacterium, but free amino acids may not be; and (vi) the influence of the disulfide bond is not taken into consideration in this calculation. Consequently, the efficiencies of incorporation of human albumin and hemoglobin in the mutant strain were calculated to be 53.0% and 82.3%, respectively, of the wild type (100%). Therefore, we

concluded that DPP11 greatly enhances peptide utilization efficiency, especially for acidic proteins.

DISCUSSION

In this study, we identified a novel Asp and Glu-specific DPP, DPP11, from *P. gingivalis* and *P. endodontalis*. DPP11 possesses essential Ser_{652} and Arg_{670} for its activity, the former of which constitutes an active triad with His_{84} and Asp_{197} conserved in the serine protease superfamily. It is curious that, although DPP11 has a similar sequence around the essential Ser of GluV8 (Fig. 2), DPP11 is more than double the size of GluV8. Currently, we suspect that DPP11 as well as DPP7 possesses a region, which is associated with other DPPs and PTP-A for co-operative degradation.

PeDPP11 was observed in both cell-associated and soluble extracellular forms, of which the 75-kDa form starting from Asp_{22} was increased in the soluble extracellular fraction. In contrast, PgDPP11 was solely observed as cell-associated, the same as DPPIV and DPP7 (Figs. 5 and S2). The existence of DPP11 is compatible with both experimental observation utilizing tryptone-based medium (16) and the entire metabolic network model of *P. gingivalis* to achieve maximal growth (41), while Glu and Asp are most intensively consumed from nutritional peptides. These previous studies also indicated that production of butyrate and propionate, estimated end-products of Glu and Asp metabolism (16, 20, 41), could become secured under a condition that includes consumption of Glu and Asp in *P. gingivalis*. Therefore, DPP11 may be one of the pathogenic factors of *P. gingivalis*.

Up to date, DPPI-III (42-44), eukaryotic (45) and bacterial (26) DPPIV, DPPV (46), DPPVI (bacterium) (47, 48), DPP6 (eukaryote) (49), DPP7 (bacterial and eukaryote types) (27, 50), DPP8-10 (51-53), fibroblast activation protein α (FAPα) (54), and Dcp (EC 3.4.15.5) (55) have been reported to be DPP members (Table S2). Dcp (EC 3.4.15.5), which liberates dipeptides from the C-terminus of polypeptides, is localized in an intracellular compartment, thus it seems to be driven for the breakdown of intracellular proteins (54). Among DPPs excluding Dcp, eukaryotic DPP7 belongs to the DPPII family (50), and FAPα, DPP6 (eukaryote) and DPP8-10 belong to the DPPIV family in respect to substrate specificity and sequence homology (49, 51-53, 56). In summary, DPPI-DPPV are expressed in eukaryotic organisms, including fungi, mammals, fishes, and plants, while DPPIV, DPPVI, and DPP7 are known as bacterial enzymes. DPPVI is expressed in the genera *Bacillus* and *Oceanobacillus*, and has specificity for D-amino acid-containing peptide moieties of some peptidoglycans (47, 48). Thus, DPPIV, DPP7, and new member DPP11 are involved with dipeptide liberation from extracellular oligopeptides consisting of L-amino acids in bacteria.

DPP11 from *P. gingivalis* is classified into the S46.001 subfamily of the S46/DPP7 family in the MEROPS database (34). Based on sequence homology, S46.001 is divided into 5 major groups (71 members) and an additional 3 members (Fig. 7*A* and Table S3). The present findings suggest that Group 3 is genuine DPP7 and Group 4 is DPP11. In studies of a Group 4 species (MER217397) from *Porphyromonas uenonis*, a bacterium isolated from human urogenital or intestinal tracts (57, 58), the homology was found to be 53.5% to PgDPP11 and 39.7% to PgDPP7, indicating its attribution to DPP11. In addition to the genus *Porphyromonas*, Group 4/DPP11 consists of members from the genera *Bacteroides* and *Parabacteroides* (Table S3). Based on the present results, it is likely that the substrate specificities of the Group-1, -2, and -5 members are also different from that of DPP7. In particular, Group-5 members are distributed only in the genus *Bacteroides*, in which at least 9 species simultaneously carry Group-3 (DPP7) and Group-4

(DPP11) genes (Table S3). Hence, it is possible that Group-5 DPP possesses substrate specificity distinct from DPP7 and DPP11. To address these issues, the proteolytic activities of several members in the S46.001 subfamily are now being examined in our laboratory.

The amino acid residues that define the substrate specificity of DPP11 were considered likely to be altered from those of DPP7, but highly conserved within every group of the S46.001 subfamily. Hence, we selected 20 amino acid residues that are perfectly conserved within all members of either DPP7/Group 3 or DPP11/Group 4, and not simultaneously present in even one member of the other group (Fig. 7*B*). Among them, Arg_{670} was the sole residue that was found to be completely conserved in all Group-4 and Group-2 members, and was also shown to be completely converted to Gly_{670} in all Group-3 as well as Group-1 and -5 members. Indeed, Arg_{670} of PeDPP11 could not be substituted by other amino acids (Fig. 6). Furthermore, the present results suggest that the guanidium side chain of Arg_{670} directly interacts with the carboxyl group of target Asp and Glu of a substrate. In turn, Gly_{670} is absolutely located in all DPP7 family members, thus a tiny and hydrophobic side chain (hydrogen) of Gly may be indispensable for the acceptance of bulky aliphatic and aromatic residues of a substrate for DPP7. The enhancing effect of NaCl on the activity of DPP7 and its negative effect on DPP11 (Fig. S3*C* and *D*) were consistent with their substrate specificities, because ions generally strengthen hydrophobic interactions and weaken hydrophilic interactions.

Takahashi and Sato (59) reported on the metabolic efficiency of dipeptides in *P. gingivalis* and other periodontopathic bacteria, i.e., *Prevotella intermedia*, *Prevotella nigrescens*, and *Fusobacterium nucleatum*. Ammonia production from *P. gingivalis* was significantly increased in the presence of aspartylaspartate and glutamylglutamate, whereas that production was limited with $(Asp)_{4\text{-}100}$ and $(Glu)_{5\text{-}100}$. This observation is now reasonably explained by the P2-position preference of DPP11 to hydrophobic residues (Fig. 4*C*), as poly-glutamic and poly-aspartic peptides are not suitable substrates for DPP11. Their study also demonstrated that $(Glu)_4$ enhanced ammonia production in *P. nigrescens*, suggesting DPP11 expression in this bacterium, though the DPP11 gene has yet to be identified (Table S3). Therefore, we speculate that DPP11 is distributed in the genus *Prevotella*, as well as the genera *Porphyromonas*, *Bacteroides*, and *Parabacteroides*.

CONCLUSION

We cloned, identified, and characterized a novel type of DPP, designated as DPP11, from *P. endodontalis* and *P. gingivalis*, that specifically cleaves at the NH_2-(Y)-Asp/Glu-|-$(X)_n$ bond, in which hydrophobic residues are preferred at the Y position. The substrate specificity of DPP11 expands the repertoire of substrates covered by DPPIV, DPP7, and PTP-A, and provides advantage to these asaccharolytic oral pathogens for survival in an oligotrophic oral environment. *P. gingivalis* DPP11 is likely keenly involved in the metabolism of abundant acidic residues in proteins and production of end-products harmful to host tissues. Hence, DPP11 may become a therapeutic target of chronic periodontitis. Our findings also suggested the distribution of DPP11 in the genera *Bacteroides*, *Parabacteroides*, and *Prevotella*, and a further variation of substrate specificity in the S46.001 DPP members.

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FOOTNOTES

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The abbreviations used are: DPP, dipeptidylpeptidase; PeDPP and PgDPP, DPP from *Porphyromonas endodontalis* and *Porphyromonas gingivalis*, respectively; FAPα, fibroblast activation protein α; PTP-A, prolyl tripeptidylpeptidase-A; Dcp, peptidyl dipeptidase; Kgp, Lys-specific gingipain; Rgp, Arg-specific gingipain; GluV8, glutamyl endopeptidase from *Staphylococcus aureus*; boc, *t*-butyloxycarbonyl-[(2*S*)-2-amino-3-(benzyloxycarbonyl)propionyl]; Z-, benzyloxycarbonyl-; MCA, 4-methylcoumaryl-7-amide.

FIGURE LEGENDS

Fig. 1. Nucleotide and deduced amino acid sequences of PeDPP11. The DNA sequence of DPP11 from *P. endodontalis* and its deduced amino acid sequence are shown. Matching of the nucleotide sequence with those of the degenerate primers DPP7deg244-276 and DPP7deg676-643 (Table S1) is indicated by asterisks (completely matched) or dots (matched with one of the degenerate bases). The nucleotide sequences (8Fcomp127-102 and 8Fcomp326-350) used for genome walking are underlined. The N-terminal amino acid sequence determined with an immuno-purified 75-kDa protein is boxed (see Fig. 3*C*).

Fig. 2. Alignment of amino acid sequences of PeDPP11, PgDPP11, and PgDPP7. The amino acid sequence of PeDPP11 was compared with those of *PGN0607*/*PgDPP11* and *PGN1479*/*PgDPP7* from *P. gingivalis* and residues 232-250 of GluV8 carrying an essential Ser_{237} (60). Hyphens represent gaps introduced for maximal matching. Common amino acid residues are marked with asterisks, while those matched in 2 DPPs are indicated by dots. Amino acids corresponding to the regions used for amplification of PeDPP11 with degenerate primers are underlined. Amino acid residues to form the active triad of PeDPP11 predicted by comparison with those of *PGN0607* and *PgDPP7* are indicated by arrows.

Fig. 3. Detection and identification of native PeDPP11 separated by DEAE-Sephacel anion-exchange chromatography. (A) A soluble extracellular fraction from *P. endodontalis* was separated using DEAE-Sephacel chromatography with a 0-0.6 M linear gradient of NaCl in buffer A, as described in EXPERIMENTAL PROCEDURES. Hydrolysis of KA- (open circle), suc-AAA- (closed circle), and ac-DNLD-MCA (square) of even fractions (10 μl) was measured. (B) Proteins at fractions 46-80 (even numbers) were separated on SDS-PAGE, then subjected to immunoblotting with anti-PeDPP11 serum (10^5-diluted). EF, extracellular fraction (10 μl). M, rainbow marker. (C) Fractions 51-53 and 70-72 (3 ml each) were immunoadsorbed to the PeDPP11 antibody resin, as described in EXPERIMENTAL PROCEDURES. Proteins bound to the resin were extracted with SDS-sample buffer, separated on SDS-PAGE, and subjected to N-terminal sequencing. LC and HC, light and heavy chains, respectively, of immunoglobulins. (D) Fraction 52 or 56 (100 μl) of DEAE-chromatography (panel A) was incubated with 15, 45, or 135 μl of a 50% suspension of a PeDPP11-antibody resin. After rotation at 0˚C for 8 h, samples were centrifuged and the remaining activity in the supernatant was determined with ac-DNLD-MCA for the sample from fraction 52 (open circle) and suc-AAA-MCA from fraction 56 (closed circle). Values are expressed as percent (mean ± SD, n=3) of the control incubated with Sepharose 4B.

Fig. 4. Determination of P1 specificity and P2 preference of DPP11. (A) Ac-DNLD- and ac-VEID-MCA were pre-incubated without (-) or with thermolysin (Th) and GluV8 (V8), as described in EXPERIMENTAL PROCEDURES. The hydrolyzing activities of recombinant PeDPP11 (open column) and PgDPP11 (closed column) were determined with the pre-treated substrates. (B) The activities of PeDPP11 (open column) and PgDPP11 (closed column) hydrolyzing peptidyl MCA substrates were determined. Asterisks indicate enzymatically prepared materials. (C) The activities of PeDPP11 (open column) and PgDPP11 (closed

column) were determined with acetyl tetrapeptidyl MCAs pre-incubated with GluV8. All values are shown as the mean ± SD (n=3).

Fig. 5. Localization of DPP11 and effect of DPP11 gene disruption in *P. gingivalis*. (A) The hydrolyzing activities toward MCA-peptides were determined using aliquots (10 μl) of cellular (open column) and extracellular (dotted column) fractions of *P. gingivalis* wild type, and cellular (hatched column) and extracellular (closed column) fractions of a DPP11 disrupted mutant. (B) Aliquots (10 μl) of the cellular factions (cell) and culture supernatants (sup) of *P. gingivalis* wild type (lane 1), disruptant (lane 2), and *P. endodontalis* (lane 3) were separated on SDS-PAGE, and subjected to immunoblotting with anti-PeDPP11 (10^5-diluted) or anti-PgDPP11 (10^4-diluted) serum. (C) *P. gingivalis* wild type (open circle) and the disruptant (closed circle) were cultured in ABCM broth supplemented with 1 μg/ml of menadione under an anaerobic condition. Cell growth was monitored by measuring the absorbance at 600 nm. A representative result of three separate experiments in triplicates is presented. (D and E) The hydrolyzing activities of the cellular fraction (10 μl) from *P. gingivalis* wild type (open column) and the disruptant (closed column) were determined with MCA-peptides carrying (D) Asp or (E) Glu at the P1 position. All values are shown as the mean ± SD (n=3).

Fig. 6. Essential role of Arg_{670} in hydrolyzing activity of DPP11. (A) The hydrolyzing activities of wild-type PeDPP11 (2 ng) and with substitution of Gly, Lys, His, Gln, or Asp (0.5 μg) were measured with LD-MCA (open column) or LE-MCA (closed column). (B) The ratio of hydrolysis of LD-MCA to that of LE-MCA was plotted against the van der Waals volumes of the amino acids at position 670. Data for the Asp substitution were not plotted, as there were no activities for either substrate. (C) The hydrolyzing activities of wild-type PeDPP11 (open symbol) and Arg670His (closed symbol) were determined with LE-MCA in 50 mM sodium acetate (circle), sodium phosphate (square), and Tris-HCl (triangle).

Fig. 7. Group-specific amino acid residues in S46.001-subfamily members. (A) Phylogenetic tree of the S46.001 subfamily of S46/DPP7 family. Numbers in parentheses indicate number of members (Table S3). (B) Twenty amino acids of PeDPP11 completely conserved in Group-3 members and simultaneously not present in any member of Group 4 and *vice versa* were compared among Group 1-5 members. Amino acids unique to each group are shown in capital letters, while others are shown together in each box with small letters. Note that the amino acid at position 670 is completely conserved among all members of Groups 1, 3, and 5 as Gly, and Groups 2 and 4 as Arg.

TABLE 1

Amino acid sequence identity among DPPIV, DPP7, and DPP11 of *P. gingivalis* and *P. endodontalis*

[a]DDBJ (this study). [b]Ref. 21. [c]MEROPS ID. [d]Protein ID.

DPPs	PeDPP11	PgDPP11	PgDPP7	PgDPPIV	PeDPPIV
P. endodontalis DPP11 (AB610284[a])	100%	57.9%	38.3%	13.8%	12.4%
P. gingivalis DPP11 (*PGN0607*[b]/MER034628[c])		100%	38.7%	11.1%	11.4%
P. gingivalis DPP7 (*PGN1479*[b]/MER014366[c])			100%	11.6%	12.6%
P. gingivalis DPPIV (*PGN1469*[b])				100%	55.1%
P. endodontalis DPPIV (MER192286[c]/ZP04390817[d])					100%

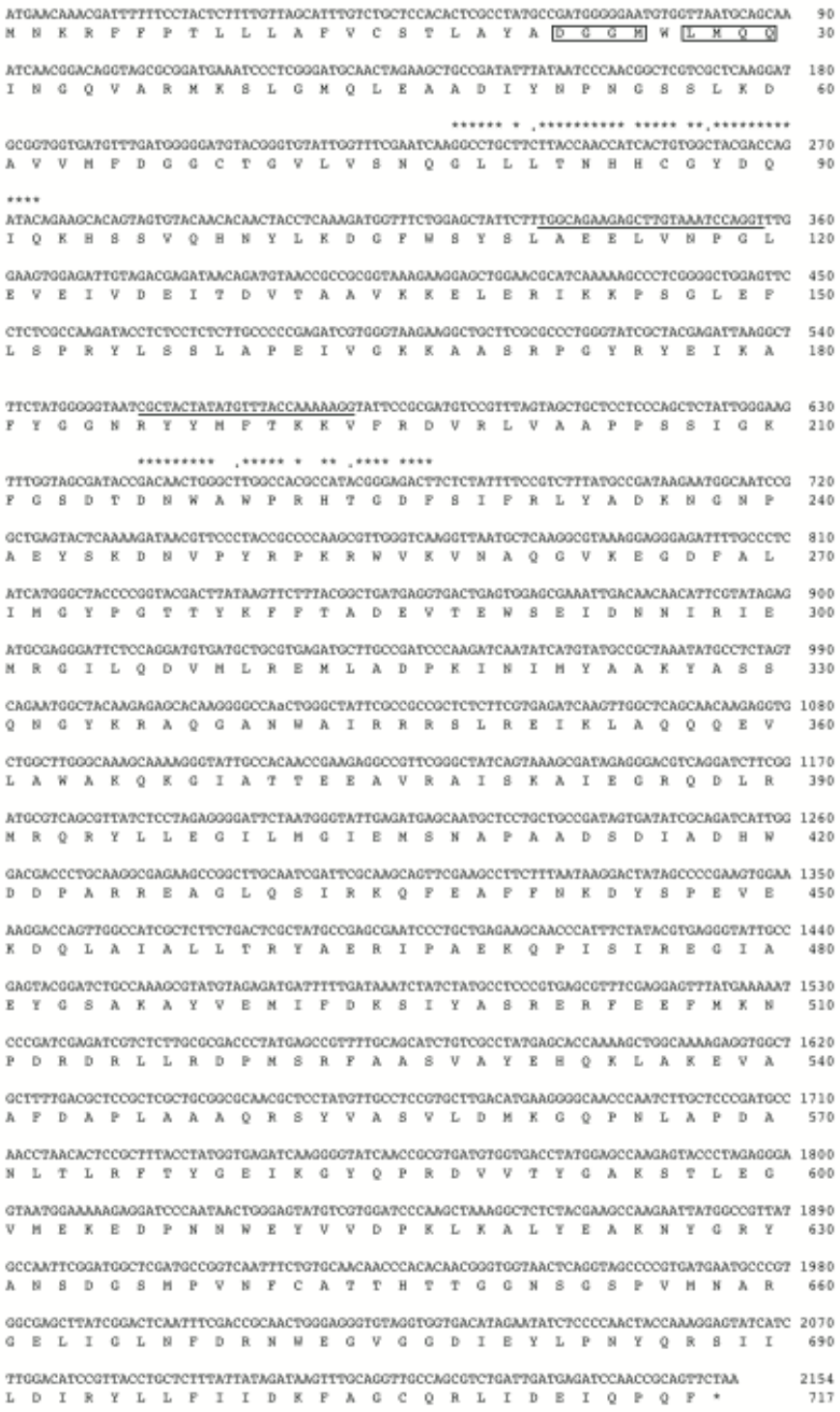
TABLE 2

Peptidase activities of recombinant PeDPP11 and fraction 52 from DEAE-Sephacel chromatography

Neuromedin B and its derivatives were separately incubated with recombinant PeDPP11 or fraction 52 for 1 h at 30˚C. Amounts of the peptide products were semi-quantitatively analyzed by MALDI-TOF MS. Substituted amino acid residues from neuromedin B are underlined. [a]Expressed as percent of fragment products losing N-terminal moiety. [b]Fraction 52 from DEAE-Sephacel chromatography (Fig. 3*A*).

Substrate	Sequence	Specimen	Degradation[a] (%)
neuromedin B	GNLWATGHFM-NH_2	PeDPP11	0
D_1-neuromedin B	DGLWATGHFM-NH_2	PeDPP11	0
D_2-neuromedin B	GDLWATGHFM-NH_2	PeDPP11	100
D_3-neuromedin B	GSDWATGHFM-NH_2	PeDPP11	0
D_5-neuromedin B	GSLWDTGHFM-NH_2	PeDPP11	0
neuromedin B	GNLWATGHFM-NH_2	fraction 52[b]	5
D_2-neuromedin B	GDLWATGHFM-NH_2	fraction 52[b]	100

Figure 1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ATCAACGGACAGGTAGCGCGGATGAAATCCCTCGGGATGCAACTAGAAGCTGCCGATATTTATAATCCCAACGGCTCGTCGCTCAAGGAT 180
I N G Q V A R M K S L G M Q L E A A D I Y N P N G S S L K D 60
****** * .********** ***** **.*********
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A V V M F D G G C T G V L V S N Q G L L L T N H H C G Y D Q 90
****
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E V E I V D E I T D V T A A V K K E L E R I K K P S G L E F 150
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L S P R Y L S S L A P E I V G K K A A S R P G Y R Y E I K A 180
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F Y G G N R Y Y M F T K K V F R D V R L V A A P P S S I G K 210
********* .***** * ** .**** ****
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A E Y S K D N V P Y R P K R W V K V N A Q G V K E G D F A L 270
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D D P A R R E A G L Q S I R K Q F E A F F N K D Y S P E V E 450
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E Y G S A K A Y V E M I F D K S I Y A S R E R F E E F M K N 510
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V M E K E D P N N W E Y V V D P K L K A L Y E A K N Y G R Y 630
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L D I R Y L L F I I D K F A G C Q R L I D E I Q P Q F * 717

Figure 2


PeDPP11
PgDPP11
PgDPP7
GluV8
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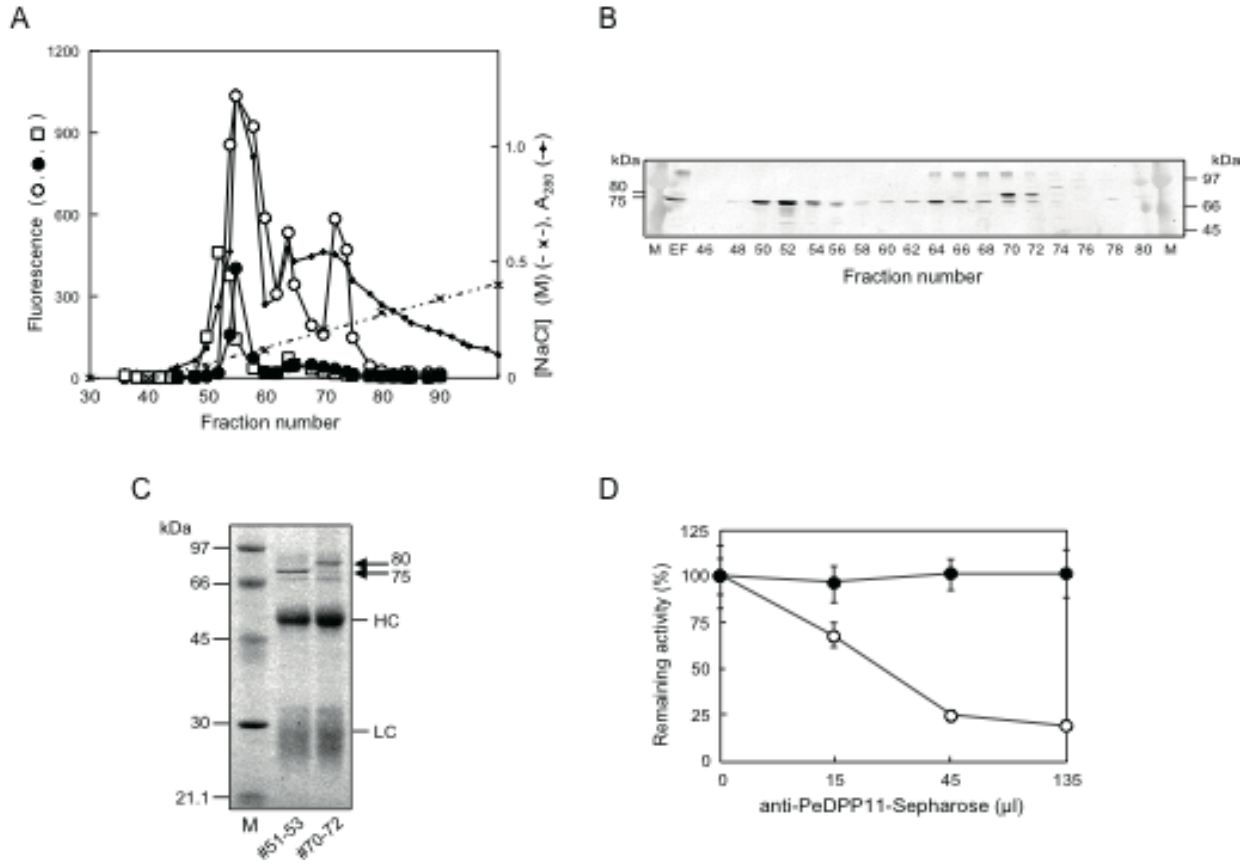
Figure 3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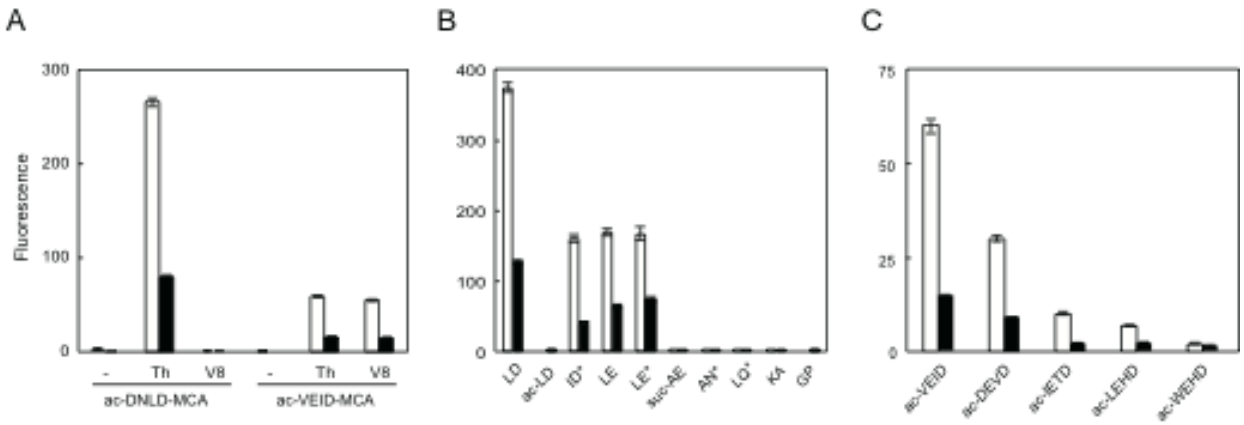
Fluorescence (○, ●, □)
[NaCl] (M) (- ×-), A_{280} (—)
Fraction number
B
kDa
80
75
97
66
45
M EF 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 M
Fraction number
C
kDa
97
66
45
30
21.1
80
75
HC
LC
M
#51-53
#70-72
D
Remaining activity (%)
anti-PeDPP11-Sepharose (μl)

Figure 4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B
C
Fluorescence
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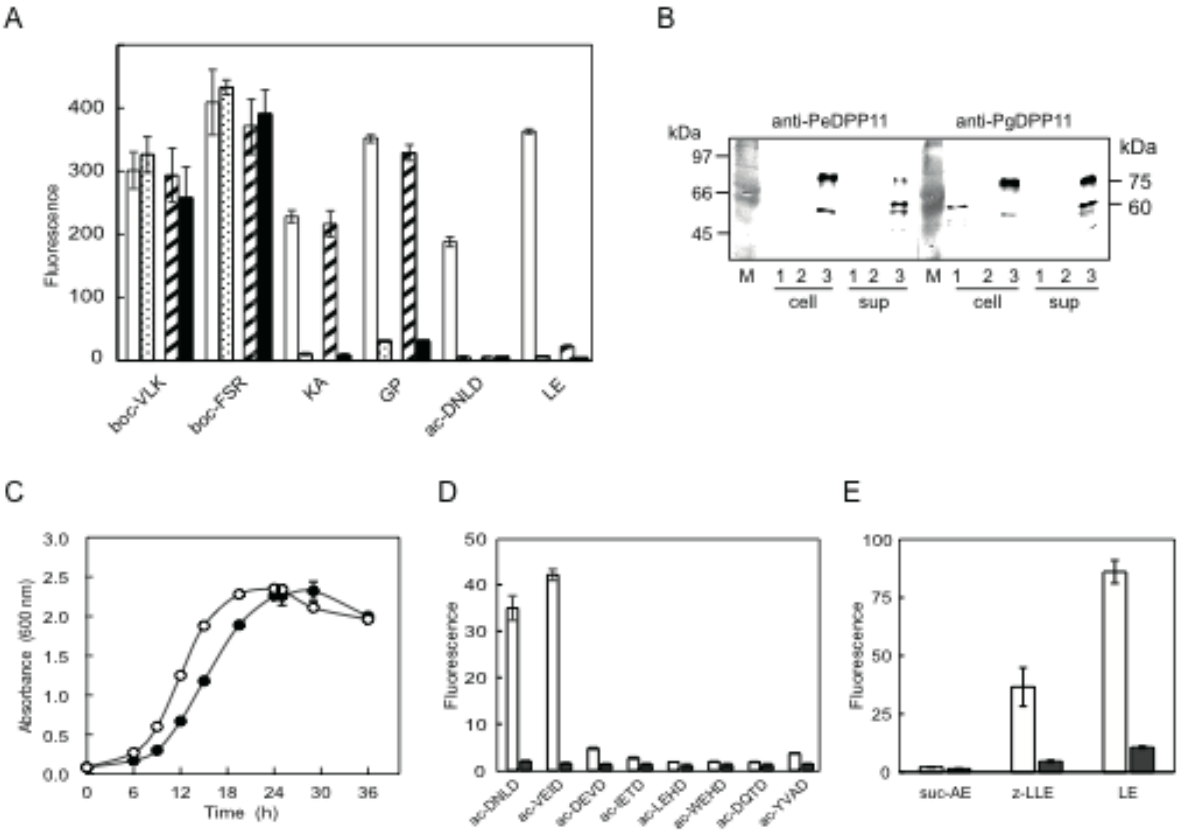
200
100
0
- Th V8
ac-DNLD-MCA
- Th V8
ac-VEID-MCA
400
300
200
100
0
LD
ac-LD
fD*
LE
LE*
suc-AE
AN*
LG*
KA
GP
75
50
25
0
ac-VEID
ac-DEVD
ac-IETD
ac-LEHD
ac-WEHD

Figure 5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Fluorescence
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300
200
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0
boc-VLK
boc-FSR
KA
GP
ac-DNLD
LE
B
kDa
anti-PeDPP11
anti-PgDPP11
kDa
97
66
45
75
60
M 1 2 3 1 2 3 M 1 2 3 1 2 3
cell
sup
cell
sup
C
Absorbance (600 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 6 12 18 24 30 36
Time (h)
D
Fluorescence
50
40
30
20
10
0
ac-DNLD
ac-VEID
ac-DEVD
ac-IETD
ac-LEHD
ac-WEHD
ac-DQTD
ac-YVAD
E
Fluorescence
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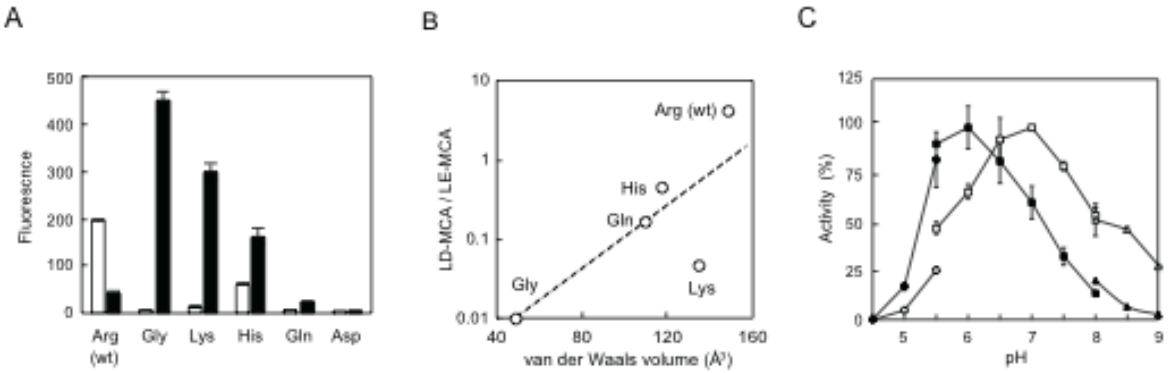
75
50
25
0
suc-AE
z-LLE
LE

Figure 6


A
B
C
Fluorescence
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400
300
200
100
0
Arg (wt)
Gly
Lys
His
Gln
Asp
LD-MCA / LE-MCA
10
1
0.1
0.01
Arg (wt)
His
Gln
Gly
Lys
40
80
120
160
van der Waals volume (Å³)
Activity (%)
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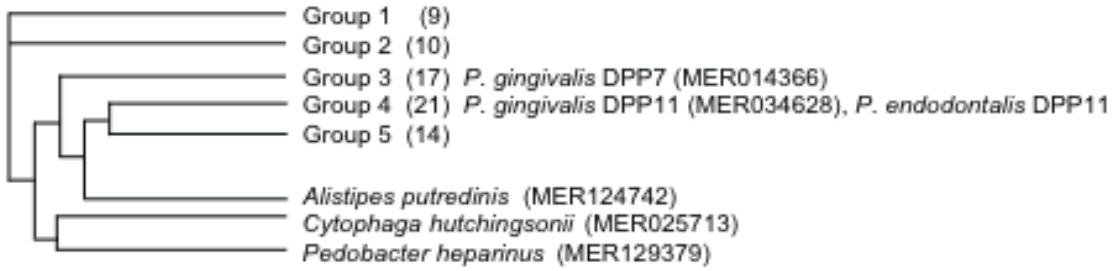
100
75
50
25
0
5
6
7
8
9
pH

Figure 7

A



B

Residue No.	107	126	192	202	282	286	305	383	407	511	521	549	597	602	606	622	645	647	670	674
Group 1	W	V	Y	G	mevl	gw	ags	yf	tkpva	pqr	lir	asnl	flmi	vilm	K	ynv	N	I	G	A
Group 2	W	vaik	tyvi	ga	li	A	avri	Y	lraim	aeqks	gaik	L	lv	mil	vni	yiqh	nc	T	R	gs
Group 3	vft	lirkv	Y	F	L	G	K	Y	E	dkq	ag	gndqe	tvaedgg	flv	knr	lfim	N	I	G	as
Group 4	W	D	K	ga	myf	eg	rl	vi	msytafl	P	M	A	L	M	D	Y	T	nt	R	G
Group 5	W	edh	kil	A	L	E	R	vi	gst	P	ams	L	tl	lm	nd	iy	N	I	G	S

SUPPORTING INFORMATION

ASP- AND GLU-SPECIFIC NOVEL DIPEPTIDYL PEPTIDASE 11 OF *Porphyromonas gingivalis* THAT ENSURES UTILIZATION OF PROTEINEOUS ENERGY SOURCES

Yuko Ohara-Nemoto, Yu Shimoyama, Shigenobu Kimura, Asako Kon, Hiroshi Haraga,
Toshio Ono, and Takayuki K. Nemoto

Contents: Tables S1-S3 and Figures S1-S3

TABLE S1

Primers used in this study

Restriction sites are underlined. Substituted bases are written in italic letters.

Primer	Usage	Sequence
DPP7deg244-276	Degenerate PCR	5'-GGCCTG(A/G)TC(G/A/T/C)TTACCAACCACCACTGCG(G/C)ATAC
DPP7deg676-643	Degenerate PCR	5'-AGTCGCCCG(A/T)GTGACGCGGCCA(G/A/T/C)ATCCAGTTGTC
8Fcomo127-102	Genome walking (upstream)	5'-ACCTGGATTTACAAGCTCTTCTGCCA
8Fcomp326-350	Genome walking (downstream)	5'-CGCTACTATATGTTTACCAAAAAGG
5PeDPP11D22	Expression	5'-TCGGGATCCCCGATGGGGGAATGTGG
3PeDPP11BglF717	Expression	5'-ATTAGATCTGAACTGCGGTTGGATCTCATCAA
5PGN0607D22Bam	Expression and gene disruption	5'-GCTGGATCCGACGAGGGTATGTGGCTGATGC
3PGN0607-P720Bam	Expression and Gene disruption	5'-ATAGGATCCGGGAACGATATTCATTTCATCCAAC
5ErmF-AM-Cla	Gene disruption	5'-GGTACCATCGATAGCTTCCGCTATTG
3ErmF-AM-Cla	Gene disruption	5'-GCAGATCGATTCTAGAGGATCCCCGA
5PeDPP11-S652A	S652A mutation	5'-*GCG*GGTAGCCCCGTGATGAATGCCCG
3PeDPP11-N651	(Mutation)	5'-GTTACCACCCGTTGTGTGGGTTG
5PgDPP11-S655A	S655A mutation	5'-*GCG*GGCAGTCCGGTCATGAATGCCAA
3PgDPP11-N654	(Mutation)	5'-GTTGCCGCCGGTTGTATGTGTGG
5PeDPP11-R670G	R670G mutation	5'-*G*GCAACTGGGAGGGTGTAGGTGG
5PeDP11-R670K	R670K mutation	5'-A*AA*AACTGGGAGGGTGTAGGTGGT
5PeDP11-R670H	R670H mutation	5'-C*A*CAACTGGGAGGGTGTAGGTG
5PeDPP11-R670D	R670D mutation	5'-*GA*CAACTGGGAGGGTGTAGGTGGT
5PeDPP11-R670Q	R670Q mutation	5'-CAGAACTGGGAGGGTGTAGGTG
3PeDPP11-D669	(Mutation)	5'-GTCGAAATTGAGTCCGATAAGCT

TABLE S2

Classification of DPP-family members

X, any amino acid. Y, aliphatic or aromatic amino acid. Z, Hydrophobic amino acids are preferable. [a]No DPP activity.

Name	Type	Specificity	Distribution	Isoform	Reference(s)
DPPI (EC 3.4.14.1)	Cysteine	NH_2-FR-|- NH_2-GF-|- NH_2-AA-|-	Animalia		42
DPPII (EC 3.4.14.2)	Serine	NH_2-XP-|- NH_2-XA-|- NH_2-XM-|-	Mammalia	Human DPP7	43, 50
DPPIII (EC 3.4.14.4)	Metallo-	NH_2-RR-|- NH_2-AR-|-	Eukaryota		44
DPPIV (eukaryote) /CD26 (EC 3.4.14.5)	Serine	NH_2-XP-|- NH_2-XA-|-	Chordata	 FAPα/seprase DPP6/DPPX[a] DPP8/DPRP1 DPP9/DPRP2 DPP10/DPRP3[a]	45 54 49, 53 51 52 53, 56
DPPIV (bacterium)	Serine	NH_2-XP-|-	Bacteriodales		26
DPPV	Serine	NH_2-AA-|- NH_2-KA-|- NH_2-HS-|- NH_2-SY-|-	Eurotiomycetes		46
DPPVI	Cysteine	NH_2-L-A-γ-D-E-|-	Bacillales		47, 48
DPP7	Serine	NH_2-XY-|-	Eubacteria		27
DPP11	Serine	NH_2-ZD-|- NH_2-ZE-|-	Eubacteria		This study

TABLE S3

Grouping of S46.001-subfamily members

S46.001 is divided into 5 major groups (71 members) and an additional 3 members in the MEROPS database. Group 3 is considered to be DPP7 and Group 4 DPP11. (-) Not reported. [a]This study (DDBJ ID). [b]Deduced only with a partial sequence (61 residues). MEROPS ID is described in a column, except for PeDPP11.

No.	Group / Species	1	2	3 (DPP7)	4 (DPP11)	5	Other
1	*Alistipes putredinis*	-	MER139860	-	-	-	MER124742
2	*Alistipes shahii*	-	MER229962	-	-	-	-
3	*Bacteroides caccae*	-	-	MER109105	MER109665	MER109454	-
4	*Bacteroides cellulosilyticus*	-	-	-	MER217673	MER217254	-
5	*Bacteroides coprocola*	-	-	-	MER136307	MER144886	-
6	*Bacteroides coprophilus*	-	-	-	MER176769	MER217170	-
7	*Bacteroides dorei*	-	-	MER180211	MER217657	MER180464	-
8	*Bacteroides eggerthii*	-	-	MER217344	MER217594	-	-
9	*Bacteroides finegoldii*	-	-	-	MER171742	MER163087	-
10	*Bacteroides fragilis*	-	-	MER039992	MER039991	MER039993	-
11	*Bacteroides interstinalis*	-	-	MER180324	MER136394	MER180879	-
12	*Bacteroides ovatus*	-	-	MER109141	MER109366	MER109242	-
13	*Bacteroides plebeius*	-	-	-	MER163085	MER136226	-
14	*Bacteroides stercoris*	-	-	MER118379	MER118465	MER124817	-
15	*Bacteroides thetaiotaomicron*	-	-	MER028076	MER028074	MER028075	-
16	*Bacteroides uniformis*	-	-	MER109068	MER109401	MER109273	-
17	*Bacteroides vulgatus*	-	-	MER061297	MER061213	MER061211	-
18	*Capnocytophaga gingivalis*	MER217135	MER217541	-	-	-	-
19	*Capnocytophaga ochracea*	MER064694	MER065040	-	-	-	-

20	*Capnocytophaga sputigena*	MER 172696	MER217591	-	-	-	-
21	*Chryseobacterium gleum*	-	MER217666	-	-	-	-
22	*Cytophaga hutchinsonii*	-	-	-	-	-	MER025713
23	*Flavobacteria bacterium BAL38*	MER109689	MER109425	-	-	-	-
24	*Flavobacteriaceae bacterium 3519-10*	MER217568	MER217518	-	-	-	-
25	*Flavobacteriales bacterium ALC-1*	-	MER118534	-	-	-	-
26	*Flavobacterium johnsoniae*	MER043795	-	-	-	-	-
27	*Flavobacterium psychrophilum*	MER109641	MER109496	-	-	-	-
28	*Kordia algicida*	MER118610	-	-	-	-	-
29	*Parabacteroides distasonis*	-	-	MER095654	MER095694	-	-
30	*Parabacteroides johnsonii*	-	-	MER217453	MER217400	-	-
31	*Parabacteroides merdae*	-	-	MER108962	MER109716	-	-
32	*Pedobacter heparinus*	-	-	-	-	-	MER129379
33	*Porphyromonas endodontalis*	-	-	-	AB610284[a]	-	-
34	*Porphyromonas gingivalis*	-	-	MER014366	MER034628	-	-
35	*Porphyromonas uenonis*	-	-	-	MER 217397	-	-
36	*Prevotella copri*	-	-	MER179133	-	-	-
37	*Prevotella melaninogenica*	-	-	MER221301	-	-	-
38	*Prevotella ruminicola*	-	-	MER203644	-	-	-
39	*Sphingobacterium spiritivorum*	MER217313	-	-	-	-	-
40	*Xanthomonas axonopodis*	-	-	MER026363[b]	-	-	-

Supplemental Figures

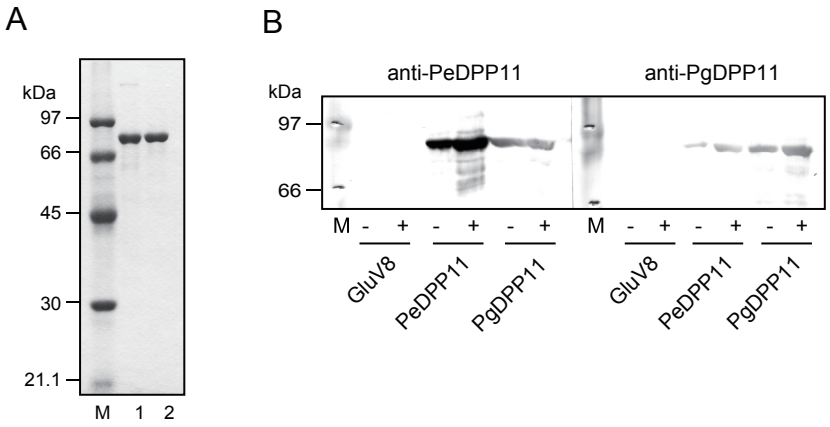


Fig. S1. *E. coli* expression of DPP11 and immunological characterization. (A) Recombinant PeDPP11 (Asp_{22}-Phe_{717}) and PgDPP11 (Asp_{22}-Pro_{720}) were expressed in *E. coli*. Purified PeDPP11 (lane 1) and PgDPP11 (lane 2) (0.8 μg) were separated on SDS-PAGE. (B) Lysates of *E. coli* harboring the GluV8, PeDPP11, or PgDPP11 expression plasmid were prepared in non-inducing (-) and inducing (+) conditions. An aliquot (5 μl) was subjected to immunoblotting with anti-PeDPP11 or anti-PgDPP11 serum (10^6-diluted). M, low-molecular-weight (A) or rainbow (B) marker.

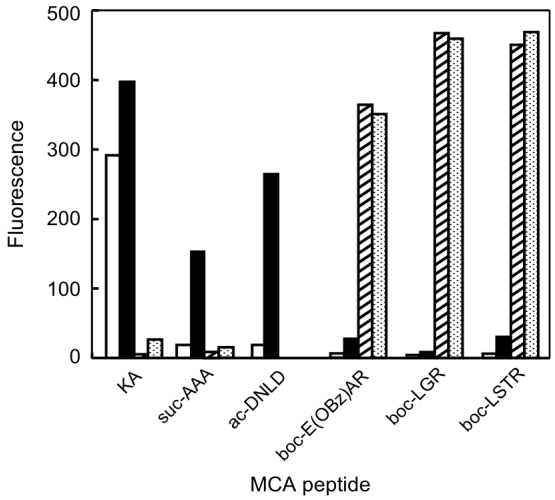


Fig. S2. Comparison of proteolytic activities in extracellular fractions between *P. endodontalis* and *P. gingivalis*. Proteolytic activities in the culture supernatants of *P. endodontalis* and *P. gingivalis* were determined with MCA-peptides at 30˚C for 2 or 24 h. Fluorescence intensity is shown as the mean of 2 samples of *P. endodontalis* after 2 (open column) and 24 h (closed column), and of *P. gingivalis* after 2 (hatched column) and 24 h (dotted column).

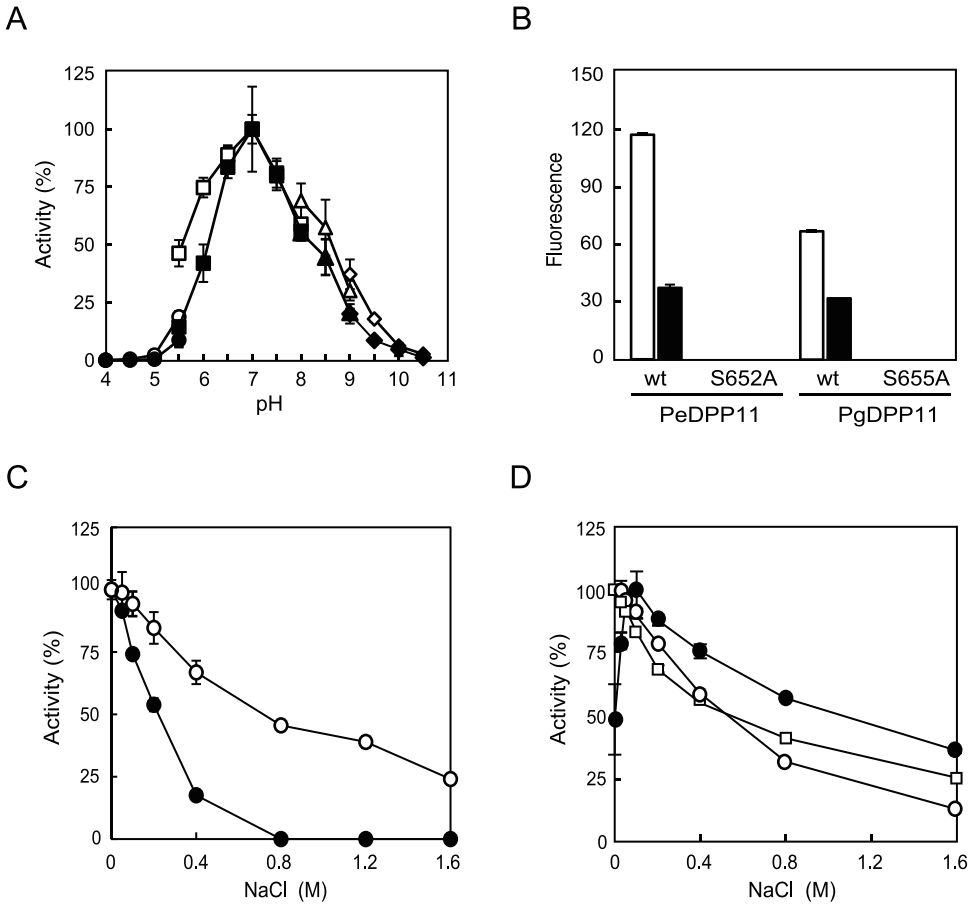


Fig. S3. Biochemical properties of DPP11. (A) The hydrolyzing activities of recombinant PeDPP11 (open symbols) and PgDPP11 (closed symbols) were determined with LD-MCA in 50 mM sodium acetate (circle), sodium phosphate (square), Tris-HCl (triangle), and glycine-NaOH (diamond). (B) The hydrolyzing activities of the wild type (wt) of PeDPP11 and PgDPP11 and their S652A and S655A mutants were determined with LD- (open column) and LE-MCA (closed column). (C) The activities of recombinant PeDPP11 (open circle) and PgDPP11 for LD-MCA were determined with 0-1.6 M NaCl. (D) The hydrolyzing activities of washed cell suspensions of *P. gingivalis* were determined with LD-MCA (open circle), KA-MCA (closed circle), and GP-MCA (square). All values are shown as the mean ± SD (n=3).