〔Original〕

The characteristics of *Prevotella oris* hemolysin and its interaction with the erythrocyte membrane

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Abstract

Prevotella oris produces a proteinous hemolysin, which is observed as a 16-kDa band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. In this study, we analyzed the N-terminal amino acid sequence of *P. oris* hemolysin. We identified 7 N-terminal amino acids, and it was deduced that *P. oris* hemolysin has a short peptide sequence. The amino acid sequence of hemolysin did not share any sequence similarity with any the other known bacterial protein or peptide.

It was also clarified that *P. oris* hemolysin binds to erythrocytes in a temperature-dependent manner before hemolysis. This hemolytic activity was inhibited when the erythrocytes treated with trypsin or glycosidase were used. Cholesterol did not affect the activity. Furthermore, a 46-kDa glycoprotein band disappeared from the profiles of the erythrocyte membranes treated with trypsin when run on a SDS-PAGE gel. This result suggests that the 46-kDa erythrocyte membrane glycoprotein may be a binding site for the hemolysin. This is the first report describing the N-terminal amino acid sequence of *P. oris* hemolysin and its binding site on the erythrocyte membrane.

Key words：*Prevotella oris*, hemolysin, N-terminal amino acid sequence, hemolysin-binding site, glycoprotein

Introduction

Prevotella oris, a nonpigmented, gram-negative, rod-shaped anaerobic bacterium, is assumed to be one of the pathogenic bacteria involved in suppurative inflammation of the oral and head regions. Previous studies, showed that *P. oris* is frequently isolated not only from the lesions of several oral infections such as periodontal disease (Yousefimashouf et al., 1993), endodontic infection (Brito et al., 2007), dentoalveolar abscess (Dymock et al., 1996), and spreading odontogenic infection (Riggio et al., 2007) but also from the lesions of systemic infections such as empyema (Civen et al., 1995) and meningitis (Frat et al.,2004). Furthermore, *P. oris* produces immunoglobulin A protease (Frandsen et al., 1986), hyaluronidase (Grenier & Michaud, 1993), β-lactamase (Iwahara et al., 2006), hemolysin (Sato et al., 2012), and coaggregates with *Porphyromonas gingivalis* (Sato and Nakazawa, 2012), suggesting that these factors may contribute to the pathogenic potential of the organism.

In our previous study, it was demonstrated that *P. oris* produces a proteinous hemolysin, determined as a 16-kDa band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Sato et al., 2012). The characteristics of hemolysin produced by oral bacteria such as *Porphyromonas gingivalis* (Deshpande & Khan,1999), *Prevotella intermedia* (Takada et al., 2003), *Aggregatibacter actinomycetemcomitans* (Kimizuka et al., 1996), and *Treponema denticola* (Chu & Holt, 1994), have been reported. These hemolysins were reported to be proteinous substances, because such activities are inactivated by heat or trypsin treatment. However, there is no report describing the function domains of hemolysin from oral bacteria. In this study, we analyzed the N-terminal

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amino acid sequence of *P. oris* hemolysin, and examined its sequence similarity with any the other known bacterial proteins and peptides.

The hemolysin of *P. oris*, *P. intermedia*, and *A. actinomycetemcomitans* has been reported to be activated by thiol compounds (Sato et al., 2012 ; Takada et al., 2003 ; Kimizuka et al., 1996). Streptolysin O, produced by *Streptococcus pyogenes*, is a member of the thiol-activated cytolysins. It binds to cholesterol in the erythrocyte cell membrane and causes hemolysis by oligomerization on the cell membrane (Bhakdi et al., 1996). Previously, perfringolysin O, listeriolysin O, and cereolysin O were identified as thiol-activated cholesterol-binding cytolysins (Palmer, 2001). However, the binding site of the thiol-activated hemolysin produced by oral gram-negative bacteria remains unknown. In this study, we investigated the interaction between hemolysin from *P. oris* and erythrocyte cell membranes to determine the hemolysin-binding site.

Materials and methods

Bacterial strain and culture conditions

We used a clinical strain of *P. oris* WK1 exhibiting strong hemolytic activity. The WK1 was isolated from the subgingival sites of patients with chronic periodontitis, and it was already confirmed as a *P. oris* species by 16S rRNA sequence analysis (Sato et al., 2012). *P. oris* was subcultured on TYHM blood agar (tryptic soy broth [BD, Difco] supplemented with 0.5% yeast extract [BD, Difco], 1.5% agar [BD, Difco], 5% v/v sheep blood, 5 μg/mL hemin [Wako], and 1μg/mL menadione [Wako]). To purify hemolysin, TYHM broth (tryptic soy broth [BD, Difco] supplemented with 0.5% yeast extract [BD, Difco], 5μg/mL hemin, and 1μg/mL menadione) was used.

Purification of hemolysin and N-terminal amino acid sequence analysis

P. oris hemolysin was purified, in the methods as described previously (Sato et al., 2012). Briefly, the hemolysin produced in the culture supernatant was subjected to ion-exchange and gel-filtration chromatography ; hemolysin fractions were then, subjected to HPLC. The purified hemolysin was desalinized using an amicon Ultra-0.5 centrifugal filter device (10 K ; Millipore, Billerica, MA, USA).

The N-terminal amino acid sequence of the purified hemolysin was determined by Edman degradation with an automatic protein sequencer (Procise 491 ; Applied Biosystems, Tokyo, Japan). Furthermore, the sequence similarity with any the other known bacterial protein and peptide was analyzed using the protein homology search data base system, BLAST (basic local alignment search tool).

Hemolysin-binding studies

Horse erythrocytes were washed 3 times with PBS and resuspended. Then, 2ml of 1% erythrocyte suspension (v/v) with 1unit *P. oris* hemolysin was incubated at 37° C, 27° C, or 4° C for 1h. Thereafter, the erythrocytes were separated by centrifugation (1,500×g for 5min) and washed three times with PBS. Subsequently, the erythrocytes were resuspended with PBS, and the preincubated supernatant (PiS) was added to untreated erythrocytes. After incubation at 37° C for 5h, the hemolytic activity in both samples was measured as described previously (Sato et al., 2012).

Effect of various treatments on hemolytic activity

The effect of cholesterol on hemolytic activity was evaluated after incubation with 1unit *P. oris* hemolysin at 37° C for 1h. The hemolysin pretreated with 10μg/mL or 50μg/mL cholesterol and horse erythrocytes were then incubated at 37° C for 5h and the activity was measured as described previously (Sato et al., 2012). Untreated hemolysin was used as the control, with an assumed hemolytic activity of 100%.

Horse erythrocytes were treated with 2mg/ml trypsin at 37° C for 2h, or 15,000 U *N*-glycosidase, 2,000,000 U *O*-glycosidase and 2,000 U neuraminidase at 37° C for 18h. After treatment, the erythrocytes were washed 3 times with PBS, and then, 2ml of 1% erythrocyte suspension (v/v) with 1unit hemolysin of *P. oris* was incubated at 37° C for 5h. After incubation, hemolytic activity was measured as described previously (Sato et al., 2012).

Effect of trypsin treatment on SDS-PAGE profiles of erythrocyte membrane proteins

To identify the hemolysin-binding site, the cell mem-

branes of horse erythrocytes treated with trypsin were subjected to SDS-PAGE. The erythrocytes were washed 3times with PBS, and then 2ml of the 1% erythrocyte suspension (v/v) was incubated at 37° C with 2mg/ml trypsin for 18h. In order to clarify the difference of band intensity on SDS-PAGE gel, the erythrocytes were treated for long time. The treated erythrocytes were washed 3times with PBS containing 6mg/ml trypsin inhibitor from soybean (Wako, Japan) and then lysed with a hypotonic buffer solution (10mM Tris-HCl, pH7.4). The erythrocytes membranes were collected by centrifugation (15,000 × g for 5min), washed 3times with a hypotonic buffer solution, and subjected to SDS-PAGE. The SDS-PAGE gel was then visualized by CBB staining or PAS staining.

Results

The N-terminal amino acids sequence of the purified hemolysin was identified as ^{1}Asp-Tyr-Glu-Pro-Phe-Glu-Pro^{7}. In this study, the amino acid peaks on the automatic protein sequencer rapidly decreased after each cycle, although the peak for ^{1}Asp was significantly higher. Finally, after identification of Pro^{7}, the N-terminal amino acids sequence could no longer be identified (data not shown). In the protein homology search by BLAST, the N-terminal amino acid sequence similarity with any the other known bacterial protein and peptide could not be detected.

The results of the hemolysin binding studies are shown in Fig. 1. The erythrocytes pre-incubated with hemolysin at 37° C were hemolyzed, and only a weak hemolytic activity was detected in PiS. However, the erythrocytes pre-incubated at 27° C showed moderate activity, whereas those pre-incubated at 4° C did not undergo hemolysis. The activity was maintained in PiS especially almost of all the activity was in PiS in case of pre-incubation at 4° C. Therefore, in this study, it was demonstrated that hemolysin bound to erythrocytes in a temperature-dependent manner.

In order to identify the hemolysin-binding site, the affinity between hemolysin and cholesterol was examined (Fig.2). The hemolytic activity was weakly inhibited by cholesterol treatment. However, there was no statistically significant difference between the results obtained using 10μg/ml and 50μg/ml cholesterol treatment.

To determine whether other membrane constituents other than cholesterol might provide a hemolysin-binding site, erythrocyte surface proteins and sugar chains were enzymatically removed. As a result, the binding activity for the erythrocytes treated with trypsin (Fig.3) and glycosidase (Fig.4) was significantly inhibited. The percent hemolysis inhibition by cholesterol treatment, trypsin treatment and glycosidase treatment was calculated 24.3%, 46.6%, 73.6% respectively (Fig.5). The inhibitory effect of treatment with trypsin or glycosidase was higher than that observed with cholesterol treatment.

Furthermore, the SDS-PAGE profiles of the erythrocyte membrane proteins with or without trypsin treat-

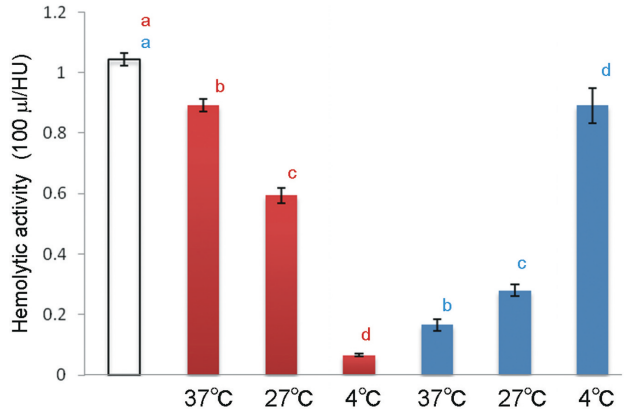

Fig.1. Hemolysin binding test
The existence of hemolysin was determined by measuring the activity in erythrocytes and reaction buffer. ■ : Erythrocyte activity when pre-incubated with hemolysin at 37° C, 27° C, or 4° C for 1 h. ■ : Activity of the pre-incubated supernatant □ : Activity without pre-incubation. The relationship between the different treatments indicates a statistically significant difference ($P < 0.05$) ; Kruskall-Wallis test.

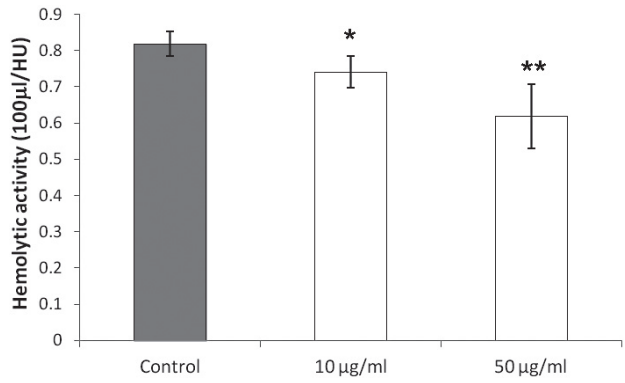

Fig.2. Effect of cholesterol on hemolytic activity
To examine the sensitivity of hemolysin for cholesterol, the activity of hemolysin treated with cholesterol was measured. ■ : Activity without treatment. □ : Activity of hemolysin treated with 10μg/mL, 50μg/mL cholesterol. Mann-Whitney *U* test, $*P<0.05$ vs. control, $**P<0.01$ vs. control

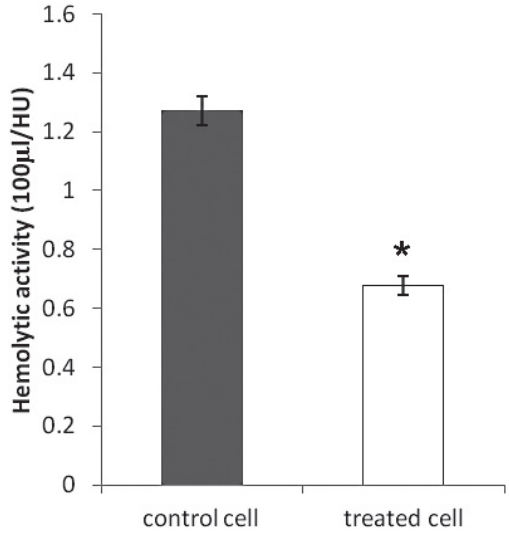

Fig.3. Hemolytic activity on erythrocytes treated with trypsin
The activity on erythrocytes treated with 2mg/ml trypsin was measured for treated cells (■). Erythrocytes not treated with trypsin were used as control cells (□). Mann-Whitney *U* test, * *P*<0.05 vs. control

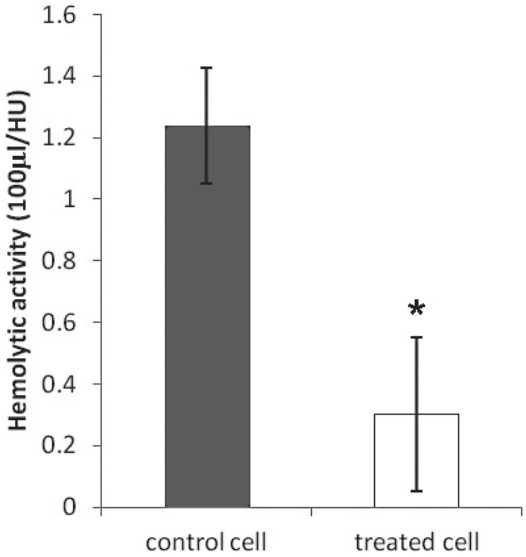

Fig.4. Hemolytic activity on erythrocytes treated with glycosidase
The activity on erythrocytes treated with 15,000 U *N*-glycosidase, 2,000,000 U *O*-glycosidase, and 2,000 U neuraminidase was measured for treated cells (■). Erythrocytes not treated with trypsin were used as control cells (□). Mann-Whitney *U* test, **P*<0.05 vs. control

ment were examined (Fig.6). When the erythrocytes were treated with trypsin, a 46-kDa and 42-kDa band disappeared from the SDS-PAGE gel following CBB staining. However, a 31-kDa band was determined, although it was not detected in the case of the erythrocytes not treated with trypsin. Furthermore, the intensity of the 23-kDa band was higher in the erythrocytes treated with trypsin. On the SDS-PAGE gel stained with PAS, a 46-kDa band also disappeared. Although several

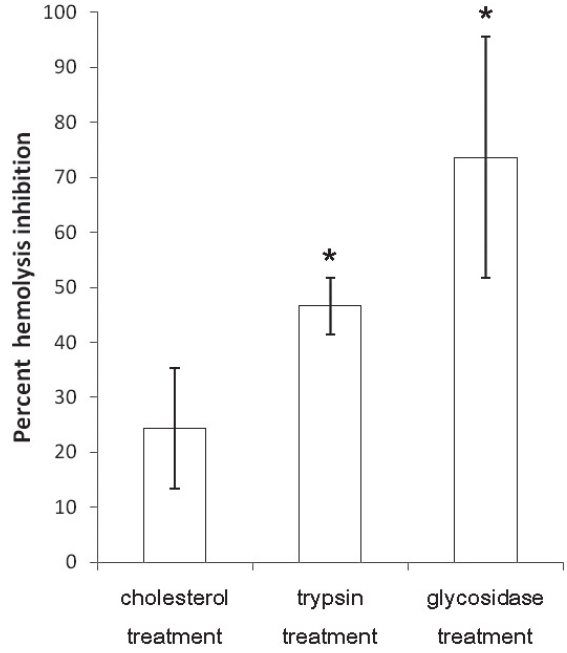

Fig.5. Percent hemolysis inhibition by various treatments
The percent hemolysis inhibition by 50μg/mL cholesterol treatment, trypsin treatment and glycosidase treatment were calculated as follows ; { (hemolytic activity of control group-hemolytic activity of test group)/hemolytic activity of control group}×100=% hemolysis inhibition
The Statistical significance was calculated using Mann-Whitney *U* test. **P*<0.05 vs. cholesterol treatment

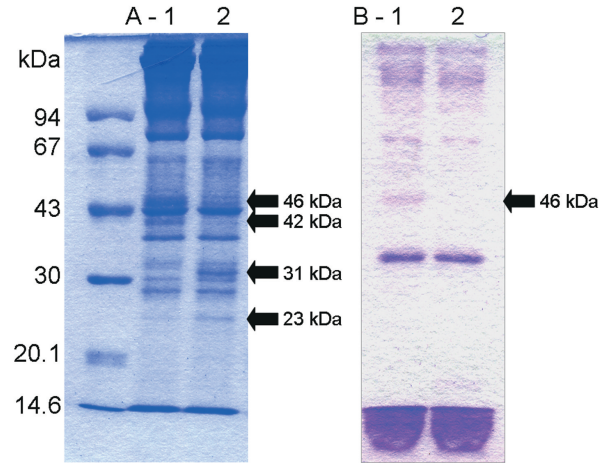

Fig.6. SDS-PAGE profiles of erythrocyte membrane proteins
Erythrocytes treated with/without trypsin were hemolyzed, and then cell membranes were collected by centrifugation. Erythrocyte membranes not treated with trypsin were loaded onto lane1. Erythrocyte membranes treated with trypsin were loaded onto lane2. The gel, A, was visualized by CBB staining ; the gel, B was visualized by PAS staining.

major bands were determined by PAS staining, there was no difference between the results in the presence and absence of trypsin except for the 46-kDa band.

Discussion

Previously, we had purified a 16-kDa proteinous

hemolysin produced by *P. oris* by using gel-filtration and ion-exchange chromatography (Sato et al., 2012). In this study, the 7 N-terminal amino acids of *P. oris* hemolysin were identified by Edman degradation. The amino acid peaks in the automatic protein sequencer rapidly decreased from cycle to cycle. In light of this phenomenon, two possibilities were suggested. One suggestion was that the amount of hemolysin is too low. However, because the peak of ^{1}Asp was sufficiently high, it was assumed that the amount of hemolysin was sufficient. The other possibility is that the protein part of hemolysin is a short peptide. In our previous study, the 16-kDa band was not observed by CBB staining, although the much clearer band was identified with silver staining. Furthermore, on the SDS-PAGE gel loaded with the partially purified hemolysin, the hemolysin band was stained by sudan black staining (data not shown). These phenomena support the possibility that *P. oris* hemolysin may be composed of a lipid and a short peptide.

Additionally, N-terminal amino acid sequencing revealed that the hemolysin of *P. oris* includes only Try and Phe, two aromatic amino acids, which have an affinity to CBB. Thus, it is assumed that *P. oris* hemolysin is not easily stained by CBB because of its amino acid composition. Furthermore, we demonstrated that purified hemolysin is not decomposed by trypsin treatment, based on SDS-PAGE analysis (Sato et al., 2012). This phenomenon supports the result of the N-terminal amino acid sequence in this study, as this hemolysin does not include Arg and Lys, which are recognized by trypsin.

In the homology search using BLAST, the N-terminal amino acids of *P. oris* hemolysin did not show any similarity to any the other known protein or peptide produced by bacteria. Although the internal amino acids sequence of crude *P. gingivalis* hemolysin has been determined (Deshpande & Khan, 1999), there is no report describing the N-terminal amino acids sequence of hemolysin from oral bacteria.

Previously, there has been no report describing sugar/lipid modification of hemolysin. Thus, wheather hemolysin from oral bacteria is a lipoprotein remains unknown. Recently, bacterial lipo-proteins have been well studied in gram-negative bacteria such as *E. coli* (Okuda & Tokuda, 2011) and *spirochete* (Haake, 2000), gram-positive bacteria (Sutcliffe & Russell, 1995), and *Mycoplasma* (Rezwan et al., 2007), indicating that many microorganisms have lipo-proteins. Furthermore, using gene analyses, the number of lipoprotein gene has been identified in pathogenic bacteria such as *Helicobacter pylori*, *Borrelia burgdorferi* and *Mycobacterium tuberculosis* (薬師 et al., 2000). Bacterial lipo-proteins exhibit a number of biological activities such as the activation of immunocytes and the induction of apoptosis (Kovacs-Simon et al., 2011). However, the cytotoxic activity of lipo-proteins has not yet been reported.

In this study, we clarified that *P. oris* hemolysin bound to erythrocytes in a temperature-dependent manner (Fig.1). The level of hemolysin binding was stronger after pre-incubation at 37° C than after pre-incubation at 27° C or 4° C. Thus, the environmental conditions in the oral cavity are appropriate for the hemolytic activities of *P. oris*. Similarly, the hemolysis rate of *P. intermedia* hemolysin is temperature-dependent (Beem et al., 1998). In contrast, *Fusobacterium necrophorum* hemolysin has been reported to bind to erythrocytes in a temperature-independent manner (Amoako et al., 1997). These phenomena may imply that the hemolysis mechanism is basically different among oral gram-negative bacterium, but details remain unknown.

Previously, we reported that the hemolysin of *P. oris* was activated by pre-treatment with thiols such as L-cysteine, DTT, and 2-mercaptoethanol (Sato et al., 2012). These phenomena are similar to the properties of streptolysin O, a product of *S. pyogenes*. The activity of streptolysin O is strongly inhibited by cholesterol, because the cholesterol on the erythrocyte membrane is a target for streptolysin O (Prigent et al., 1976 ; Bhakdi et al., 1985). However, the activity of *P. oris* hemolysin was not inhibited by cholesterol pre-treatment (Fig.2). Thus, the major binding site of *P. oris* hemolysin is not likely to be a cholesterol on the erythrocyte membrane. In contrast, the activity of the erythrocytes treated with trypsin or glycosidase was inhibited, suggesting that the binding site of this hemolysin may be a glycoprotein (Fig.3, 4).

To identify the hemolysin-binding site, the SDS-PAGE profiles of erythrocyte membranes were examined (Fig.6). When the erythrocytes were treated with

trypsin, a 46-kDa glycoprotein band disappeared from the SDS-PAGE gel when visualized with CBB and PAS staining. Furthermore, a 42-kDa protein band also disappeared. However, it is considered that the 42-kDa protein may be a scaffold protein as a part of the erythrocyte membrane, because this protein was not visualized by PAS staining. Additionally, a 31-kDa band was detected following trypsin treatment. It is being investigated whether this protein is a product from the 46-kDa protein. The intensity of the 23-kDa band was higher in the erythrocyte membrane treated with trypsin. It is assumed that the recovery of the erythrocytes membrane proteins was affected by trypsin treatment. Therefore, the 46-kDa glycoprotein is most likely to behave as a binding site for *P. oris* hemolysin. Further investigations are in progress to identify the 46-kDa glycoprotein.

Previously, glycophorin, one of the main proteins on the erythrocyte surface, was identified as a receptor for *E. coli* α-hemolysin (Aitziber et al., 2001) and *Vibrio cholera* El Tor hemolysin (Zhang et al.,1999). Furthermore, it has been shown that the receptor for *Aeromonas sobria* hemolysin is a glycoprotein on the intestine cell (Wang et al., 1999). In the case of oral bacteria, there are only few reports describing the hemolysin receptor, although it has been proposed that the erythrocyte receptor for *Fusobacterium necrophorum* hemolysin is phosphatidylcholine (Amoako et al., 1998).

Because the erythrocytes treated with glycosidase had not undergone hemolysis by *P. oris* hemolysin, the sugar chain of the 46-kDa protein may be involved in hemolysin binding. However, the saccharides comprising the cell surface sugar chain (glucose, galactose, mannose, arabinose, xylose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid) did not affect the hemolytic activity (data not shown). Thus, there is the possibility that *P. oris* hemolysin does not have an affinity against the monosaccharide but instead against the conformation of the sugar chain.

Previously, we reported the release of GAPDH from erythrocyte membranes following treatment of the cell with *P. oris* hemolysin (Sato et al., 2012). GAPDH is a glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1, 3-diphosphoglycerate. Furthermore, recent studies demonstrated that GAPDH has many other important roles including the cellular response to oxidative stress (Hara et al., 2006), apoptosis (Huang et al., 2009), transcriptional (Zheng et al., 2003) and posttranscriptional gene regulation (Backlund et al., 2009), chromatin structure (Demarse et al., 2009), maintenance of DNA integrity (Azam et al., 2008), vesicular transport (Tisdale, 2001), and receptor-mediated cell signaling (Raje et al., 2007). 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 (Tsai et al., 1982 ; Mallozzi et al., 1995). Therefore, a significant release of free GAPDH into the cytosol may alter cellular homeostasis, and thereby lead to hemolysis. However, we consider that there is not direct interaction between *P. oris* hemolysin and GAPDH, because cell membrane GAPDH is one of a scaffold protein as a part of the erythrocyte membrane. After the hemolysin binding to 46-kDa erythrocytes glycoprotein, GAPDH may be released from cell membrane through some intracellular reactions. Further studies are in progress to understand the detailed mechanism of hemolysin-driven cell damage due to *P. oris*.

In this study, we examined the N-terminal amino acids sequence of *P. oris* hemolysin and its interaction with erythrocytes. Our findings are useful for better understanding the mechanisms underlying *P. oris* hemolysin-driven cell damage.

Conclusion

It was suggested that h *P. oris* hemolysin may have a short peptide. This hemolysin binds to erythrocytes membrane in a temperature-dependent manner before hemolysis. Further, there is the possibility that the hemolysin- binding site is a 46-kDa glycoprotein on the erythrocytes membranes.

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経歴

平成14年４月	北海道医療大学歯学部	入学
平成20年３月	同	卒業
平成20年４月	北海道医療大学歯科内科クリニック	臨床研修歯科医
平成21年３月	同	修了
平成21年４月	北海道医療大学大学院歯学研究科	入学
平成25年３月	同	修了