Review Article

Exopeptidases and gingipains in *Porphyromonas gingivalis* as prerequisites for its amino acid metabolism

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**Summary** *Porphyromonas gingivalis*, an asaccharolytic bacterium, utilizes amino acids as energy and carbon sources. Since amino acids are incorporated into the bacterial cells mainly as di- and tri-peptides, exopeptidases including dipeptidyl-peptidase (DPP) and tripeptidyl-peptidase are considered to be prerequisite components for their metabolism. We recently discovered DPP11, DPP5, and acylpeptidyl oligopeptidase in addition to previously reported DPP4, DPP7, and prolyl tripeptidyl peptidase A. DPP11 is a novel enzyme specific for acidic P1 residues (Asp and Glu) and distributed ubiquitously in eubacteria, while DPP5 is preferential for the hydrophobic P1 residue and the first entity identified in prokaryotes. Recently, acylpeptidyl oligopeptidase with a preference for hydrophobic P1 residues was found to release N-terminally blocked di- and tri-peptides. Furthermore, we also demonstrated that gingipains R and K contribute to P1-basic dipeptide production. These observations implicate that most, if not all, combinations of di- and tri-peptides are produced from extracellular oligopeptides even with an N-terminal modification. Here, we review *P. gingivalis* exopeptidases mainly in regard to their enzymatic characteristics. These exopeptidases with various substrate specificities benefit *P. gingivalis* for obtaining energy and carbon sources from the nutritionally limited subgingival environment.

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**Abbreviations:** DPP, dipeptidyl peptidase; PtpA, prolyl tripeptidyl peptidase A; AOP, acylpeptidyl oligopeptidase; Rgp, gingipain R; Kgp, gingipain K; MCA, 4-methycoumaryl-7-amide; Z, benzoyloxycarbonyl.

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Exopeptidases in Porphyromonas gingivalis for amino acid metabolism

Contents

1. Introduction ........................................................................................................... 23
2. Peptidases involved in degradation of extracellular proteinaceous nutrients .......... 23
   2.1. Gingipains ....................................................................................................... 23
       2.1.1. Rgp ........................................................................................................ 24
       2.1.2. Kgp ........................................................................................................ 24
   2.2. Exopeptidases ................................................................................................. 24
       2.2.1. DPP4 ..................................................................................................... 24
       2.2.2. DPP5 ..................................................................................................... 25
       2.2.3. DPP7 ..................................................................................................... 25
       2.2.4. DPP11 ................................................................................................... 25
       2.2.5. Prolyl tripeptidyl peptidase A (PtpA) .................................................. 26
       2.2.6. Acylpeptidyl oligopeptidase (AOP) ....................................................... 26
   2.3. Absence of exopeptidases with preference to hydrophilic residues ................. 26
3. Subcellular localization and comparison of enzymatic properties among exopeptidases ........................................................................................................... 26
4. Exopeptidases as potential virulence factors of P. gingivalis ................................. 26
5. Conclusion ............................................................................................................. 27
   Additional note ....................................................................................................... 27
   Conflict of interest ............................................................................................... 27
   Acknowledgements .............................................................................................. 27
   References ............................................................................................................ 27

1. Introduction

Porphyromonas gingivalis, a Gram-negative black-pigmented anaerobe, is a major causative agent of chronic periodontitis [1], which leads to permanent tooth loss [2]. Recently, much attention has been paid to this bacterium and other periodontopathic ‘red complex species’ (Tannerella forsythensis, Treponema denticola) [3], because of their close relationships to systemic diseases, such as atherosclerotic cardiovascular disorder [4—6], decreased kidney function [7], and rheumatoid arthritis [8].

A common feature among these bacteria is that they do not ferment glucose or sucrose (asaccharolytic), but rather utilize amino acids as energy and carbon sources [9—11]. In P. gingivalis, nutritional extracellular proteins are initially degraded to oligopeptides by potent cysteine endopeptidases, i.e., gingipains R (Rgp) and K (Kgp) [12—14], then oligopeptides are degraded to di- and tri-peptides, the main incorporated forms in P. gingivalis [15,16]. As for the amino acid transport system, an analysis of the P. gingivalis genome indicated the existence of two types of oligopeptide transporters [10], which are considered to mediate di- and tri-peptide incorporation. In addition, a sodium ion-driven serine/threonine transporter with a sequence similar to that of the Escherichia coli serine transporter has been reported [17]. In this context, exopeptidases consisting of dipeptidyl peptidases (DPPs), tripeptidyl peptidase, and acylpeptidyl oligopeptidase (AOP) producing di- and tri-peptides from oligopeptides are viewed as important for P. gingivalis to acquire proteinaceous nutrition from the mixed-species environment of the subgingival sulcus.

Initially, DPP4, DPP7, and prolyl tripeptidyl peptidase A (PtpA) were the only exopeptidases identified in P. gingivalis. These share substrates according to their altered specificities, as DPP4 is highly specific for Pro at the penultimate position from the N-terminus (P1 position), though it accepts Ala to a lesser extent [18,19], DPP7 is preferential to P1 hydrophobic amino acids [20], and PtpA liberates tripeptides with P1-position Pro, an activity that is able to compensate DPP4 and DPP7, which are unable to accept oligopeptides with Pro at the third position [21]. Although these three exopeptidases could not sufficiently explain the entire metabolism of extracellular oligopeptides, no other members were added to the list of P. gingivalis exopeptidases for a period of 10 years. Even though Asp and Glu are located in central routes of metabolism in P. gingivalis [10,15,22] (Fig. 1), oligopeptides with acidic amino acid residues do not seem to be efficiently produced. Moreover, none of DPPs [19,21,23] and PtpA [20] are incapable of utilizing N-terminally blocked polypeptides, such as the various serum proteins present in gingival crevicular fluid.

The majority of these disadvantages have been overcome by recent discoveries such as a novel Asp/Glu-specific DPP, DPP11 [23], DPP5, which is specific for hydrophobic P1 amino acid [24], and AOP in P. gingivalis [25]. Furthermore, we demonstrated that gingipains are able to produce dipeptides from oligopeptides [24]. These peptidases with various substrate specificities benefit P. gingivalis for colonization in nutrition-limited subgingival environments.

2. Peptidases involved in degradation of extracellular proteinaceous nutrients

2.1. Gingipains

P. gingivalis produces substantial quantities of cysteine endopeptidases that cleave peptide substrates with basic residues at the P1 position [12—14]. These peptidases, termed Rgp and Kgp, have been shown to be major virulence factors of the species. Gingipains are able to degrade many human proteins including complement system proteins, cytokines, and integrin. Their potent activities are implicated in most phases of the pathogenesis of periodontal
disease, from adherence and colonization through nutrient acquisition and neutralization of host defense.

The gingipain null (rgpA-, rgpB-, kgp-) mutant KDP136 was reported unable to grow in defined medium with human albumin as the sole carbon source [26]. Comparative studies of the virulence of isogenic mutants lacking RgpA, RgpB, and Kgp indicated that Kgp contributed more to the pathogenicity of \textit{P. gingivalis} in murine lesion and periodontal models [27], though Rgp activity was always found to be at a level higher than Kgp activity [28]. The roles of gingipains as virulence factors have been the focus point of many reviews [29–31], and will not be further described in this article.

2.1.1. Rgp

\textit{Rgp} (EC 3.4.22.37) is classified in MEROPS as clan CD, family C25, peptidase C25.001 [32]. Its specificity is highly limited to the Arg|-Xaa bond with no particular preference for P2 and P1’ sites [14,33]. Two closely related genes, \textit{rgpA} and \textit{rgpB}, are present in the \textit{P. gingivalis} genome, and possibly developed by gene duplication and rearrangement. The \textit{rgpA} gene translation product, RgpA, is composed of 1703 amino acids, consisting of the propeptide, a catalytic domain, four adhesin domains, and a C-terminal domain. RgpB, encoded by \textit{rgpB}, is composed of 736 amino acids, consisting of the propeptide, and catalytic and C-terminal domains. Heterogeneous molecules with Arg-specific activity have been observed in both \textit{P. gingivalis} bacterial cells and culture supernatant samples containing vesicles. The heterogeneity in the Rgp molecules was implicated in the existence of the two genes, auto-proteolysis occurring at the domain junctions, complex formation between the catalytic and adhesion domains, and LPS association [34].

2.1.2. Kgp

Kgp (EC 3.4.22.47), classified as clan CD, family C25, peptidase C25.002, is specific for the P1 Lys residue [35,36] and encoded by a genomic single gene, \textit{kgp} [37]. The translation product of \textit{kgp} is composed of 1723 amino acids, producing a multi-domain protein similar to RgpA, and consists of the propeptide, a caspase-like catalytic domain, four adhesin domains, and a C-terminal domain. Kgp possesses an additional putative immunoglobulin-like domain located between the caspase-like and first adhesion domains. Soluble and cell-associated forms of Kgp are detected in the same manner as those of Rgp [33].

2.2. Exopeptidases

Extracellular oligopeptides produced by gingipains are converted into di- or tri-peptides in the periplasm space (Fig. 1), then incorporated into bacterial cell across the inner membrane. These peptides are finally hydrolyzed in the cytoplasm into single amino acids by dipetidase, aminopeptidase, and carboxyl peptidase, and further converted into metabolic end-products, such as ammonia and butyrate [15,16], which are considered to be virulence factors that cause host tissue damage [38–40]. Accordingly, periplasmic exopeptidases, i.e., DPP4, DPP5, DPP7, DPP11, and AOP that produce di- and tri-peptides are considered to play critical roles in both cell growth and pathogenicity.

2.2.1. DPP4

The serine exopeptidase DPP4 hydrolyzes the peptide bond at the carboxyl side of Pro (NH2-Xaa1-Pro2–Xaa3–) and is classified as clan SC, family S9, subfamily S9B, peptidase
Exopeptidases in *Porphyromonas gingivalis* for amino acid metabolism

S09.013. Pro2 residue can be substituted by Ala or hydroxyproline, though the rate of hydrolysis is substantially lower than that of Pro2. *P. gingivalis* DPP4 is composed of 723 amino acids with calculated masses of 82,018 (precursor) and 80,235 (mature form) [41]. The growth of dpp4-, dpp7, or ptpA single-knockout strains was shown to be comparable with that of wild-type strain W83, while that of the triple-knockout mutant was retarded [42]. *P. gingivalis* DPP4 also hydrolyzes biologically active peptides that include substance P, fibrin inhibitory peptide, and β-casomorphin [19], and binds to fibronectin, thus it mediates bacterial adhesion to host cells [43]. A recent study also demonstrated that DPP4 was closely associated with biofilm formation in a murine subcutaneous abscess model [44].

### 2.2.2. DPP5

DPP5, classified as peptidase S9.012, was first isolated from *Aspergillus fumigatus* culture supernatant samples [45] and, until our discovery of *P. gingivalis* DPP5, species carrying this exopeptidase were restricted to the fungi *A. fumigatus*, *Aspergillus orizae* (koji fungus, used for fermentation of soybeans), and *Microsporum canis*, which causes the skin disease dermatophytosis [46]. *A. fumigatus* DPP5 has been reported to be frequently detected in mycelium of patients with invasive aspergillosis and aspergillosis, and was reported to be one of the major antigens in immunocompetent patients with aspergillosis [47].

In a report published in 2014, we noted that a *P. gingivalis* ATCC 33277 dpp4-dpp7-dpp11 triple-knockout strain (NDP511) still possessed some hydrolyzing activities for Met-Leu-, Lys-Ala-, Gly-Phe-, and Ser-Tyr-4-methylcoumaryl-7-amide (MCA), indicating the expression of an unidentified DPP with a preference for the hydrophobic P1 residue [24]. In order to identify this speculated DPP, we searched for candidates among the 2090 protein-coding sequences of this strain. Consequently, it was found that PGN_0756, tentatively annotated as prolyl oligopeptidase, exhibited DPP activity preferential for Ala and hydrophobic residues at the P1 position. Its amino acid sequence was 28.5% identical to *A. fumigatus* DPP5. Indeed, the substrate specificity of PGN_0756 was similar to that of fungal DPP5. Hence, we concluded that PGN_0756 represents *P. gingivalis* DPP5 and should be classified as clan SC, family S9, subfamily C, S09.075 [32]. The discovery of bacterial type DPP5 revealed that this enzyme is distributed from eubacteria, archaea members, and eukaryotes including fungi, as well as higher animals and plants [25].

Although DPP5 and DPP7 showed a hydrophobic P1 preference, the former has no apparent amino acid preference at the P2 position (N-terminus) [25], in contrast to the hydrophobic P2 preference of DPP7 [48]. Thus, DPP5 and DPP7 share substrates based on the differences in their P2 position specificities. For example, DPP7 is able to hydrolyze dipeptidyl MCA substrates harboring non-hydrophobic residues at the P1 position to some extent, such as Leu-Gln- and Leu-Arg-MCA, which are scarcely hydrolyzed by DPP5. In contrast, Lys-Ala- and Gly-Phe-MCA are predominantly hydrolyzed by DPP5.

We previously reported that *Porphyromonas endodontalis*, a bacterium frequently detected in infected root canals of patients with acute symptoms, showed Lys-Ala-MCA-hydrolyzing activity much greater than that of *P. gingivalis* [49]. Also, results of a biochemical study that used recombinant peptidases demonstrated that this difference was mainly caused by an 18-fold increase in $k_{cat}/K_m$ of *P. endodontalis* DPP5 as compared to that of *P. gingivalis* DPP5 [50].

### 2.2.3. DPP7

DPP7 is classified in MEROPS as clan SC, family S46, subfamily S9B, peptidase S46.001. *P. gingivalis* DPP7 was the first S46-family peptidase reported that hydrolyzes the peptide bond at the carboxyl side of aliphatic or aromatic residues (Yaa) (NH$_2$-Xaa$^1$-Yaa$^2$-$\cdots$-Xaa$^3$-$\cdots$), but does not cleave substrates with a blocked N-terminus and long oligopeptides, such as insulin B chain, azocasein, and type I collagen [20]. We recently found that *P. gingivalis* DPP7 is also preferential for hydrophobic residues at the P2 as well as P1 positions, which are mainly mediated by Phe$^{664}$ [51].

Unlike DPP4 and DPP5, the species distribution of DPP7 as well as DPP11 seems to be limited to the bacterial kingdom, and DPP7 proteins of *P. gingivalis* [20,51], *P. endodontalis* [49], and *Pseudomonas mexicana* WO24 [52] have been biochemically characterized to date. Recently, Ogawara’s group was first to report the crystal structure of *P. mexicana* DPP7 (named DAPBII). They demonstrated that Gly$^{675}$ of DAPBII is located at the bottom of a wall of the S1 subsite, which is in accordance with our finding that Gly$^{675}$ of *P. gingivalis* DPP7, equivalent to Gly$^{675}$, is critical for hydrophobic P1 specificity [51].

Because of confusion in nomenclature, human and mouse DPP7 (also known as QPP and DPPII, respectively) are post-Pro aminopeptidase belonging to clan SC, S28.002 [53,54], and hence, essentially distinct from *P. gingivalis* DPP7 [20,51].

### 2.2.4. DPP11

In *P. gingivalis*, the gene coding DPP11 (PGN_0607 [55]/PG_1283 [10]) was reported to be an isof orm of authentic DPP7 (PGN_1479) [20] or only described as a hypothetical protein [54]. However, we revealed that the gene encodes a novel DPP that hydrolyzes the peptide bond on the carboxyl side of acidic residues (NH$_2$-Xaa$^1$-Asp/Glu$^2$-$\cdots$-Xaa$^3$-$\cdots$) [23] and newly classified it separately from DPP7 as peptidase S46.002 in clan SC, family S46, subfamily S9B [32].

DPP11 was unexpectedly discovered during our attempt to identify the Lys-Ala-MCA-hydrolyzing entity of *P. endodontalis* most similar to DPP7 [23]. Accordingly, degenerated PCR cloning was performed with primers designed from the nucleotide sequences of PGN_1479, PGN_0607 and their homologues from other species [20]. As a result, a gene with an open-reading frame encoding 717 amino acids (uploaded as AB610284) was cloned, and its amino acid sequence was shown to be 38.3% and 57.9% identical to that of authentic *P. gingivalis* DPP7 and the DPP7 isoform, respectively. However, the AB610284 and PGN_0607 recombinant proteins did not hydrolyze any of the 80 commercially available dipeptidyl-MCA substrates examined. Finally, we determined that they were novel enzymes, designated as DPP11, with preference for acidic amino acid P1 residues (Asp and Glu) [23]. Also, hydrophobic residues were found
to be preferable at the N-terminus (P2 position). DPP11 does not hydrolyze N-terminally modified substrates, such as acetyl-Leu-Asp-MCA, the same as DPP4 and DPP7. DPP11 orthologues are widely distributed throughout the bacterial kingdom [51].

The acidic P1-position preference of *P. gingivalis* DPP11 is primarily mediated by Arg\textsuperscript{673}. Asp at the P1 position is more preferential than Glu [23]. In some species, such as *Shewanella putrefaciens*, Arg\textsuperscript{673} is substituted by Ser, in which the Asp preference is converted toward Glu [51]. Arg\textsuperscript{673} of DPP11 is replaced by Gly\textsuperscript{666} in DPP7 of all species, which allows acceptance of a large hydrophobic residue at the P1 position [23].

We found that a *dpp11*-knockout strain lost most of its Asp/Glu-dependent DPP activity, while growth was partially (30%) retarded [23]. Because orthologues of peptidase family S46 comprising DPP11 as well as DPP7 are not present in humans, DPP11 may be a potential drug target for chronic periodontitis.

2.2.5. Prolyl tripeptidyl peptidase A (PtpA)
Different from DPPs, which are unable to hydrolyze oligopeptides with Pro at the third position from the N-terminus, PtpA, classified as Clan SC, family S9, subfamily B; S09.017, hydrolyzes the Pro\textsuperscript{3}-Xaa\textsuperscript{2} bond and liberates an N-terminal tripeptide (NH\textsubscript{2}-Xaa\textsuperscript{1}-Xaa\textsuperscript{2}-Pro\textsuperscript{3}) [21]. An unblocked N-terminus is absolutely required for cleavage and no cleavage occurs with substrates with Pro at the P2 position [56]. Synthetic substrates Ala-Ala-Pro- and Gly-Ala-Pro-p-nitroanilide are suitable for measurement of the activity [21]. PtpA is possibly related to degradation of type I collagen carrying the tri-peptidyl repeat (-Gly-Xaa-Pro-\textsubscript{n}). Reflecting the similarity of the substrate specificities, the amino acid sequence of *P. gingivalis* PtpA is partially homologous (23.5%) to that of *P. gingivalis* DPP4. The crystal structure of *P. gingivalis* PtpA was previously reported [56].

2.2.6. Acylpeptidyl oligopeptidase (AOP)
The *dpp4-dpp5-dpp7*-triple knockout strain NDP211 was found to have lost most DPP activities except that for DPP7 and a small level of Met-Leu-MCA-hydrolyzing activity. However, further disruption of *dpp11* in NDP211, termed NDP212, reversed that Met-Leu-MCA-hydrolyzing activity [24], which strongly suggested induction of an unidentified DPP under emergent conditions. Our search revealed that an S9-family peptidase, PGN\textsubscript{1349}, was responsible for hydrolisis of Met-Leu-MCA in NDP212 [25]. Maximal activity of PGN\textsubscript{1349} was achieved with benzylxoycarbonyl-(Z)-VKM-MCA. Thus, the Met-Leu-MCA-hydrolyzing activity observed in the *quatro-dpp* knockout strain NDP212 was not mediated by DPP, but rather by the non-DPP peptidase PGN\textsubscript{1349}. Since PGN\textsubscript{1349} preferentially liberates di- and tri-peptides from acylated oligopepti- tides, we designated it acylpeptidyl oligopeptidase (AOP). Details of the biochemical and enzymatic properties of AOP will be reported elsewhere. Expression of AOP is likely beneficial for the organism, because it provides N-terminally unblocked serum proteins toward DPPs and PtpA.

2.3. Absence of exopeptidases with preference to hydrophilic residues

*P. gingivalis* exopeptidases recognize P1-position Pro, acidic, or hydrophobic residues in their substrate peptides. Hydrophobic interaction is also involved in the P2-position preference for DPP7 and DPP11 [48]. In contrast, *P. gingivalis* does not seem to possess a DPP to liberate N-terminal dipeptides composed of hydrophilic residues, since it does not hydrolyze Thr-Ser- or Gly-Gly-MCA [24]. In fact, no DPP for hydrophilic amino acids has been found in any organisms to date [32]. The hydrophilic interaction between DPP and the dipeptidyl portion of a substrate may be not sufficient to form an enzyme—substrate complex, because the interaction is likely weakened by surrounding water and hydrophilic macromolecules.

3. Subcellular localization and comparison of enzymatic properties among exopeptidases

All four DPP and AOP activities have been detected in *P. gingivalis* cells, though not in culture medium, indicating that they are present as so-called cell-associated forms. Furthermore, subcellular fractionation demonstrated that DPP5 and DPP11 are localized in the periplasmic space of the cell (Fig. 1) [23,24]. Indeed, DPPs do not have the C-terminal domain, which is required for outer membrane localization of Rgp and Kgp mediated by the type IX secretion system [57]. Periplasmic localization of *P. gingivalis* DPPs, PtpA, and AOP has also been suggested based on proteome analysis [58]. The differing subcellular localization between DPPs and gingipains seems to be compatible with the process of extracellular polypeptide processing.

We determined and compared the enzymatic parameters of recombinant forms of DPPs and AOP (Table 1). The *k*\textsubscript{cat}/*K*\textsubscript{m} values for the best substrates for their respective peptidases were the highest in DPP4 followed by DPP11, while DPP5 and DPP7 possessed moderate values, and AOP had the lowest. Interestingly, this order was in parallel with substrate confinement in these exopeptidases, from Pro-specific DPP4, to Asp/Glu-specific DPP11, hydrophobic P1- and P2-specific DPP7, hydrophobic P1-specific DPP5, and the most relaxed hydrophobic P1-specific AOP. This coincidence is reasonable, because DPP4 may fold into the most specific steric structures for its substrate, whereas AOP, which is able to liberate di- and tri-peptides with and without N-terminal modification, may fold into a more open structure that is able to accept several types of substrates.

4. Exopeptidases as potential virulence factors of *P. gingivalis*
All four DPP activities have been detected in *P. gingivalis* strains ATCC 33277, ATCC 49417, W83, W50, HW24D1, HNA99, HG1690, and 16-1 [50], and the exopeptidases discussed in this article contribute to bacterial growth. The findings presented here suggest that the activities of these exopeptidases influence both bacterial colonization in the oral cavity and clearance from the cardiovascular system.
after entering the blood circulation from a periodontal lesion.

It has been reported that *P. gingivalis* DPP4 promotes the degradation of collagen and gelatin mediated by host proteases, and also inhibits binding between gingival fibroblasts and fibronectin [43]. In addition, DPP4 is involved in biofilm formation by *P. gingivalis*, which is closely related to bacterial virulence [44]. Hence, it is reasonable to postulate that other DPPs also contribute to biofilm formation and mixed-species colonization in subgingival plaque. Furthermore, evaluation of the roles of *P. gingivalis* DPPs in systemic diseases, especially type-2 diabetes mellitus and cardiovascular diseases, would be interesting. Our preliminary data indicate that the human peptide hormone incretin GLP-1, which induces secretion of insulin from the pancreas, is degraded by *P. gingivalis* bacterial cells and DPP4 in vitro. This is now an important point of focus for further studies.

5. Conclusion

*P. gingivalis* expresses various exopeptidases, i.e., DPP4, DPP5, DPP7, DPP11, PtpA, and AOP, in periplasmic space, which produce di- and tri-peptides from most oligopeptides. This oligopeptide processing step is important as an extracellular event in the metabolism of asaccharolytic *P. gingivalis*. Rgp and Kgp are also involved in dipeptide production. An organized subcellular localization of various exopeptidases and gingipains is a rational explanation for processing of proteinaceous nutrients present in the subgingival environment, thus providing a means of efficient survival for the bacterium.

Additional note

To activate studies of DPPs of *P. gingivalis* and other oral bacteria, we contacted the Peptide Institute (Osaka, Japan) to produce DPP substrates. As a result, Leu-Asp- and Met-Leu-MCA, substrates for DPP11 and DPP7, respectively, have recently become available (The Peptide Institute, Supplemental Product List 28-3, 2015).

Conflict of interest

The authors have no conflict of interest to declare.

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