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Original Article

Purification and characterization of hemolysin from *Prevotella oris*

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ABSTRACT

We observed hemolytic activity in culture supernatant of *Prevotella oris*. Results from growth-phase experiments show that hemolysin production increased during the logarithmic growth phase and decreased during the stationary phase. The hemolysin produced by *P. oris* was purified from the culture supernatant by ultrafiltration, diethylaminoethyl (DEAE) and carboxymethyl (CM) ion-exchange chromatography, and gel filtration chromatography; further, we investigated the purified hemolysin characteristics, including its ability to lyse human, horse, sheep, and rabbit erythrocytes. The purified hemolysin was observed as a single, 16-kDa band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The specific activity was obtained by concentrating the purified hemolysin by 9200 fold. Although hemolysin was inactivated by heat treatment, ethylenediaminetetraacetic acid (EDTA), L-cysteine, dithiothreitol (DTT), and 2-mercaptoethanol enhanced its activity. Further, treatments using trypsin, $MgCl_2$, $CaCl_2$, and cholesterol did not affect its hemolytic activity. A pH of 6.0 was optimal for inducing the hemolysin activity. To the best of our knowledge, this is the first report describing the purification and characterization of hemolysin produced by *P. oris*.
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1. Introduction

Prevotella oris is a nonpigmented, gram-negative, rod-shaped anaerobic bacterium frequently isolated from the lesions of several oral infections, such as periodontal disease [1], endodontic infection [2], dentoalveolar abscess [3], bacteremia [4], and spreading odontogenic infection [5]. In addition, *P. oris* has also been isolated from the lesions of systemic infections such as 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 the pathogenic potential of the organism.

Prevotella species, including *P. oris*, require hemin for growth [11–13]. In general, the bacteria require an iron concentration of 0.05–0.5 μM for growth [14]. However, in humans, the concentration of free iron should be maintained at 10^{-18} M [15], which is much lower than that required by the bacteria. Furthermore, the iron supply in the oral cavity is limited by various mechanisms [16,17]. Some bacteria produce siderophores to sequester iron from lactoferrin and transferrin. However, no studies have identified siderophore-producing bacteria from the oral regions. Therefore, hemolysin, which lyses erythrocytes to release hemoglobin, may be vital for in vivo survival of oral bacteria.

Hillman et al. [18] reported that hemolytic bacteria of the following 5 genera were frequently isolated from subgingival plaques of patients with periodontal diseases: *Actinomyces*, *Streptococcus*, *Staphylococcus*, *Prevotella*, and *Aggregatibacter*. Furthermore, it was also shown that crevicular fluid collected from the periodontitis sites had higher iron concentration than that in the gingivitis sites [19]. Moreover, studies have shown that hemolysin damages host tissues because of its cytotoxic effects on many cell types. Therefore, hemolysin is regarded as a putative virulence factor in many gram-positive and gram-negative pathogenic bacteria; however, the biological mechanism underlying hemolysin-driven cell damage is still unknown.

The role of *P. oris* in human oral and systemic infections is still unclear, and the hemolysin produced by *P. oris* has not been studied in detail. The purpose of this study was to purify and characterize hemolysin from *P. oris* to better understand its pathogenic potential in various oral and systemic infections.

2. Materials and methods

2.1. Bacterial strain and culture conditions

We used a strongly hemolytic strain of *P. oris*, WK1 was isolated from the subgingival sites of patients with chronic periodontitis. Before initiating the present study, 16 S rRNA sequence analysis

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was performed to confirm whether the strain belonged to the *P. oris* species. The *P. oris* WK1 strain was grown anaerobically (10% CO_2, 10% H_2, and 80% N_2) in TYHM broth (tryptic soy broth [BD, Difco] supplemented with 0.5% yeast extract [BD, Difco], 5 μg/mL hemin, and 1 μg/mL menadione) or on TYHM blood agar (TYHM supplemented with 5% v/v sheep blood and 1.5% agar [BD, Difco]). To obtain a culture of *P. oris*, we suspended colonies of *P. oris* obtained on TYHM blood agar in 20 mL of TYHM broth, incubated anaerobically at 37 °C for 24 h, diluted 50-fold with TYHM broth, and further incubated anaerobically at 37 °C until an optical density of 0.6 at 600 nm was obtained (approximately 18 h).

2.2. Hemolysin tube assay

Hemolytic activity against erythrocytes was determined using the method described by Deshpande and Khan [20], with some modifications. Horse, human, sheep, and rabbit erythrocytes were used. Human erythrocyte samples were collected from 8 volunteers (blood types: A, B, AB, and O; 2 persons each). Ethical approval was obtained from the Research Ethics Committee of the School of Dentistry, Health Sciences University of Hokkaido. Erythrocyte samples were washed 3 times with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 and pH adjusted to 6.8) and diluted with the same buffer. Next, 1% v/v erythrocytes (cell count, 7×10^7/mL), 5% v/v bacterial samples, and 5% v/v inhibitor or PBS were mixed (final concentration) and incubated at 37 °C for 5 h. After centrifugation (1500 × *g* for 5 min), the absorbance of the supernatant was measured at 540 nm by using a UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). One hemolytic unit (HU) was defined as the amount of hemolysin required to release 50% hemoglobin in a standardized erythrocyte suspension.

2.3. Relationship between bacterial growth phase and hemolytic activity

A 1-mL aliquot of the culture supernatant was harvested from the main culture broth of *P. oris* every 2 h to study the growth curve and determine the hemolytic activity of the bacteria. After determining the turbidity by measuring the absorbance at 600 nm, the aliquot was centrifuged at 10,000 × *g* for 20 min, and the hemolytic activity in the supernatant was determined.

2.4. Purification of hemolysin

The cell-free supernatant obtained by 20-min centrifugation of the bacterial culture at 10,000 × *g* was used as the crude hemolysin preparation and was subjected to ultrafiltration using an Ultrafilter Q0100 (10-kDa filter, 0.39 MPa; Advantec, Tokyo, Japan). The 20-fold concentrated crude preparation was applied to a Sephadex G-100 column (2.6 cm × 90 cm; GE Healthcare, Tokyo, Japan). The column was eluted using 50 mM phosphate buffer (50 mM NaH_2PO_4 and 50 mM Na_2HPO_4; pH 5.0) at a flow rate of 0.7 mL/min. The absorbance at 280 nm and the hemolytic activity of the eluted fraction were monitored. The active fractions were pooled and applied to a diethylaminoethyl (DEAE)-Sephadex A-25 column (2.6 cm × 5 cm; GE Healthcare). After washing the column with the same buffer containing 0.4 M NaCl, the fractions were eluted using a buffer containing 1.0 M NaCl. Next, the active fraction was dialyzed against 50 mM phosphate buffer (50 mM NaH_2PO_4 and 50 mM Na_2HPO_4; pH 4.0) and loaded onto the carboxymethyl (CM)-Sephadex A-50 column (1.0 cm × 5 cm; GE Healthcare). The column was eluted with the same buffer at a flow rate of 1.0 mL/min and the flow-through from the column was collected. After concentration with Amicon Ultra centrifugal filter units (10 K; 3000 × *g* for 15 min; Millipore, Billerica, MA, USA), the active fraction was applied to a Sephadex G-100 Superfine column (1.0 cm × 35 cm; GE Healthcare) with PBS at a flow rate of 50 μL/min. All the above purification steps were performed at 4 °C. After a second chromatography step using the Sephadex G-100 Superfine column, the active fractions were collected and concentrated with Amicon Ultra-0.5 centrifugal filter devices (10 K; 14,000 × *g* for 10 min; Millipore). The sample was then further purified twice by high-pressure liquid chromatography (HPLC) using the Shodex PROTEIN KW-802.5 column (Showadenko, Japan) with PBS at flow rate of 80 μL/min. After HPLC, the active fractions were collected as the purified hemolysin.

The molecular weight and purity of the purified hemolysin were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel, with molecular weight markers from Amersham-Pharmacia Biotech (Low-molecular-weight marker kit), was observed after silver staining (fixation [50% methanol, 5% acetic acid; for 60 min], rinse [H_2O, twice for 15 min each], sensitization [0.02% sodium thiosulfate, for 1 min], rinse [H_2O, twice for 1 min each], stain [0.1% silver nitrite, for 30 min], rinse [H_2O, twice for 1 min each], develop [2% Na_2CO_3, 0.015% formaldehyde; until brown bands appeared], and stop [2% acetic acid, for 10 min]).

2.5. Determination of protein concentration

The protein concentration after each purification step was determined using the method by Lowry et al. [21], which uses bovine serum albumin as the standard. During the chromatographic purification, the protein concentration was estimated by measuring the absorbance at 280 nm.

2.6. Effects of various treatments and pH on hemolytic activity

The purified hemolysin was heated to temperatures between 40 °C and 80 °C for 10 min, cooled on ice, and immediately assayed for hemolytic activity. After incubating the purified hemolysin with 100 μg/mL trypsin (4790 USP units/mg) at 37 °C for 1 h, 300 μg/mL trypsin inhibitor from soybean (3000–6000 USP units/mg) was added, and the hemolytic activity was assayed. The concentrations of EDTA, $MgCl_2$, $CaCl_2$, cholesterol, and sulfur-containing compounds such as L-cysteine, DTT, and 2-mercaptoethanol were determined, and their effect on hemolytic activity was evaluated after 10-min incubation with purified hemolysin at room temperature. The following final concentrations of each reagent were tested for their ability to stimulate the hemolytic activity of the purified hemolysin: 0.1 mM EDTA, 5 mM $MgCl_2$ and $CaCl_2$, 20–50 mM sulfhydryl compounds, and 10 μg/mL cholesterol. The assay was performed as described previously. The purified, untreated hemolysin was used as the control, with an assumed hemolytic activity of 100%. To determine the optimum pH for the induction of hemolytic activity, the activity of purified hemolysin was measured at different pH values ranging from 5.0 to 7.5.

3. Results

P. oris WK1 exhibited a typical zone of β-hemolysis on the TYHM agar supplemented with sheep, horse, or rabbit blood. Hemolysis was observed after 24 h of bacterial growth and increased until the fifth day of growth (data not shown). The hemolytic activity of the culture supernatant increased rapidly during the early logarithmic growth phase in the TYHM broth and reached the maximum growth curve at 16 h; however, the growth of *P. oris* WK1 reached a stationary phase after 30 h (Fig. 1). The hemolytic activity was sustained at a high level for 10 h, and then, it gradually decreased.

To study the localization of the hemolytic activity, cell membrane and intracellular fractions were prepared from sonication extracts of the bacterial cells. Except for the culture supernatant, hemolytic activity was not detected in any of the other fractions.

The hemolysin of *P. oris* was purified from the culture supernatant by using a combination of ultrafiltration, gel-filtration chromatography, and ion-exchange chromatography. A summary of these steps is shown in Table 1. The active fractions collected by Sephadex G-100 gel filtration chromatography were further applied to the DEAE-Sephadex A-25 column to effectively isolate hemolysin from other proteinaceous substances (a 74.5-fold increase in specific hemolytic activity was observed). Because hemolysin was not adsorbed by the CM-Sephadex A-50 column at pH 4.0, the hemolytic fractions were collected from the flow-through. Next, gel-filtration chromatography was performed twice with a Sephadex G-100 Superfine column; the chromatogram is shown in Fig. 2. The peak fractions with hemolytic activity (fractions 24–28) were collected and concentrated, and then applied twice to an HPLC column. The chromatogram by HPLC showed a single, symmetric peak by absorbance at 280 nm (Fig. 3). After these steps, the specific activity of the purified hemolysin was increased 9200-fold, with a final yield of 1.1% (Table 1).

SDS-PAGE analysis of the purified hemolysin showed a single band after silver staining (Fig. 4), although it was not visualized by Coomassie brilliant blue (CBB) staining. By comparing the relative mobility of the marker proteins, the molecular mass of the hemolysin was found to be 16-kDa.

Intriguingly, when the purified hemolysin was treated with proteinase K, the 16-kDa band disappeared on the SDS-PAGE gel after silver staining (Fig. 5). On the other hand, treatment with trypsin did not affect the intensity of the purified hemolysin band on the SDS-PAGE gel (Fig. 5).

We studied the effect of the hemolysin purified from the culture supernatant on the erythrocytes from various species. The purified hemolysin showed strong hemolytic activity against horse erythrocytes, moderate activity against human erythrocytes (blood type O), and weak activity against sheep and rabbit erythrocytes (Table 2). Human erythrocytes of type A, B, and AB also generated similar patterns as that by type O; however, type O erythrocytes were generally used for determining hemolytic activity (data not shown).

The effects of various treatments, including heat, chemical reagents, and pH, on the hemolytic activity of the hemolysin produced by *P. oris* were determined. The purified hemolysin was inactivated by heat treatment. At temperatures more than 50 °C, the hemolytic activity decreased significantly, and all activity was lost between 60 °C and 70 °C (Table 3). EDTA, L-cysteine, DTT, and 2-mercaptoethanol enhanced the hemolytic activity, and trypsin, $MgCl_2$, $CaCl_2$, and cholesterol did not affect the hemolytic activity (Table 3). The purified hemolysin showed activity over a wide pH range (5.0–7.5). The optimum pH for the hemolytic activity of hemolysin was 6.0 (Table 3).

4. Discussion

It has been shown that hemolysin is produced by members of the black-pigmented *Prevotella* species, such as *P. intermedia* [22–26], *P. nigrescens* [24,25], and *P. melaninogenica* [27]. However, hemolytic activity derived from the nonpigmented *Prevotella*

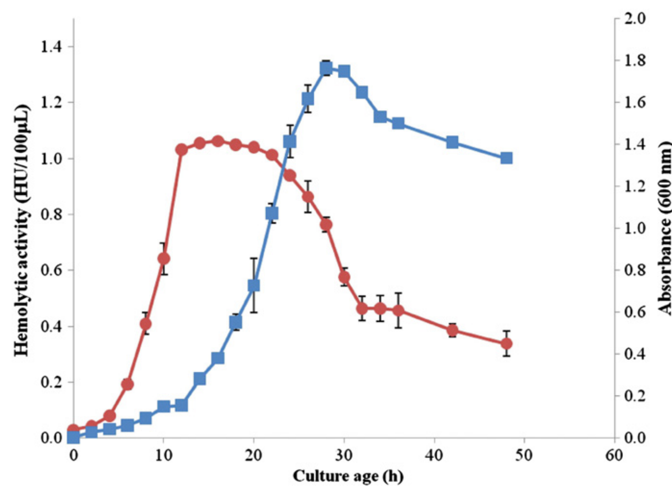

Fig. 1. Hemolytic activity during the different phases of bacterial growth *Circle*, hemolytic activity (HU/100 μL); *square*, cell density (absorbance at 600 nm).

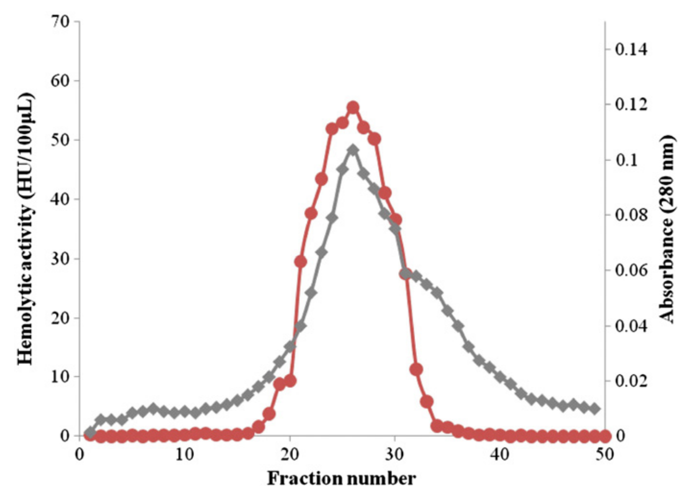

Fig. 2. Second chromatogram after the Sephadex G-100 Superfine column *Circle*, hemolytic activity (HU/100 μL); *square*, absorbance at 280 nm.

Table 1
Purification of hemolysin.

Purification step	Total volume (mL)	Total activity (HU)	Total protein (mg)	Specific activity (HU/mg)	Recovery of activity (%)
Culture supernatant	9430.0	83,639.0	81,679.0	1.0	100.0
Ultrafiltration	565.0	83,447.0	19,909.7	4.2	99.8
Sephadex G-100	2520.0	63,756.0	5090.4	12.5	76.2
DEAE-Sephadex	180.0	26,820.0	28.8	931.3	32.1
CM-Sephadex	28.0	22,540.0	10.1	2231.7	26.9
First Sephadex G-100 Superfine	4.0	3611.5	0.5	6945.2	4.3
Second Sephadex G-100 Superfine	2.5	1314.3	0.2	6571.5	1.6
Shodex PROTEIN KW-802.5	2.4	920.0	0.1	9200.0	1.1

DEAE-diethylaminoethyl; CM-carboxymethyl.

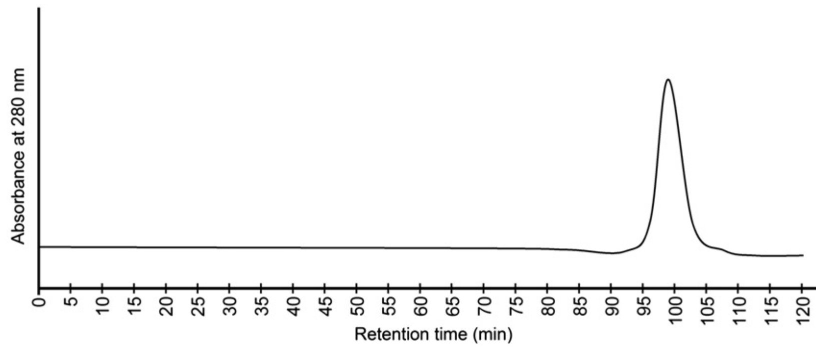

Fig. 3. High-pressure liquid chromatography (HPLC)-derived chromatogram of hemolysin.

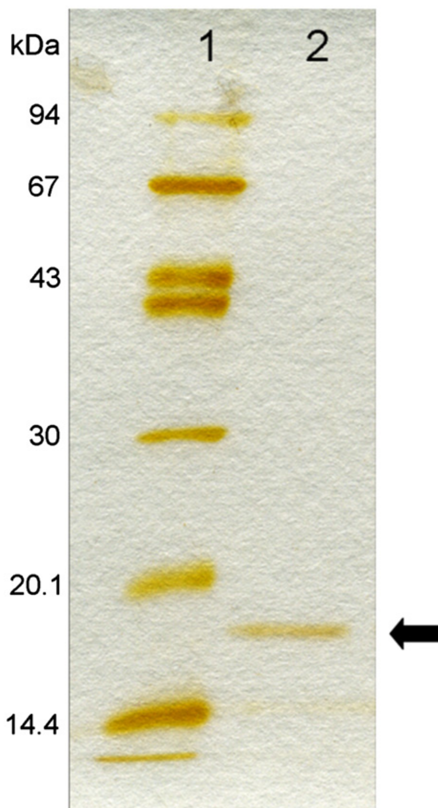

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified hemolysin with silver staining (1) Molecular weight markers and (2) purified hemolysin. The arrow indicates the 16-kDa hemolysin.

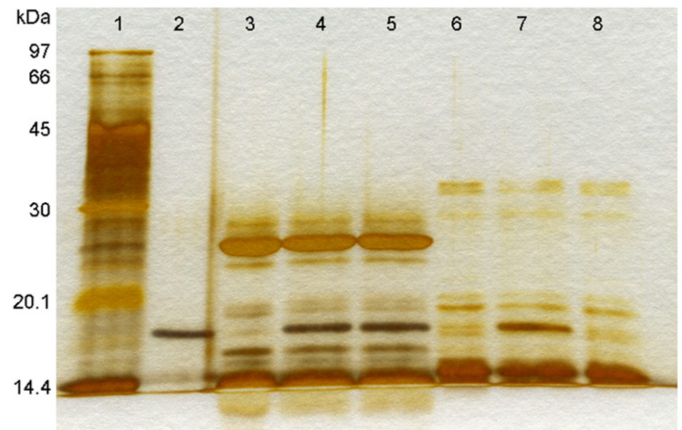

Fig. 5. Effect of treatment with trypsin and proteinase K on the 16-kDa band by silver staining (1) Molecular weight markers; (2) purified hemolysin; (3) 100 μg/mL trypsin; (4) mixture of hemolysin and trypsin without incubation; (5) mixture of hemolysin and 100 μg/mL trypsin with incubation at 37 °C for 60 min; (6) 100 μg/mL proteinase K; (7) mixture of hemolysin and proteinase K without incubation; and (8) mixture of hemolysin and 100 μg/mL proteinase K with incubation at 37 °C for 60 min.

Table 2
Relative activity on the erythrocytes of different species.

Erythrocyte	Relative activity (% ± SD)
Horse	100.0 ± 3.0
Human	63.7 ± 2.2
Sheep	31.4 ± 1.8
Rabbit	34.2 ± 3.2

SD-standard deviation.

species, including *P. oris*, has not yet been described. To the best of our knowledge, ours is the first study to demonstrate the hemolytic activity of the culture supernatant of *P. oris*, as well as purification and characterization of hemolysin produced by *P. oris*.

The culture supernatant obtained from the early logarithmic growth phase had a much higher hemolytic activity, compared to the low activity of the culture supernatant obtained from the stationary phase. A similar pattern of hemolysin production has been reported for *P. intermedia* [22], *P. nigrescens* [25], *Porphyromonas gingivalis* [20], and *Aggregatibacter actinomycetemcomitans* [28]. However, the underlying reason for this phenomenon remains unknown. Periodontal pathogens such as *P. gingivalis, P. intermedia*, and *Fusobacterium nucleatum* produce autoinducer-2 (AI-2) [29]. Further, Fong et al. [30] reported that *A. actinomycetemcomitans* produces AI-2, which increases its leukotoxin production and iron uptake. Levels of AI-2 peak during the mid-exponential phase, and decrease significantly in the late log and stationary phases during *A. actinomycetemcomitans* culture. The production of AI-2 by *P. oris* remains unclear; therefore, further research is required to investigate the underlying mechanism of hemolysin production by *P. oris*.

In this study, the hemolytic activity of *P. oris* was detected in the culture supernatant, but not in the bacterial cell fractions. However, it has been shown that the hemolysins from *P. intermedia* were mainly detected in the outer membrane fraction, suggesting that hemolysins are localized to bacterial cells surfaces [22–24,26]. The trypsin-like cysteine proteases, gingipains produced by *P. gingivalis* have been shown as important virulence factors in adult periodontitis and are localized on the cell surface in almost all strains [31]. Moreover, the *A. actinomycetemcomitans* leukotoxin is a nonsecretory toxin associated with bacterial cells [32]. Although the localization of hemolysin in *P. oris* has not yet been described in detail, it is apparent that hemolytic activity is absent in, and on, the bacterial cell.

Table 3
Effect of various treatments and pH on the activity of hemolysin.

Treatment	Relative activity (% ± SD)
None	100.0 ± 1.5
40 °C	69.8 ± 4.9
50 °C	38.3 ± 0.7
60 °C	8.2 ± 0.2
70 °C	2.0 ± 0.8
pH 5.0	84.0 ± 0.7
pH 5.5	85.6 ± 0.7
pH 6.0	100.0 ± 1.3
pH 6.5	91.0 ± 1.7
pH 7.0	56.4 ± 1.8
pH 7.5	44.8 ± 1.3
Trypsin (100 μg/mL)	108.2 ± 1.4
Cholesterol (10 μg/mL)	72.8 ± 2.1
EDTA (0.1 mM)	197.9 ± 2.0
$MgCl_2$ (5 mM)	83.5 ± 2.7
$CaCl_2$ (5 mM)	113.4 ± 2.9
L-cysteine (20 mM)	140.3 ± 2.1
DTT (25 mM)	192.5 ± 0.9
2-mercaptoethanol (50 mM)	170.3 ± 1.4

SD-standard deviation; EDTA-ethylendiaminetetraacetic acid; DTT-dithiothreitol.

Generally, ammonium sulfate/polyethylene glycol precipitation methods are used for the isolation of enzymes from bacterial culture supernatants [20]. However, during the isolation of hemolysin produced by *P. oris*, these precipitation methods resulted in a significant loss of activity (data not shown). In this study, hemolysin was efficiently purified from the culture supernatant of *P. oris* by ion-exchange and gel-filtration column chromatography.

The molecular weights of hemolysin from *P. gingivalis* and *A. actinomycetemcomitans* hemolysin are 45-kDa [20] and 12-kDa [28], respectively. However, there have been no reports regarding the molecular weight of hemolysin produced by *Prevotella* species. In this study, the molecular weight of the purified hemolysin from *P. oris* was 16-kDa, as determined by SDS-PAGE analysis with silver staining, which is similar to the molecular weight of the hemolysin from *A. actinomycetemcomitans*.

Previously, it was reported that the black-pigmented *Prevotella* spp., *P. gingivalis* and *A. actinomycetemcomitans* produce proteinaceous hemolysin, and the hemolytic activity was inhibited by trypsin treatment. In case of *P. oris*, pre-treatment with trypsin did not inhibit the activity of the purified hemolysin and did not affect the results of the SDS-PAGE analysis (Table 3 and Fig. 5). Although the purified hemolysin could not be observed by CBB staining after SDS-PAGE analysis, hemolysin band disappeared from the silver stained SDS-PAGE gel after treating with proteinase K before SDS-PAGE analysis. These results suggest that the hemolysin produced by *P. oris* has a structure different from that in other bacterial species. The hemolysin of *P. oris* contains proteinaceous substances, but their concentration may have been too low to be seen on SDS-PAGE gel with CBB staining. The hemolysin produced by *P. oris* may consist of conjugated proteins such as lipoproteins, although further studies are required to clarify the constituents of hemolysin in detail.

The erythrocytes from different species differ in their susceptibility to the hemolysin from *P. oris*. Similar results have been reported for *P. intermedia* and *A. actinomycetemcomitans* [22,28]. Amoako et al. [33] also reported that the distribution of receptors against the hemolysin produced by *F. necrophorum* provide an explanation for these differences in erythrocyte susceptibility. Currently, we are studying receptors against hemolysin produced by *P. oris*.

It has been reported that several gram-positive bacteria such as *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria* produce cytolytic proteinaceous toxins called oxygen-labile or thiol-activated cytolysins. In gram-negative bacteria, the hemolysin from *P. intermedia*, designated as prevolysin O [26], and that from *A. actinomycetemcomitans* [28] possess similar properties. In the present study, it was shown that hemolysin produced by *P. oris* also showed thiol-activated cytolytic activity.

Previous studies have shown that streptolysin O binds to cholesterol, and treatment with cholesterol decreases the cytolytic activity of streptolysin O [34,35]. In this study, cholesterol levels did not affect the activity of the purified hemolysin, suggesting that the mechanism of membrane damage caused by the hemolysin produced by *P. oris* differs from that caused by streptolysin O.

A wide variation has been reported in the optimum pH of hemolysins produced by various bacterial species [26]. The hemolysin of *P. oris* requires mildly acidic conditions for hemolysis, and maximal activity was achieved at pH 6.0. 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 [36].

Previously, little was known about the pathogenic factors of *P. oris*. To the best of our knowledge, this is the first report describing hemolysin produced by *P. oris*, which was purified from the culture supernatant and characterized as a 16-kDa, heat-labile proteinaceous, thiol-activated compound.

The results of the present study suggest that hemolysin produced by *P. oris* possesses a potential for cytotoxicity in various oral and systemic infections. Further studies are required to clarify the molecular structure of *P. oris* hemolysin and the mechanisms underlying hemolysis, so that effective strategies can be developed to avoid the growth of *P. oris*, as well as the development of other pathological conditions.

Conflict of interest

No potential conflicts of interest are disclosed.

Acknowledgments

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