Hemolysin of Prevotella oris : Purification and characteristics

by Toshiya Sato

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Review

Hemolysin of *Prevotella oris*: Purification and characteristics

Toshiya Sato, Herastuti Sulistyani, Arihide Kamaguchi, Hiroshi Miyakawa, Futoshi Nakazawa*

Department of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobetsu, Hokkaido 061-0293, Japan

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ABSTRACT

Hemolysin, which lyses erythrocytes to release hemoglobin, is an important virulence factor associated with infectious diseases. *Prevotella oris* is frequently isolated from oral and systemic infectious lesions, suggesting that this bacterium is closely related to purulent inflammation. *P. oris* hemolysin was purified from culture supernatant by ion-exchange and gel-filtration chromatography. It was subsequently characterized as a proteinous, thiol-activated compound with a stronger hemolytic activity toward human erythrocytes than that toward sheep and rabbit erythrocytes. Hemolysin binding experiments showed that *P. oris* hemolysin binds to erythrocyte membranes in a temperature-dependent manner before hemolysis. Further, it was suggested that the glycoprotein in erythrocyte membranes is the potential binding site of *P. oris* hemolysin. Interestingly, we found that glyceraldehyde-3-phosphate dehydrogenase was released from human erythrocyte membranes when the cells were lysed by *P. oris* hemolysin. In this review, we have discussed the purification, characteristics, and mechanisms underlying hemolysis-driven cell damage due to *P. oris* hemolysin, including hemolysin from other oral bacteria.

Contents

1. Introduction

Prevotella oris is a nonpigmented, gram-negative, rod-shaped anaerobic bacterium frequently isolated from infectious oral lesions, such as those found in periodontal disease [1], endodontic infection [2], dentoalveolar abscess [3], bacteremia [4], and spreading odontogenic infection [5]. The bacterium is also found in systemic infection lesions such as those found in empyema [6], cervical spinal epidural abscess, and meningitis [7]. Previous studies have shown that *P. oris* produces immunoglobulin A protease [8], hyaluronidase [9], and β-lactamase [10], which suggests that these factors may contribute to its potential pathogenicity. Furthermore, *P. oris* coaggregates with *Porphyromonas gingivalis*, indicating that *P. oris* promotes colonization of *P. gingivalis* during the early stages of biofilm formation [11].

It is known that periodontopathogens produce lipopolysaccharide, proteinases, hyaluronidase, and hemolysin [12]. These virulence factors lead to the destruction of periodontal tissues and are involved in the nutrient acquisition required for bacterial growth. Iron is an essential nutrient for bacterial growth. *Prevotella* species, including *P. oris*, require hemin for growth [13–15], of which iron is the primary component. Consistent with this, iron is an essential nutrient for bacteria; in general, bacteria require an iron concentration of 0.05–0.5 μM for growth [16]. However, in humans, the concentration of free iron is maintained at 10^{-21} M [17], which is

* Corresponding author. Tel.: +81 133 23 2484; fax: +81 133 23 1385.
E-mail addresses: toshiya@hoku-iryo-u.ac.jp (T. Sato), herastuti@hoku-iryo-u.ac.jp (H. Sulistyani), kamaguti@hoku-iryo-u.ac.jp (A. Kamaguchi), miya@hoku-iryo-u.ac.jp (H. Miyakawa), nakazawa@hoku-iryo-u.ac.jp (F. Nakazawa).

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much lower than that required by bacteria. Furthermore, the iron supply in the oral cavity is limited by iron-binding proteins such as transferrin and lactoferrin [18,19]. Some bacteria such as *Escherichia coli* produce siderophores to sequester iron from lactoferrin and transferrin [16]. However, no studies have identified siderophore-producing bacteria in the oral cavity. Therefore, hemolysin, which lyses erythrocytes to release iron-containing hemoglobin, may be vital for the *in vivo* survival of oral bacteria.

Previously, Hillman et al. reported that *Actinomyces*, *Streptococcus*, *Staphylococcus*, *Prevotella*, and *Aggregatibacter*, each of which possesses hemolytic activity, were all isolated from the subgingival plaques of patients with periodontal diseases [20]. Additionally, the characteristics of hemolysin produced by oral bacteria such as *P. gingivalis* [21], *Prevotella intermedia* [22–26], *Prevotella nigrescens* [24,25], *Prevotella melaninogenica* [27], *Treponema denticola* [28], *Aggregatibacter actinomycetemcomitans* [29], *Fusobacterium necrophorum* [30], and *Streptococcus milleri* group [31] have been reported. Furthermore, it has been shown that crevicular fluid collected from periodontitis sites had a higher iron concentration than that collected from gingivitis sites [32]. Moreover, studies have shown that hemolysin damages host tissues because of its cytotoxic effects on many cell types such as fibroblasts, leukocytes, and renal tubular cells [33–35]. Therefore, hemolysin is regarded as a putative virulence factor in many pathogenic bacteria. In this article, we have discussed the purification and characteristics of *P. oris* hemolysin to better understand its pathogenic potential in various oral and systemic infections.

2. Distribution and purification of *P. oris* hemolysin

In our previous study, it was demonstrated that the culture supernatant of *P. oris* contained hemolytic activity; we also purified and characterized hemolysin produced by *P. oris* [36]. The hemolytic activity in the culture supernatant of *P. oris* during the early logarithmic growth phase was much higher than that during the stationary phase [36]. A similar pattern of hemolysin production has been reported for *P. intermedia* [22], *P. nigrescens* [25], *P. gingivalis* [21], *T. denticola* [37], and *A. actinomycetemcomitans* [29]. However, the underlying reason for this phenomenon remains unknown. The autoinducer-2 (AI-2) produced by *A. actinomycetemcomitans* increases its leukotoxin production and iron uptake [38]. Levels of AI-2 peak during the mid-exponential phase and decrease significantly in the late log and stationary phases during *A. actinomycetemcomitans* culture [38]. Periodontopathogens such as *P. gingivalis*, *P. intermedia*, and *Fusobacterium nucleatum* also produce AI-2 [39], although the relationship between hemolysin production and AI-2 in these bacteria is not clear. The production of AI-2 by *P. oris* remains unclear; therefore, further research is required to investigate the underlying mechanism of hemolysin production of *P. oris*.

The hemolytic activity of *P. oris* was detected in the culture supernatant, but not in the bacterial cell fractions [36]. However, it has been shown that the hemolysins from *P. intermedia* and *T. denticola* were mainly detected in cell-associated fraction, suggesting that hemolysins are localized to the surface of bacterial cells [22–24,26]. Gingipains, which are trypsin-like cysteine proteases produced by *P. gingivalis*, are important virulence factors in adult periodontitis and are localized on the cell surface in almost all strains [40]. Moreover, the *A. actinomycetemcomitans* leukotoxin is a nonsecretory toxin associated with bacterial cells [41]. Although the localization of hemolysin in *P. oris* has not been described in detail thus far, it is apparent that hemolytic activity is absent in, and on, the bacterial cell.

Generally, ammonium sulfate/polyethylene glycol precipitation methods are used for the isolation of proteins from bacterial culture supernatants [21,26]. However, during the isolation of hemolysin produced by *P. oris*, these precipitation methods resulted in a significant loss of activity [36]. Furthermore, the activity of *P. oris* hemolysin was gradually lost at 4 °C and easily lost at room temperature. The *F. necrophorum* hemolysin is also easily inactivated during experiments [42]. A suitable stabilizer is required to purify the *P. oris* hemolysin without loss of activity. In our previous study, the *P. oris* hemolysin was purified by ion-exchange and gel-filtration column chromatography and ultimately yielded a single peak on high-performance liquid chromatography [36]. Although specific activity increased 9200-fold, recovery of activity was only 1.1% [36]. The molecular mass of the purified hemolysin was 16 kDa, as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1). Previously, there have been no reports regarding the molecular weight of hemolysin produced by *Prevotella* species except *P. oris*. Deshpande and Khan effectively purified *P. gingivalis* hemolysin by using a nickel–nitrilotriacetic acid agarose column [21]. Kimizuka et al. purified *A. actinomycetemcomitans* hemolysin using a combination of isoelectric focusing and gel-filtration chromatography [29]. Chu and Holt purified *T. denticola* hemolysin, cystalysin, using a combination of ammonium sulfate fractionation and preparative electrophoresis [28]. The mass weights of hemolysin from *P. gingivalis* and *A. actinomycetemcomitans* are 45 kDa [21] and 12 kDa [29] on SDS-PAGE, respectively (Table 1). The molecular mass of *T. denticola* hemolysin is 45 kDa on PAGE in the presence and absence of SDS [28] (Table 1).

3. Characteristics of *P. oris* hemolysin

Previously, it has been reported that the black-pigmented *Prevotella* species [26], *T. denticola* [28], *F. necrophorum* [30], *P. gingivalis* [21], and *A. actinomycetemcomitans* [29] produce proteinous hemolysin, because the hemolytic activity was inhibited by proteinase treatment. Furthermore, the cloning, sequencing, and characterization of *P. gingivalis* hemolysin [43], *P. intermedia* hemolysin [23], *P. melaninogenica* hemolysin [27], and *T. denticola* cystalysin [44] have been described. Whether these

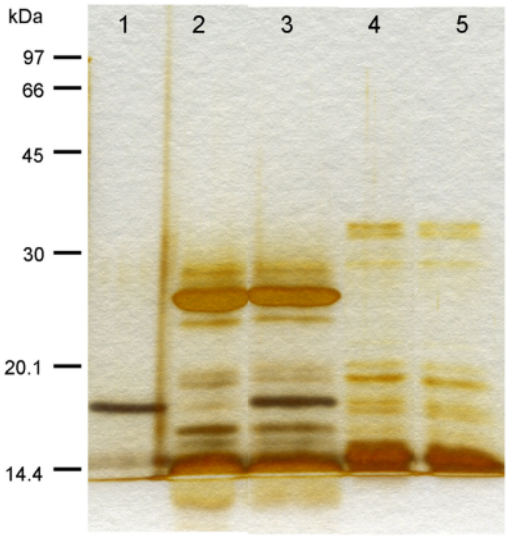

Fig. 1. Effect of trypsin or proteinase K treatment on *Prevotella oris* hemolysin. The *P. oris* hemolysin was purified from culture supernatant using ion-exchange and gel-filtration chromatography. After treatment with trypsin or proteinase K, the purified hemolysin is subjected to SDS-PAGE. The gel was visualized by silver staining. Lane 1, purified hemolysin; Lane 2, 100 μg/ml trypsin; Lane 3, mixture of hemolysin and 100 μg/ml trypsin with incubation at 37 °C for 60 min; Lane 4, 100 μg/ml proteinase K; and Lane 5, mixture of hemolysin and 100 μg/ml proteinase K with incubation at 37 °C for 60 min. (Modified from Sato et al. [36].).

Table 1
Characteristics of hemolysin from oral gram-negative bacteria.

Bacterial species	Hemolysin name	Molecular weight	Effect of thiols	Effect of cholesterol	Reference
Prevotella intermedia	Prevolysin O	–	Enhanced	–	[26]
Prevotella oris	–	16 kDa	Enhanced	Not affected	[36]
Porphyromonas gingivalis	–	45 kDa	Inhibited [21]/Enhanced [43]	Inhibited	[21,43]
Aggregatibacter actinomycetemcomitans	–	12 kDa	Enhanced	Inhibited	[29]
Treponema denticola	Cystalysin	46 kDa	Cystein-dependent	Not-affected	[28]

hemolysins contain sugar or lipid modifications is still unknown, and the details of the molecular structure of these hemolysins are limited. Interestingly, Fifis et al. proposed that *F. necrophorum* hemolysin, leukocidin, and phospholipase B together form a molecular complex [45]. We also showed that *P. oris* produces a proteinous hemolysin [36]. As shown in Fig. 1, when the purified hemolysin was treated with proteinase K, the 16-kDa hemolysin band disappeared on the SDS-PAGE gel with sliver staining. However, treatment with trypsin did not affect the intensity of this band (Fig. 1). Furthermore, the hemolysin band of *P. oris* on SDS-PAGE gel was not visualized by Coomassie Brilliant Blue staining [36]. The *P. oris* hemolysin may consist of conjugated proteins, although further studies are required to clarify the constituents of the hemolysin in detail.

It has been reported that several gram-positive bacteria such as *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria* produce cytolytic proteinous toxins called thiol-activated cytolysins [46]. These toxins have several common characteristics. They are water-soluble, single-chain polypeptides with molecular masses ranging from 47 to 60 kDa, and they lyse eukaryotic cells by triggering the formation of pores in the cell membrane. The members of this family show 30–60% similarity in the primary amino acid sequence. In gram-negative bacteria from the oral region, the *P. intermedia* hemolysin designated as prevolysin O [26], *T. denticola* cystalysin [28], and *A. actinomycetemcomitans* hemolysin [29] possess similar properties (Table 1). In our previous study, *P. oris* hemolysin also showed thiol-activated cytolytic activity [36]. In the process of cytolysis triggered by the thiol-activated cytolysin family (TACY family), cholesterol is the major target receptor, and small amounts of free cholesterol can inhibit lytic activity [47]. The activity of *P. oris* hemolysin is weakly inhibited with cholesterol, suggesting that the mechanism of membrane damage differs from that by the TACY family [36]. Although the activity of *T. denticola* cystalysin is not affected by cholesterol [28], that of *A. actinomycetemcomitans* hemolysin is inhibited [29] (Table 1). Therefore, it is possible that the mechanisms of hemolysin-induced cytotoxicity differ from one oral gram-negative bacterium to another.

A wide variation has been reported in the optimum pH of hemolysins/cytolysins produced by various bacterial species [26,48]. The hemolysin of *P. oris* requires mildly acidic conditions for hemolysis, and maximal activity was achieved at pH 6.0 [36]. This result suggests that the hemolytic activity in lesions with acute inflammation might be stronger than that at a healthy site, because the pus from infected tissue is generally mildly acidic [49].

4. Diversity of hemolytic activity among strains

Several investigators have reported the characteristics of hemolysin. However, there are discrepancies in the strength and sensitivity of activity to different agents or treatments. For example, in the case of *P. gingivalis*, it has been reported that *P. gingivalis* hemolysin is of a hot-cold type [50,51], but it has also been reported to not be of the hot-cold type [52]. Although it has been reported that *P. gingivalis* hemolysin is thiol independent [50], it is enhanced by dithiothreitol [43] and some oxygen-labile bacterial toxin is reactivated by reductants, whereas other investigators have reported that it is inhibited by dithiothreitol [21,51,52]. Furthermore, it has been shown that *P. gingivalis* hemolysin is inhibited by Nα-*p*-tosyl-L-lysine (TLCK) [52]. TLCK is an inhibitor of cysteine protease from *P. gingivalis*, but studies have reported that it is not affected by TLCK [21,43]. Because investigators used different strains in their respective studies, it is likely that the characteristics of hemolysin are unique to each strain. In the case of *P. oris* hemolysin, the strain WK 1 isolated from subgingival plaques with chronic periodontitis shows stronger hemolysis than the strain JCM 8540, the sole strain available from bacterial culture collections, by measuring the diameter of the hemolysis zone around the colony on blood agar plates. Additionally, Okamoto et al. showed that there is a wide spectrum of hemolytic activities across *P. intermedia* and *P. nigrescens* isolates [53]. Thus, much more research is needed to further define the relationship between the strength of hemolytic activity and pathogenicity in oral infections.

5. Mechanisms underlying hemolysin-driven cell damage

Hemolysis mediated by *P. oris* hemolysin requires incubation at 37 °C. Before hemolysis, *P. oris* hemolysin binds to erythrocytes in a temperature-dependent manner. The amount of hemolysin binding is stronger after preincubation at 37 °C than that after preincubation at 27 °C or 4 °C. These data indicate that the environmental conditions in the oral cavity are appropriate for hemolytic activities of *P. oris*. Similarly, the hemolysis rate of *P. intermedia* hemolysin and the binding between erythrocytes and *T. denticola* hemolysin are also temperature dependent [22,28]. On the other hand, the hemolysin of *F. necrophorum* has been reported to bind to erythrocytes in a temperature-independent manner [42].

Erythrocytes from different species differ in their susceptibility to hemolysin from *P. oris* [36]. *P. oris* hemolysin showed strong activity against horse erythrocytes, moderate activity against human erythrocytes, and weak activity against sheep and rabbit erythrocytes. Similar results have been reported for *P. intermedia* and *A. actinomycetemcomitans* [22,29]. Amoako et al. reported that the distribution of receptors against the *F. necrophorum* hemolysin is different in each mammalian erythrocyte, and this irregularity provides an explanation for erythrocyte susceptibility to hemolysin [54].

The hemolysin activity on erythrocytes treated with trypsin or glycosidase was inhibited, suggesting that the binding site of this hemolysin may be a glycoprotein. Previously, glycophorin, one of the main proteins on the erythrocyte surface, was identified as a receptor for *E. coli* α-hemolysin [55] and *Vibrio cholerae* El Tor hemolysin [56]. Furthermore, a glycoprotein on intestinal cells was identified as the receptor for *Aeromonas sobria* hemolysin [57]. In the case of oral bacteria, there are few reports describing a hemolysin receptor; however, it has been proposed that the erythrocyte receptor for *F. necrophorum* hemolysin is phosphatidylcholine [54]. Further studies are needed to identify the *P. oris* hemolysin receptor and to better understand the interaction between the hemolysins of oral bacteria and erythrocytes.

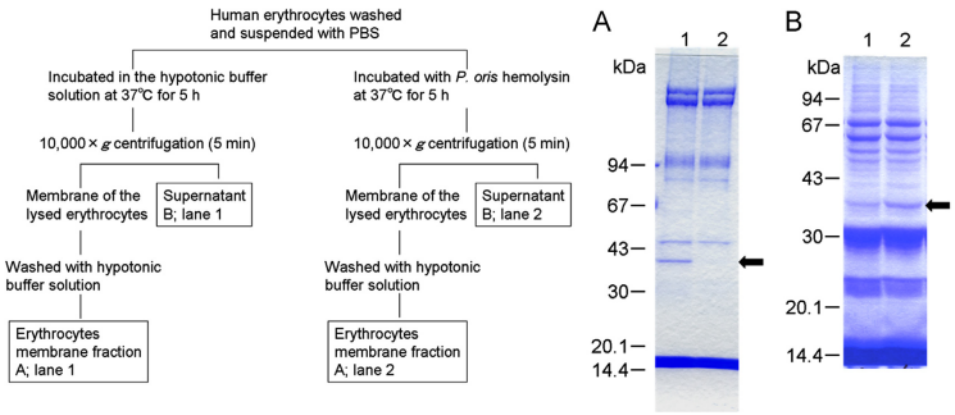

Fig. 2. SDS-PAGE profiles of human erythrocytes before and after treatment with *Prevotella oris* hemolysin. Human erythrocytes were incubated for 5 h with *P. oris* hemolysin that was purified from the culture supernatant of *P. oris*. Membranes of the lysed erythrocytes were subsequently separated from the supernatant by centrifugation. The treated membranes were washed 3 times with hypotonic buffer solution (10 mM Tris–HCl, pH 7.4) and subjected to SDS-PAGE analysis. Additionally, a portion of the erythrocyte membrane and supernatant that was lysed in the hypotonic buffer solution was subjected to SDS-PAGE analysis as a control. An SDS–polyacrylamide linear gradient (5–20%) gel was used for the treated membrane, and a 12% gel was used for the supernatant. The gels were visualized by CBB staining. A: Lane 1, erythrocyte membranes without hemolysin treatment; Lane 2, the erythrocyte membrane with hemolysin treatment. B: Lane 1, the supernatant without hemolysin treatment; Lane 2, the supernatant with hemolysin treatment. The arrows show GAPDH bands, which were identified by analysis of *N*-terminal amino acid sequence. (Modified from Sato et al. [60]).

Little is known regarding the mechanisms of hemolysis following hemolysin binding. In a previous report, erythrocytes treated with *P. gingivalis* hemolysin were generally smaller, and there appeared to be extracellular debris around the erythrocytes [21]. Additionally, pore-like structures were found on the surface of erythrocytes [21]. Furthermore, *T. denticola* cystalysin, cysteine desulfhydrase, leads to impressive spikes and protrusions in the erythrocyte membranes and finally to the formation of countless irregular holes [28]. Cystalysin causes the oxidation and sulfation of hemoglobin to methemoglobin and sulfhemoglobin [58]. It has been suggested that methemoglobin and sulfhemoglobin synthesized by cystalysin disrupt spectrin within the erythrocyte membrane, which in turn triggers membrane destruction [59].

In our study, we observed release of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from erythrocyte membranes [60]. As shown in the flow chart in Fig. 2, we compared the protein profiles of erythrocyte membranes under various conditions. GAPDH was not present in erythrocyte membranes after lysis with *P. oris* hemolysin (Fig. 2A, lane 2). On the other hand, the amount of GAPDH was higher in the supernatants of erythrocytes under these conditions (Fig. 2B, lane 2) when compared with the control (Fig. 2B, lane 1).

GAPDH is a glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. However, recent studies demonstrated that GAPDH has many other important roles, including the cellular response to oxidative stress [61], apoptosis [62], transcriptional [63] and posttranscriptional gene regulation [64], chromatin structure [65], maintenance of DNA integrity [66], vesicular transport [67], and receptor-mediated cell signaling [68]. The glycolytic activity of GAPDH is inhibited upon binding to the polyanionic *N*-terminus of anion exchanger/Band 3 and activated when it is released from Band 3 to the cytosol [69,70]. Therefore, a significant release of free GAPDH into the cytosol may alter cellular homeostasis and thereby lead to hemolysis. Mallozzi et al. reported that the translocation of GAPDH in human erythrocytes was induced by free radicals [70]. Moreover, Zhang and Honda demonstrated the disappearance of GAPDH from human erythrocyte membranes following hemolysis triggered by a thermostable hemolysin of *Vibrio parahaemolyticus* and *V. cholerae* El Tor hemolysin [71]. To the best of our knowledge, there have been no other reports describing hemolysis accompanied by the release of GAPDH from erythrocyte membranes. We have shown here that *P. oris* hemolysin triggers the release of GAPDH from human erythrocyte membranes during the process of hemolysis. Further studies are in progress to understand the detailed mechanism of hemolysin-driven cell damage caused by this bacterium.

6. Conclusion

Hemolysin of *P. oris* was purified from the culture supernatant using ion-exchange and gel-filtration column chromatography and was characterized as a 16-kDa, heat-labile, proteinous, thiol-activated compound. This hemolysin may bind to the glycoprotein of erythrocyte membranes in a temperature-dependent manner before hemolysis. Additionally, it appears that during the hemolytic process, the release of GAPDH from the cell membrane to the cytoplasm may be involved in cell damage. However, much more remains to be discovered regarding the mechanisms underlying the pathogenesis associated with *P. oris* hemolysin.

Conflict of interest

Authors declare no conflict of interest.

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