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Short communication

Distribution and characterization of hemolytic activity by an oral anaerobe from the *Streptococcus milleri* group

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Some oral anaerobes from the *Streptococcus milleri* strain group were found to secrete human specific hemolytic toxin, which was detected when bacteria were cultured in Todd-Hewitt broth and Brain Heart Infusion broth. The toxin elicited by the *Streptococcus intermedius* strain was partially fractionated by ammonium sulfate precipitation. Preincubation with glutathione or cysteine showed significant inhibiting effects; however, no effects were seen with dithiothreitol or β-mercaptoethanol, and cholesterol was a weak inhibitor. Five kinds of protease inhibitor had no effect on the hemolytic activity, and rabbit preimmune and immune sera against the bacterial cells showed weak inhibition at a similar level. Digestion with trypsin, chymotrypsin, proteinase-K, subtilisin and pronase-P brought about a rise in activity, followed by a decrease during long-term incubation. Other enzymes tested showed no effects. Further, the presence of the intermedilysin gene in the portion with hemolytic activity was not identified by polymerase chain reaction.

Key words: human specific hemolysis; *Streptococcus milleri*; thiol-insensitive

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Streptococcus milleri are commonly found organisms in certain areas of the human body that are closely associated with a wide variety of local and systemic suppurative infections. The etiologic role of streptococcus species in systemic infections has been increasingly elucidated, and the *S. milleri* group has been demonstrated to have some virulence factors (1, 4).

Several hemolytic factors of streptococcus species have been reported. Group A, B, C and G hemolytic streptococci secrete streptolysin O (SLO) and S (SLS), which have membrane-disrupting characteristics. Further, these are known to be possible toxic agents for the causes of pharyngitis, tonsillitis, scarlet fever, and rheumatic fever (2). *Streptococcus pneumoniae* produce a hemolytic toxin, pneumolysin, that appears to be associated with bacterial pneumonia, and is one of the principal causes of bacterial meningitis and otitis media in young children (3). In the oral cavity, three *Streptococcus mutans* isolates among 47 taken from dental plaque showed beta-hemolysis (8). However, the possible role of the elaborated toxin remains unclear.

In the present study, we tested 209 *S. milleri* group strains: 66 isolates from the mouths of young adults (9), 66 isolates from children (10), 71 isolates from systemic purulent infections found in various locations in the human body (5), as well as 6 reference strains, NCTC 10708, FW 73, ATCC MG 9895, ATCC 33397, ATCC 27335 and ATCC 27823. Additionally, we tested four strains of *Streptococcus sanguis*, 9H, OMZ9, K44Y, and ATCC 10556, one of *Streptococcus parasanguis*, ATCC 15911, one of *Streptococcus gordonii*, ATCC 10558, one of *Streptococcus oralis*, ATCC 35037, one of *S. mutans*, GS5, and one of *Actinomyces naeslundii*, KWS81. All cells were cultured anaerobically (85% N_2, 10% H_2 and 5% CO_2) at 37°C overnight in Todd-Hewitt broth and Brain Heart Infusion broth for the screening of hemolytic activity. Culture media were purchased from BBL Microbiology Systems (Cockeysville, MD).

Erythrocytes were derived from various sources. Horse, sheep and rabbit defibrinated blood samples were purchased from Nippon Bio-Test Laboratories Inc. (Tokyo, Japan), and defibrinated cow blood sample came from Nippon Bio-Material Center Co. (Tokyo, Japan). Peripheral blood from two male and two female Wistar rats and dd-line mice were collected in the presence of 0.1% heparin (Kyudo Co., Kumamoto, Japan). Human blood was provided by the

Japanese Red Cross (Kagoshima Blood Center, Kagoshima, Japan). Erythrocytes were washed with phosphate buffered saline (10 mM sodium-potassium phosphate buffer, pH 6.8, 140 mM NaCl and 3 mM KCl) several times, followed by re-suspension in phosphate buffered saline at final concentration of 4% (vol/vol). The final solution contained $2.0 \times 10^5/\mu l$ erythrocytes.

A hemolytic assay was carried out in 96-well flat-bottom microtiter plates (No. 3915, Falcon, Nippon Becton Dickinson Co., Tokyo, Japan). The culture supernatant (50 μl) was gently mixed with an equal volume of 2% erythrocyte solution and stored at room temperature (23°C). Absorbance at 630 nm was measured for 1 h using a Microelisa Auto Reader, MR580 (Dynatech Instruments, Inc., Torrance, CA). Culture media was used instead of bacterial culture supernatants as a negative control. All assays were performed in duplicate and the averages were taken as the results. An individual strain was determined to be positive when absorbance by its reaction solution after 1 h of incubation was less than 30% of the negative control.

Among all of the tested strains, 100 produced hemolysin with an activity that was specific for human erythrocytes, regardless of ABO typing. No hemolysis was demonstrated toward erythrocytes from other species. No difference in hemolytic sensitivity was demonstrated in strains from the 40 human donors, comprising 10 of each blood type (A, B, AB and O). Hemolysis occurred rapidly following a short induction at room temperature (data not shown). The *S. milleri* group was classified as *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius* (7), and the rate of toxigenicity was 2.4%, 22.5% and 100.0%, respectively.

For further characterization of the present toxin, *S. intermedius* 1208-1 (serotype g, biotype IIc) was used, which was previously isolated from the mouth of a healthy child and had no Lancefield antigen (10). The bacteria was anaerobically grown in Todd-Hewitt broth or Brain Heart Infusion broth at 37°C. During the 3-day culture, absorbance at 600 nm, pH and hemolytic activity in culture media were determined at appropriate times. The growth rates in Todd-Hewitt broth and Brain Heart Infusion broth were similar; however, the toxic activity in Todd-Hewitt broth was approximately twice of that in Brain Heart Infusion broth throughout the growing period, and pH reached 6.3 in Todd-Hewitt and 5.6 in Brain Heart Infusion broth in the late-stationary phase.

To prepare a highly concentrated toxin, 2 liters of Todd-Hewitt broth culture supernatant of 1208-1 was brought to 50% saturation by the slow addition of powdered ammonium sulfate. After gentle mixing for 2 h at 4°C, the precipitate was collected by centrifugation at $10{,}000 \times g$ for 30 min at 4°C. The acquired precipitate was then dissolved in 20 ml of 20 mM Tris-acetic acid buffer (pH 5.5) and 150 mM NaCl solution, and dialyzed against the same buffer at 4°C. This concentrated hemolytic solution is hereafter referred to as concentrated toxin solution.

To estimate the effectors, concentrated toxin solution (5 μl) was mixed with various materials in each well (50 μl). After incubation at room temperature for 10 min, 2% erythrocyte solutions (50 μl) were added for the hemolytic assay. The effectors tested were four reducing agents, cholesterol, five proteinase inhibitors and rabbit sera. The reducing agents were dithiothreitol, β-mercaptoethanol, glutathione and cysteine. The proteinase inhibitors employed were benzamidine, ovoinhibitor, Na-P-tosyl-L-lysine chloromethyl ketone, phenylmethylsulfonyl fluoride, and Protease Inhibitor Cocktail. Ovoinhibitor was purchased from Takara Syuzo Co. (Ohzu, Japan) and the other reagents were from Nacalai Tesque Co. (Kyoto, Japan). Protease Inhibitor Cocktail was from Sigma Chemical Co. (St. Louis, MO).

When concentrated toxin solution was preincubated separately with the four reducing agents, dithiothreitol and β-mercaptoethanol had no effect, whereas glutathione and cysteine caused complete inactivation at a concentration of 12 μM (Table 1). Used alone at a concentration of 12 μM no agent caused hemolysis. Hemolytic activities in the presence of cholesterol at concentrations of 200 μM, 100 μM, 50 μM, 30 μM, 20 μM and 10 μM were 0%, 34.2%, 59.6%, 66.3%, 67.1% and 84.0%, respectively. For comparison, 150 units of SLO (Sigma), whose activity was nearly the same as 5 μl of concentrated toxin solution, was tested in the presence of 10 mM dithiothreitol, and activity in the presence of 3 μM, 1 μM, 500 nM and 60 nM cholesterol was 0%, 36.7%, 79.6% and 85.0%, respectively. Ethanol for cholesterol solubilization (20%) in a reaction solution (100 μl) did not significantly lyze the human erythrocytes.

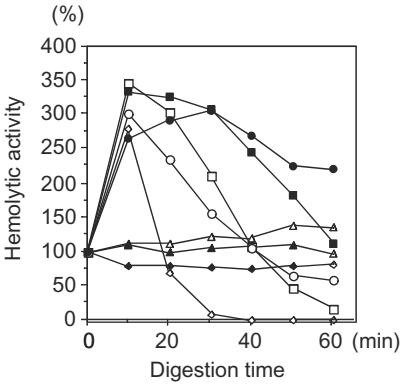

Fig. 1. Effects of proteolytic enzymes on hemolytic activity. Concentrated toxin solution was incubated with each enzyme (1 mg/ml) at 37°C for suitable periods and hemolytic activity was determined. Tested enzymes were trypsin (○), chymotrypsin (●), elastase (△), papain (▲), proteinase-K (□), subtilisin (■), pronase-P (◇) and collagenase (◆). Values on the x-axis indicate enzyme digestion time.

To determine if hemolysis was derived from proteinase activity, five proteinase inhibitors were examined. First, 5 μl of concentrated toxin solution was incubated with one of 0.5 mg/ml of ovoinhibitor, a 100-fold dilution of Protease Inhibitor Cocktail, or 5.0 mM of another reagent in a total volume of 50 μl for 10 min. Next, 50 μl of 2% erythrocyte solution was added to make a total volume of 100 μl and was incubated for up to 1 h. All of these procedures were carried out at room temperature. These treatments did not have a large effect on hemolysis (113∼86%). Hemolysis was not revealed in this condition except with concentrated toxin solution.

Rabbit antiserum against 1208-1 cells was prepared as described previously (11).

Table 1. Effect of reducing agents on the hemolytic activity

	Hemolytic activity (%)[a]	
Effector	12 μM	6 μM
Reducing agents		
dithiothreitol	104.3	97.1
β-mercaptoethanol	107.4	101.8
Glutathione	2.6	76.3
cysteine	0.0	93.4
–[b]	100.0	–

[a]Each assay was carried out duplicate and the average indicated.
[b]Hemolytic assay as positive control was carried out in the absence of effector.

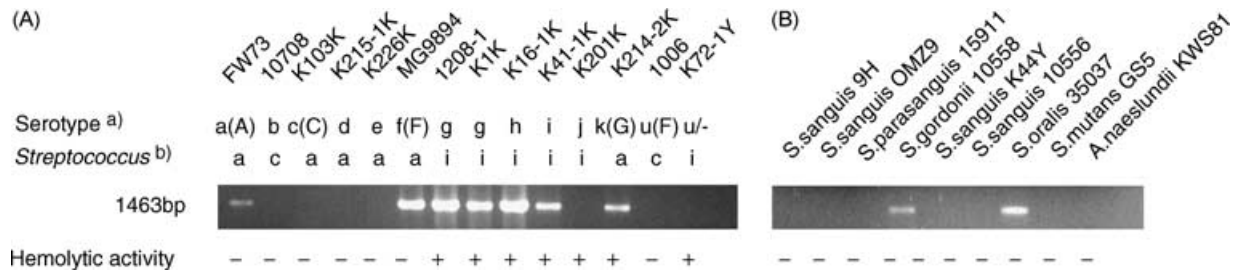

Fig. 2. Distribution of intermedilysin gene and hemolytic activity among streptococci. Amplification of the intermedilysin gene among milleri group streptococci (A), and other streptococci (B) was performed by polymerase chain reaction. Arrow indicates the 1463-bp fragment from intermedilysin gene. [a]This serotype among the milleri group streptococci has been described previously (10, 11). [b]*S. anginosus* (a), *S. constellatus* (c), *S. intermedius* (i).

Concentrated toxin solution 5 μl was preincubated with 40 μl of anti 1208-1 serum or preimmune serum, each with 5 μl of buffer, in a total volume of 50 μl for 10 min. Next, 50 μl of 2% erythrocyte solution was added and incubated for up to 30 min. The concentrated toxin solution solution preincubated with anti 1208-1 serum showed 65% activity and that preincubated with preimmune serum showed 69% activity, as compared to the same reaction containing no concentrated toxin solution. These findings indicate that a cell-associated form of the toxin does not exist. Further, rabbit sera samples without concentrated toxin solution showed no hemolytic activity.

Next, erythrocyte solutions (2% (vol/vol)) were incubated with a reducing agent (30 μM), cholesterol (100 μM), benzamidine (10 mM), ovoinhibitor (1.0 mg/ml), Na-P-tosyl-L-lysine chloromethyl ketone (10 mM), phenylmethylsulfonyl fluoride (2.5 mM), Protease Inhibitor Cocktail (100-fold dilution) or rabbit sera (40% (vol/vol)) at room temperature for 10 min. After several washes with phosphate buffered saline, the treated erythrocytes were assessed for hemolytic susceptibility. These various treatments showed little hemolytic effect on erythrocytes.

To estimate the effect of several hydrolytic enzymes, 100 μl of concentrated toxin solution and each enzyme solution with phosphate buffered saline was mixed to make a 200-μl sample, and then incubated at 37°C for the appropriate periods. The enzymes used and their concentrations were as follows: 1 mg/ml of trypsin (Wako Pure Chemical Industries Co., Osaka, Japan), chymotrypsin, elastase, papain, proteinase-K, subtilisin (Sigma), pronase-P (Kaken Chemical Co., Tokyo, Japan), collagenase (Sigma) and lysozyme (Seikagaku Kogyo Co., Tokyo, Japan); 20 mU/ml of endo-β-galactosidase (Seikagaku), α-galactosidase, β-glucosidase, and neuraminidase (Sigma) and 200 μg/ml of mutanolysin (Sigma). Concentrated toxin solution was treated with an equal volume of phosphate buffered saline at 37°C for the same period and used for the positive control reaction. Treated concentrated toxin solution 10 μl specimens and 40 μl of phosphate buffered saline were transferred into reactive wells, and then mixed with 50 μl of 2% erythrocyte solution for the hemolytic assay.

Interestingly, digestion with trypsin, proteinase-K, subtilisin or pronase-P for 10 min caused maximum activities, whereas longer digestion times resulted in a reduction of hemolytic activity (Fig. 1). Pronase-P was the most sensitive enzyme, digestion for 40 min causing a complete loss of activity. Chymotrypsin was also a sensitive enzyme, though weaker, as maximum hemolytic activity was seen after digestion for 30 min, and the activity after 1 h was more than twice that of the primary activity. The acquired maximum activities were between 2.5 and 3.5-fold with digestion with these five sensitive enzymes. On the other hand, digestion with elastase, papain and collagenase had no effect on hemolytic activity. Further, none of the enzymes tested showed hemolytic activity under the same experimental conditions, except for concentrated toxin solution. Treatment of the six carbohydrate hydrolyzed enzymes had no significant effect, as activity after 1 h pretreatment with these agents was 91–110% of the positive control. Reactions in the absence of concentrated toxin solution showed no hemolysis.

S. intermedius elicited a human-specific cytolysin, intermedilysin (6). Twenty-three strains were tested for the present toxin using a polymerase chain reaction technique with a primer pair composed of ILY-NFw and ILY-CBw, which have been described previously. Hemolytic activity toward human erythrocytes was found in nine of the strains (Fig. 2), but the activity was not directly related to the presence of the intermedilysin gene. As a result, further comparisons of intermedilysin and the present toxin are necessary.

The characteristics for human erythrocyte specificity, dithiothreitol independence and weak inhibition by cholesterol were similar, but there were also some differences. First, results from the concentrated toxin solution used in the present study were independent of salt concentration as the solution dialyzed against distilled water possessed approximately 70∼80% activity even after 1 month at 4°C, whereas intermedilysin was sensitive to low salt concentration. In addition, we attempted to purify the toxin molecule. Column procedures that used ion exchange or hydrophobic interaction chromatographs produced no active fractions regardless of salt concentration and pH value. We therefore consider that the hemolytic activity observed in this study was not derived from a homogeneous molecule. In spite of these differences, the possibility can not be ruled out that the toxin observed in the present study was a derivative of intermedilysin.

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