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Temperature-dependent hemolytic activity of membrane pore-forming peptide toxin, tolaasin

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Tolaasin, a pore-forming peptide toxin produced by *Pseudomonas tolaasii*, causes brown blotch disease on cultivated mushrooms. Hemolysis using red blood cells was measured to evaluate the cytotoxicity of tolaasin. To investigate the mechanism of tolaasin-induced cell disruption, we studied the effect of temperature on the hemolytic process. At 4 °C, poor binding of the tolaasin molecules to the erythrocyte membrane was observed and most of the tolaasin molecules stayed in the solution. However, once tolaasin bound to erythrocytes at 37 °C and the temperature was decreased, complete hemolysis was observed even at 4 °C. These results indicate that tolaasin binding to cell membrane is temperature-sensitive while tolaasin-induced membrane disruption is less sensitive to temperature change. The effect of erythrocyte concentration was measured to understand the membrane binding and pore-forming properties of tolaasin. The percentage of hemolysis measured by both hemoglobin release and cell lysis decreased as erythrocyte concentration increased in the presence of a fixed amount of tolaasin. The result shows that hemolysis is dependent on the amount of tolaasin and multiple binding of tolaasin is required for the hemolysis of a single cell. In analysis of dose-dependence, the hemolysis was proportional to the tenth power of the amount of tolaasin, implying that tolaasin-induced hemolysis can be explained by a multi-hit model. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hemolysis; peptide toxin; pore formation; *Pseudomonas tolaasii*; tolaasin

Introduction

Tolaasin forms membrane pore and causes a brown blotch disease on various cultivated mushrooms [1]. It is a lipodepsipeptide toxin produced by *Pseudomonas tolaasii* and consists of eighteen amino acids with molecular mass of 1,985 Da. The amino acid sequence of Tol I is β-hydroxyoctanoyl-Δ-But-D-Pro-D-Ser-D-Leu-D-Val-D-Ser-D-Leu-D-Val-L-Val-D-Gln-L-Leu-D-Val-Δ-But-D-alloThr-L-Ile-L-Hse-D-Dab-L-Lys, where Δ-But, L-Hse, and D-Dab denote 2,3-dehydro-2-aminobutyric acid, L-homoserine, and D-2,4-diaminobutyric acid, respectively. Two analogues of tolaasins, Tol I and Tol II, were isolated from *P. tolaasii* Paine, and their primary structures were determined [2]. Both analogues have a β-hydroxyoctanoic acid covalently linked to *N*-terminus. Eight and seven analogues including Tol I and Tol II were identified and their structures were determined from a Japanese strain and NCPPB2192 of *P. tolaasii*, respectively [3,4]. Since tolaasin has two positive charges at *C*-terminus, it is a cationic peptide which has hydrophobic domain at *N*-terminus.

Rainey *et al*. [5] investigated the biological spectrum of tolaasin toxicity. The mechanism of tolaasin-induced structural disruption of cell membrane has been explained by both pore-forming and biosurfactant properties of tolaasin [6]. Ion channel formation of tolaasin was demonstrated in a planar lipid bilayer and two types of ion channels were characterised [7,8]. Recently, a relatively simple and time-saving purification procedure consisting of a four-step chromatography was reported for the purification of tolaasin [9]. Although the basic characteristics of tolaasin-induced membrane pore have been investigated by measuring tolaasin-induced hemolysis and the effects of various osmotic protectants and metal ions on hemolysis [5,10,11], the detailed mechanism of tolaasin-induced cell disruption is not well understood.

The modes of action for pore-forming peptide toxins consist of several steps, such as toxin binding to the cell membrane, aggregation of peptides and formation of membrane pore, collapse of transmembrane electrochemical gradients, increment of water and ion flow across membrane, and cell swelling or osmotic lysis [12]. It is believed that tolaasin-induced cell disruption also follows these steps. Since the cytotoxic mechanism of many pore-forming toxins has been successfully studied by measuring the temperature-dependence of their lytic activity [13–17], we examined the effect of temperature on the pore formation of tolaasin to understand the mechanism of tolaasin-induced cell disruption. In this study, tolaasin-induced hemolysis was measured and membrane-binding properties of tolaasin were analyzed at various temperatures. We have found that the membrane binding of tolaasin is dependent on temperature, but the hemolysis by membrane-bound tolaasins shows poor dependency.

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Materials and Methods

Preparation and Purification of Tolaasin

Tolaasin was purified according to the methods described by Cho *et al*. [9]. Briefly, tolaasin molecules in the culture supernatant of *P. tolaasii* 6264 were collected by ammonium sulfate precipitation and ultracentrifugation. The precipitated crude tolaasin preparation was suspended in 10 mM sodium phosphate buffer (pH 7.0) and subjected to Sephadex G-75, Dowex 1X8-50, and carboxymethyl-cellulose column chromatographies. The purified tolaasin was resuspended and dialyzed for 8 h against a 10 mM sodium phosphate buffer. The concentration of tolaasin was determined by the Lowry method [18]. The purity of tolaasin preparation was evaluated by SDS-PAGE with Tris-Tricine precast gel (Bio-Rad Laboratories, Hercules, CA) and HPLC using Protein-Pak™ 60 column (Waters Co.). The purified peptides were stored at −80 °C. Molecular masses of the peptides were determined by MALDI-TOF mass spectrometry (Axima-CFRplus, Shimadzu/Kratos). Tricine [*N*-{Tris(hydroxymethyl)methyl}-glycine], Tris-Tricine precast gel, and molecular weight markers for peptide gel electrophoresis were purchased from Bio-Rad Laboratories. Materials for chromatographies and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Hemolysis

Cytotoxicity of tolaasin was evaluated by measuring hemolytic activity with rat erythrocytes [5,10,11]. Defibrinated rat erythrocytes were washed with HBS (HEPES-buffered saline; 5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] and 150 mM NaCl, pH 7.4) three times and diluted with the same buffer to make a 10% erythrocyte solution. The tolaasin preparation properly diluted with HBS was added to the erythrocyte solution and incubated for 30 min at 37 °C. Hemolysis was monitored by measuring the absorbance changes at 600 nm and hemoglobin release was at 540 nm using a spectrophotometer (U-2000, Hitachi Ltd., Tokyo). One hemolytic unit (HU) of tolaasin was defined as the amount of tolaasin which is able to induce complete hemolysis of 1% erythrocyte within 30 min. The amount of one unit was equivalent to 0.53 μg ml^{-1} of the purified tolaasin, which is approximately 26.5 nM [8]. To measure the tolaasin-induced hemoglobin release, erythrocytes were incubated with tolaasin for 1 h at 37 °C and centrifuged for 10 s at 3000 rpm, and the supernatant was monitored by measuring the absorbance at 540 nm.

Results

Temperature-dependence of Tolaasin-induced Hemolysis

The cytotoxicity of tolaasin could be dependent on membrane structure and fluidity of the host cells. Temperature is one of the major components controlling these factors. Tolaasin-induced hemolysis was measured at various temperatures ranging from 4 to 40 °C (Figure 1(A)). In the presence of 1 HU tolaasin, erythrocytes were completely hemolyzed within 30 min at 37 °C. Hemolysis was very slow at 30 °C and started after a 2 h incubation. When erythrocytes were incubated at temperatures below 20 °C, hemolysis was not observed even after 3 h incubation. In the presence of 2 HU, however, hemolysis became faster and occurred within 2 h at 10 °C. No hemolysis was observed with both doses at 4 °C (Figure 1(B)). These data demonstrate the temperature-dependence of tolaasin toxicity and the increase in hemolytic activity of tolaasin at high temperatures.

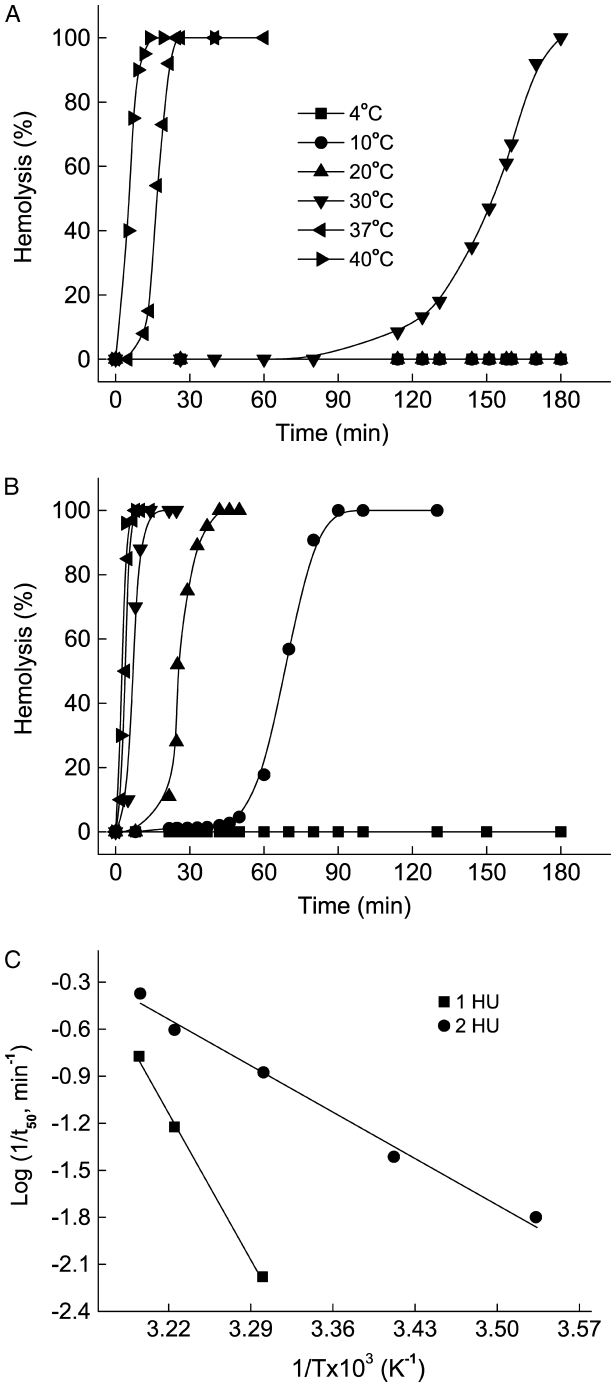

Figure 1. Temperature-dependence of hemolysis. Time courses of hemolysis were obtained at various temperatures in the presence of 1 HU (A) and 2 HU (B) tolaasin. (C) Kinetics of temperature-dependent hemolysis. Logarithmic reciprocal value ($1/t_{50}$) of time t_{50} which hemolyzed 50% of erythrocytes was plotted against $1/T \times 10^3$ from the data shown in A & B.

The velocity of hemolysis calculated from the data shown in Figures 1(A) and (B) was expressed with time t_{50}, a time for 50% hemolysis. As temperature increased, hemolysis occurred faster and t_{50} became a smaller value. When Arrhenius dependence was measured by plotting $1/t_{50}$ *versus* 1/T, a straight line was obtained at each dose of tolaasin (Figure 1(C)). These results imply that there is a strong correlation between temperature and tolaasin-induced hemolysis.

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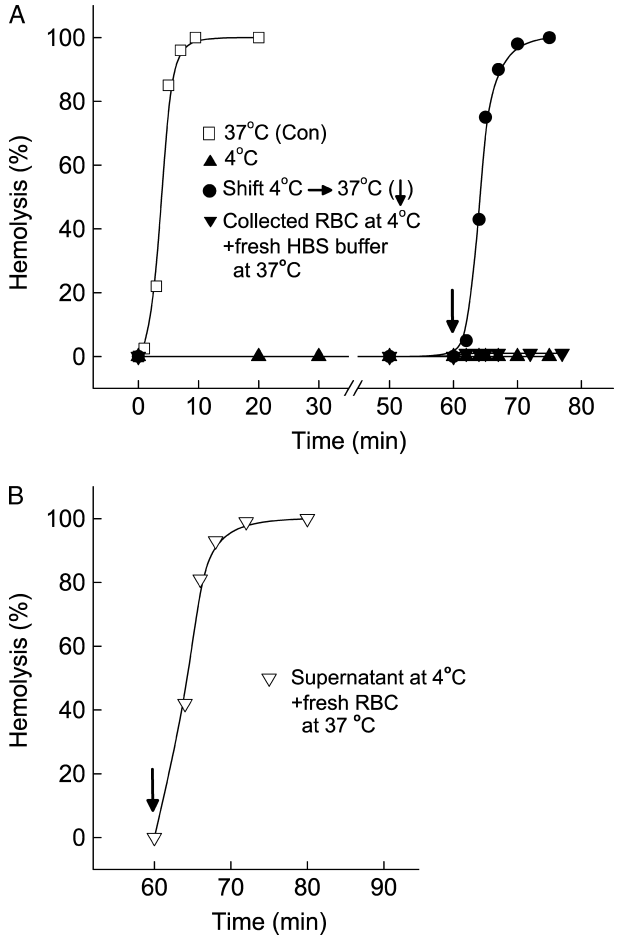

Figure 2. Temperature-dependent binding of tolaasin to erythrocytes. (A) Erythrocytes were incubated with 2 HU tolaasin and hemolysis was measured at 37 °C (□) or 4 °C (▲). Incubation temperature was shifted from 4 to 37 °C (●) at the indicated time by an arrow (↓). In separate experiments, after erythrocytes were incubated with tolaasin at 4 °C for 40 min, the erythrocytes were collected by centrifugation, resuspended with tolaasin-free HBS, and incubated at 37 °C (▼). (B) Fresh erythrocytes were added to the supernatant collected from 4 °C incubation as indicated by an arrow and hemolysis was measured.

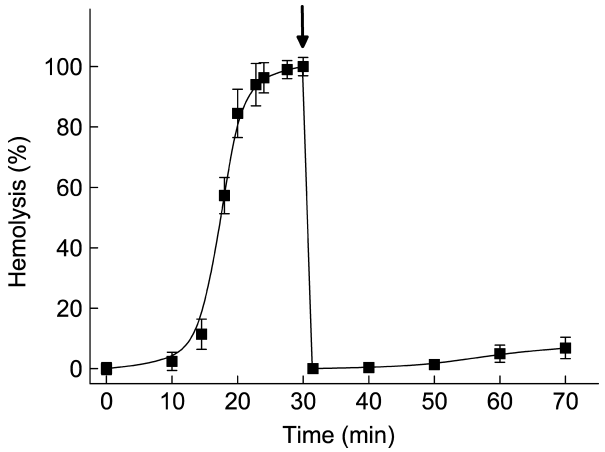

Figure 3. Irreversible binding of tolaasin to erythrocytes. After erythrocytes were hemolyzed, fresh erythrocytes (1%) were subsequently added at the indicated time (↓). Hemolysis of the added erythrocytes was monitored.

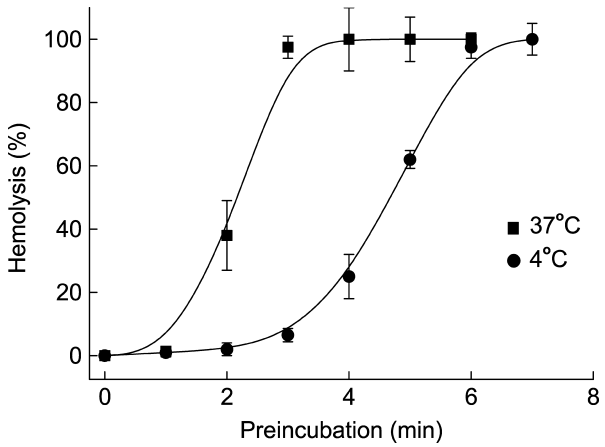

Figure 4. Temperature-dependence of membrane binding and pore formation of tolaasin. Erythrocytes were preincubated with 2 HU tolaasin at 37 °C in the presence of osmotic protectant, 30 mM PEG 2000. Tolaasin molecules were allowed to bind to the membranes of erythrocytes for the indicated time period (preincubation) shown on the *x*-axis. The erythrocytes were collected and resuspended with tolaasin-free HBS and incubated at either 37 °C (■) or 4 °C (●). After 10 min of incubation, the amount of hemolysis was measured from each preparation and plotted on the *y*-axis.

Temperature-dependence of Membrane Binding

The hemolytic activity of tolaasin was completely suppressed at 4 °C. This may be due to either the inhibition of membrane binding or pore formation of tolaasin. Therefore, the membrane binding properties of tolaasin were investigated at low temperatures. In the control experiment with 2 HU tolaasin, hemolysis was completed after 10 min at 37 °C (Figure 2(A), □). Although the hemolytic activity of tolaasin was suppressed at 4 °C, a fast hemolysis was obtained by increasing the temperature from 4 °C to 37 °C and it was similar to that of the control (●). In order to test whether the tolaasin molecules bind to the erythrocyte membrane at 4 °C, erythrocytes were incubated with tolaasins for 40 min at 4 °C, collected by centrifugation, resuspended in tolaasin-free HBS buffer, and incubated at 37 °C. No hemolysis was observed with the incubated erythrocytes (▼). However, when the collected supernatant at 4 °C was added to fresh erythrocytes, fast hemolysis was observed at 37 °C (Figure 2(B)), implying that most of the tolaasin molecules remained in the medium without binding to the erythrocyte membrane at 4 °C. These results indicate that no tolaasin binds to cellular membrane at 4 °C.

The binding of tolaasin on the erythrocyte membrane was irreversible. After erythrocytes were hemolyzed, fresh erythrocytes were added to the reaction medium containing tolaasin and cell debris. No additional hemolysis was observed, implying that free tolaasin molecules were not available for the hemolysis of freshly added erythrocytes (Figure 3). Once tolaasins were bound to the cell membrane, they were not released into the solution from the cell debris.

Hydrophilic polymers, such as polysaccharides and polyethylene glycol (PEG), play a role as osmotic protectants and suppress the hemolysis of erythrocytes by pore-forming toxins [19]. PEG 2000 is known to block tolaasin-induced hemolysis [5,11] by increasing osmotic pressure of extracellular medium and preventing cell swelling. To characterise the temperature-dependence of tolaasin-induced hemolysis, 2 HU tolaasin was added to the erythrocyte solution and incubated for a fixed period from 1 to 10 min at 37 °C in the presence of PEG 2000 (Figure 4). After the indicated period

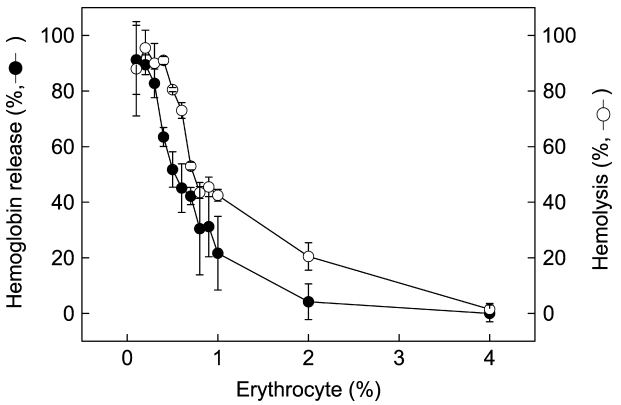

Figure 5. Effect of erythrocyte concentration on tolaasin-induced hemolysis. A fixed amount of tolaasin (0.75 HU) was added to various amounts of erythrocytes. Tolaasin-induced hemoglobin release (●) and hemolysis (○) were monitored at various amounts of erythrocytes. In order to measure the release of hemoglobin, the mixture was centrifuged after 1 h incubation at 37 °C and the absorbance of the supernatant at 540 nm was measured. The percentage of hemolysis was monitored by observation of the changes in absorbance at 600 nm.

of pre-incubation, supernatant containing PEG 2000 was removed by centrifugation, tolaasin-bound erythrocytes were resuspended with tolaasin-free HBS, and the erythrocyte solution was further incubated at either 4 °C (●) or 37 °C (■) for 10 min. At both temperatures, hemolyses were observed, and the membrane-bound tolaasin molecules were able to destroy the membrane structure regardless of the incubation temperature. Interestingly, hemolysis at a low temperature required pre-incubation for more than 4 min, and it required the binding of more tolaasin molecules. Once enough molecules of tolaasin were bound to the membrane, hemolysis occurred even at 4 °C. Taken together, these data suggest that membrane binding of tolaasin is dependent on temperature but the disruption of membrane by tolaasin-pore is less sensitive to temperature.

Multiple Binding of Tolaasin to the Erythrocyte Membrane

The effect of erythrocytes concentration on hemolysis was evaluated with a fixed amount of tolaasin (0.75 HU) by changing the erythrocyte concentration from 1% to 4% (Figure 5). After 1 h incubation at 37 °C, hemolyses were monitored by the observation of absorbance changes at both 600 and 540 nm representing cell lysis and hemoglobin release, respectively. Hemoglobin release was measured from the supernatant containing the released hemoglobin after the removal of erythrocytes by centrifugation. When the concentration of erythrocytes was increased gradually from 0.1% to 4%, the fraction of the released hemoglobin to the corresponding total was sharply decreased by increasing erythrocyte concentration in the presence of the fixed amount of tolaasin (●). Similarly, the percentage of hemolysis was also decreased with the increase in erythrocyte concentration (○). As the concentration of erythrocytes increased, the amount of tolaasin became insufficient for the complete hemolysis. These results suggest that multiple binding of tolaasin is required for the hemolysis of a single cell. Multi-hit processes have been described to explain the cytotoxicity of various pore-forming toxins [17,20–22].

To estimate the number of hits necessary for the hemolysis of an erythrocyte, the effect of tolaasin concentration was investigated

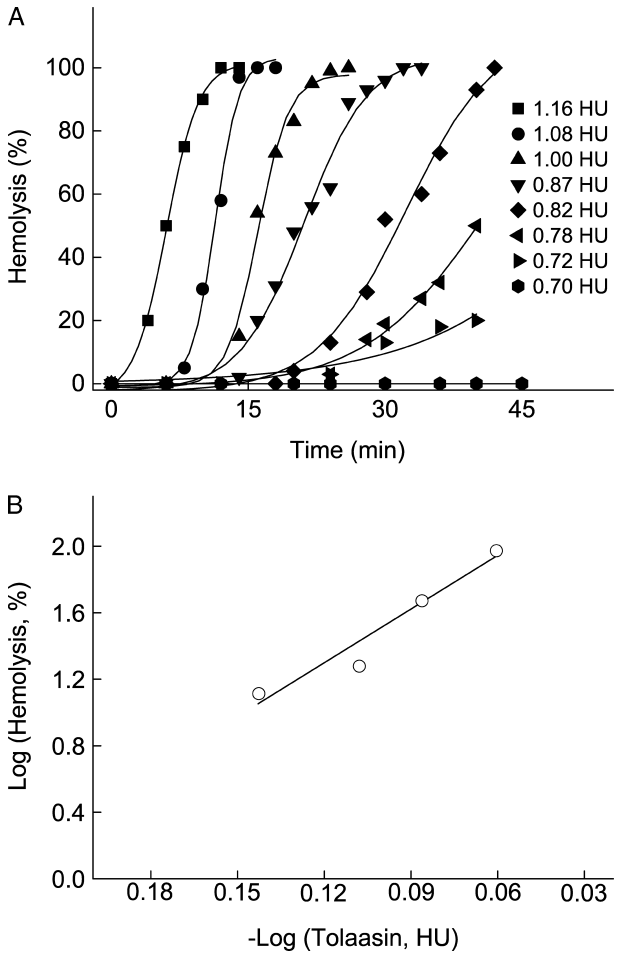

Figure 6. Correlation of hemolysis with the amount of tolaasin. (A) Dose-dependence of tolaasin-induced hemolysis. Time-dependent hemolysis was measured at various concentrations of tolaasin. Tolaasin doses ranging from 0.7 to 1.16 HU were added to 1% rat erythrocyte solution. (B) Logarithmic values of hemolysis *versus* that of tolaasin concentration were plotted. The percentages of hemolysis at doses ranging from 0.72 to 0.87 HU were obtained at 30 min incubation from the data shown in A.

in the presence of a fixed amount of erythrocytes (Figure 6). When the amount of tolaasin was increased from 0.7 to 1.16 HU, the hemolytic activity was dependent on the concentration of tolaasin (Figure 6(A)). The logarithmic value of the percentage of hemolysis *versus* that of tolaasin concentration was plotted. A linear relationship was obtained and the slope of the line for tolaasin-induced hemolysis was ten (Figure 6(B)). The results showed that the amount of hemolysis was proportional to the tenth power of the amount of tolaasin, implying that tolaasin-induced hemolysis can be explained by the multi-hit model.

Discussion

Tolaasin disrupts cellular membrane by forming pores on the membrane of various cells, such as fungal, plant, and animal cells. Although the basic characteristics of tolaasin-induced pore formation and biosurfactant properties have been investigated by the measurement of tolaasin-induced hemolysis and surface-tension study [5,6,10,11], the detailed mechanism of tolaasin-induced cell disruption has remained unconfirmed. Hemolytic activities of various pore-forming toxins, such as haemolysin

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III [13], streptolysin S [14], streptolysin O [15], El Tor toxin [16], and *Vibrio* cytolysin [17], were modulated by temperature and their temperature-dependencies were used to investigate the molecular mechanism of toxin-induced cell disruption. The hemolytic process by these toxins consists of several steps, and each step can be differentially affected by temperature. Since tolaasin-induced cell disruption probably undergoes similar lysis steps, we examined the effect of temperature on the pore formation of tolaasin to understand the mechanism of tolaasin-induced cell disruption.

Rainey *et al*. [5] reported the effect of temperature on tolaasin-induced hemolysis, and they determined the optimum temperature of tolaasin-induced hemolysis. Although temperature seems to play a major role in tolaasin-induced hemolysis, the mechanism of temperature-dependency has not been pursued in detail. When the hemolysis was measured at various temperatures, we found that it consists of a temperature-sensitive membrane binding step and less temperature-sensitive pore formation and lysis step (Figures 1 and 2). Similarly, temperature-dependencies of peptide toxins were investigated by use of haemolysin III [13] and *Vibrio* cytolysin [17]. The temperature-dependence of these toxins is suggested to be caused by changes in membrane fluidity. With various toxins, low temperature-induced inhibition of hemolysis has been attributed to a decrease in fluidity of target membrane [23–25]. Zitzer *et al*. [16] demonstrated, however, that the effects of temperature on cytolysin activity are not mediated by changes in fluidity of the target membrane since membrane binding of toxin was successful even at low temperature.

Interestingly, Lo Cantore *et al*. [26] measured the temperature-dependence of tolaasin toxicity compared to that of another *Pseudomonas* lipodepsipeptide toxin, white line-inducing principle (WLIP), at temperatures, 25 and 37 °C. The hemolytic activity of tolaasin was decreased at 25 °C; however, that of WLIP was not changed at both temperatures. In addition, to decrease the membrane fluidity of erythrocytes, structural changes of tolaasin molecules probably occurred since the cytotoxicity of tolaasin reduced at 25 °C. Our data also showed that membrane binding of tolaasin was severely reduced at 4 °C. The poor membrane binding of tolaasin could be explained by two possibilities, decrease in membrane fluidity of erythrocytes and change in peptide structure. When temperature-dependent decrease in membrane fluidity was measured in the temperature ranges from 4 to 40 °C, two phase-transitions in rat erythrocyte membrane at 17.4 and 34.5 °C were revealed by electron spin resonance (ESR) study using 12-doxyl stearic acid [27]. These transitions might be due to changes in the conformation of membrane proteins, phospholipids, or both in the membrane. Reduced membrane fluidity at low temperature is likely to hinder the binding of tolaasin peptide on the erythrocyte membrane. Structural changes of tolaasin molecules should be investigated in detail to understand the poor membrane binding at low temperature. Solubility of tolaasin peptide may not significantly decrease at low temperature, since no precipitation was observed from concentrated tolaasin solutions (100 HU/ml, 2.65 μM) under refrigeration. Meanwhile, even though the lysis step is less sensitive to temperature changes, hemolysis requires more tolaasin molecules or longer incubation time at 4 °C than at 37 °C. In Figure 4, when the tolaasin-bound erythrocytes were incubated at low temperature, the pre-incubation time required for complete hemolysis was doubled compared with time needed at 37 °C.

The binding of tolaasin on the erythrocyte membrane seems to be irreversible. If tolaasin binds to the membrane reversibly, some molecules will be released from the tolaasin-bound erythrocyte membrane depending on the partition coefficient toward lipid membrane *versus* HBS buffer and the released tolaasins may cause hemolysis of the subsequently added fresh erythrocytes. However, no significant hemolysis was observed when fresh erythrocytes were added to the solution containing cell debris of completely hemolyzed erythrocytes (Figure 3). This result represents that free tolaasin molecules were not available in the solution because tolaasin binding to the membrane is irreversible. A slow hemolysis of less than 10% was observed at 40 min after the addition of fresh erythrocytes. It might be due to the time-dependent autolysis of erythrocytes since 6.8 ± 3.5% hemolysis was measured by 40 min incubation at 37 °C in the absence of tolaasin.

In the presence of a fixed amount of tolaasin, the effect of erythrocyte concentration on the hemolysis was evaluated and the tolaasin-induced hemolysis was found to be mediated by a multi-hit process (Figure 5). The multi-hit model proposed by Inoue *et al*. [20] was used to explain the mechanism of cell disruption induced by streptolysin O and θ-toxin. Some pore-forming toxins and complements, including *Vibrio metschnikovii* cytolysin [17], *V. damsela* cytolysin [21], and C8–C9 complement [22], have been known to follow the multi-hit model. If tolaasin-induced hemolysis was mediated by a one-hit model, in which one toxin molecule is sufficient for lysis of a single erythrocyte, the amount of hemoglobin released would be constant no matter what concentrations of erythrocyte were added.

Logarithmic correlation between hemolysis and concentration of toxin could be used to estimate the number of hits necessary to lyse cells. The slope of the correlation curve represents the number of hits according to Inoue *et al*. [20]. The number of hits required for hemolysis by several toxins was reported to range from two to five [20,21,28]. In this study, the number of tolaasin molecules for the hemolysis of one erythrocyte was calculated as ten (Figure 6(B)). Tolaasin molecules have to multimerize to form an ion channel, but there has been no clear demonstration of how many monomers are needed to form a functional channel. Our results suggest that the percentage of hemolysis is proportional to the tenth power of the HU of tolaasin. The difference in the number of hits, between two and five for the toxins described above and ten for tolaasin, may be represented by different molecular multimerizations. Recently, the average numbers of monomers constituting the active conducting unit (*M*) for WLIP and tolaasin I in artificial liposome were reported as eleven and seven, respectively [29]. It is difficult to compare directly to our results since we obtained the number of hits by measuring hemolysis rather than by using artificial liposomes.

The present study investigated the effect of temperature on tolaasin-induced hemolysis to explore the mechanism of tolaasin-induced cell disruption. In conclusion, when tolaasin-induced hemolysis was measured at various temperatures and analyzed in terms of binding properties, we found that the membrane binding of tolaasin is dependent on temperature, but the formation of membrane pores as well as lysis of erythrocytes is less sensitive to temperature. Furthermore, multiple binding of tolaasin molecules is required for the hemolysis of single cells, suggesting that tolaasin-induced hemolysis follows a multi-hit process.

Acknowledgement

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