Inactivation of *Vibrio vulnificus* hemolysin through mutation of the N- or C-terminus of the lectin-like domain

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Abstract

Vibrio vulnificus is an etiological agent causing serious systemic infections in the immunocompromised humans or cultured eels. This species commonly produces a hemolytic toxin consisting of the cytolysin domain and the lectin-like domain. For hemolysis, the lectin-like domain specifically binds to cholesterol in the erythrocyte membrane, and to form a hollow oligomer, the toxin is subsequently assembled on the membrane. The cytolysin domain is essential for the process to form the oligomer. Three-dimensional structure model revealed that two domains connected linearly and the C-terminus was located near to the joint of the domains. Insertion of amino acid residues between two domains was found to cause inactivation of the toxin. In the C-terminus, deletion, substitution or addition of an amino acid residue also elicited reduction of the activity. However, the cholesterol-binding ability was not affected by the mutations. These results suggest that mutation of the C- or N-terminus of the lectin-like domain may result in blockage of the toxin assembly.

***Keywoeds*:** *Vibrio vulnificus*, Hemolysin, Cell-free translation, Site-directed mutagenesis

1. Introduction

Vibrio vulnificus is a facultative anaerobic gram-negative bacterium inhabiting ubiquitously at estuarine and marine environments (Janda et al., 1988; Chakraborty et al., 1997). However, this bacterium is an etiological agent causing fatal septicemia in humans by consumption of contaminated seafood, whereas most of the patients have underlying diseases such as hepatic cirrhosis, hepatitis or diabetes (Strom and Paranjpye, 2000; Miyoshi 2006). *V. vulnificus* also causes serious systemic infections called vibriosis in cultured eels (Tison et al., 1982). This species commonly produces a hemolytic/cytolytic toxin termed *V. vulnificus* hemolysin (VVH) (Shinoda et al., 1985; Miyoshi et al., 1993), which has been reported to cause cytolysis of various eukaryotic cells, as well as erythrocytes (Yamanaka et al., 1990; Miyoshi et al., 1993; Kashimoto at al., 2010). For hemolysis, the toxin binds specifically to cholesterol and is subsequently assembled on the membrane to form an oligomer (Yamanaka et al., 1987; Kim and Kim, 2002). Although VVH is a single polypeptide consisting of 451 amino acid residues (Yamamoto et al., 1990), it is divided into two functional domains, the cytolysin domain (Gln^{1} to Ala^{318}) essential for the toxin assembly and the lectin-like domain (His^{319} to Leu^{451}) mediating the toxin binding (Olson and Gouaux, 2005). Indeed, our recent study demonstrated that replacement of Thr^{438} in the lectin-like domain caused significant change of the affinity to cholesterol (Senoh et al., 2008). The VVH precursor is encoded in the *vvhA* gene (1416 bp), while it constitutes an operon with the *vvhB* gene (507 bp) (Yamamoto et al., 1990). Senoh et al. (2008) reported that the product of *vvhB* functioned as a chaperone to support the maturation of VVH.

The overall three-dimensional structures of bacterial hemolytic/cytolytic toxins are considerably similar each other even though their primary structures are not fairly related (Tilley and Saibil, 2006; Iacovache et al., 2010). *Vibrio cholerae*, a human pathogen causing

cholera or enteric diseases, also produces a hemolytic toxin, of which amino acid sequence is 17 % identical with VVH (Yamamoto et al., 1990). Based on the crystal structure of *V. cholerae* hemolysin (Olson and Gouaux, 2005), the tertiary structure model of VVH was constructed by using the SWISS-MODEL Workspace (Arnold et al., 2006). The model revealed that two domains were connected linearly and the C-terminal leucine was located close to the joint of the domains (Miyoshi et al., unpublished). This suggests that mutation of the N- or C-terminus of the lectin-like domain may result in inactivation of VVH because of change of the linear arrangement of the two domains. Kashimoto et al. (2010) recently found that mutation of Phe^{314} located near to the joint of the domains caused remarkable reduction of the cytolytic activity. However, the mutated toxin was found to bind sufficiently to the membrane. In the present study, several mutated toxins were prepared by using the cell-free translation system, and their hemolytic activities and cholesterol-binding abilities were compared.

2. Materials and methods

2.1. Bacterial strain and growth

V. vulnificus strain CDC B3547 isolated from human leg ulcer was used in the present study. The bacterium was cultivated overnight at 37 ^{o}C in Luria-Bertani broth (1 % trypton, 0.5 % yeast extract, 3.0 % NaCl, pH 7.5) with shaking.

2.2. Toxin production by using the cell-free system

As described by Senoh et al. (2008), the wild type or mutated toxin was produced with the rapid translation system (Roche Diagnostics, Mannheim, Germany) using the polymerase chain reaction (PCR)-amplified liner DNA fragments. To prepare the DNA fragments, in the

first PCR, an appropriate primer set carrying the 20 or 21 bp overlap region for the second PCR was designed from the nucleotide sequence of *vvhA* or *vvhB* of strain CDC B3547 (GenBank accession number, AB124803). The bacterial genomic DNA extracted was heat-treated at 94 ^{o}C for 2 min, and the PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) was carried out for 30 cycles as following: 1 min denaturation at 94 ^{o}C, 1 min annealing at an appropriate temperature, 2-3 min extension at 68 ^{o}C.

In the second PCR, the sequences for T7 promoter and His_6-Tag were added to the upstream, and the sequence for the T7 terminator was added to the down stream. The product of the first PCR was mixed with the RTS *E. coli* Linear Template Generation Set and His_6-tag, and the admixture was subjected to the second PCR. After denaturation at 94 ^{o}C for 4 min, the reaction was carried out for 30 cycles as following: 1 min denaturation at 94 ^{o}C, 1 min annealing at 60 ^{o}C, and 2-3 min extension at 68 ^{o}C.

Thereafter, the final cell-free translation was performed using the products of the second PCR as the linear template DNAs. The amplified DNA fragments corresponding to *vvhA* (1.0 μg) and *vvhB* (0.5 μg) were mixed with the RTS 100 *Escherichia coli* HY Kit, and the translation was carried out at 25 ^{o}C for 6 hr according to the manufacture's manual.

For Western blotting, the crude toxin preparation was applied to a Phenyl-Sepharose HP 5/5 column, and the toxin was partially purified as described (Senoh et al., 2008). The toxin thus prepared was treated at 100 ^{o}C for 5 min with an equal volume of the mixture of 2 % SDS and 5 % 2-mercaptoethanol (SDS-ME). An aliquot of the heat-treated sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on the PhastSystem using a PhastGel Gradient 10-15 (GE Healthcare, Buckinghamshire, England), and the proteins were transferred to a polyvinylidene difluoride membrane. Thereafter, the toxin was detected with the rabbit IgG antibody against VVH purified from strain CDC B3547 (Oh et al., 1993) and the anti-rabbit IgG antibody conjugated with horseradish peroxidase (Cosmo-Bio, Tokyo,

Japan), and the density of the protein band detected was analyzed by using NIH Image version 2.1.

2.3. Hemolytic activity

The hemolytic activity was assayed according to the method of Shinoda et al. (1985) with some modifications. The toxin preparation (50 μl) was diluted serially with 20 mM Tris-HCl buffer (pH 7.5) containing 0.9 % NaCl and 0.01 % bovine serum albumin (TBS), and each of the toxin samples was allowed to act on 50 μl of 1 % sheep erythrocytes suspended into TBS at 37 ^{o}C. At 2 hr-incubation, the reaction was terminated by the addition of 0.4 ml of ice-cold TBS, and the supernatants were collected after centrifugation at 1000 x g for 5 min. Thereafter, the amount of hemoglobin liberated from the disrupted erythrocytes was quantified by measuring absorbance at 540 nm, and the amount of the toxin causing 50 % hemolysis was determined, and then, the relative activity of each mutated toxin was estimated.

2.4. Binding to cholesterol particles

The toxin preparation (10 μl) was allowed to act on cholesterol (1 to 10 μg in 1 μl of ethanol) in a total of 0.1 ml of TBS for 30 min at 0 or 37 ^{o}C. Thereafter, the cholesterol particles were collected by centrifugation (12000 x g for 5 min), rinsed twice with TBS and dissolved in an equal volume of SDS-ME by incubation at 37 ^{o}C for 30 min. An aliquot of the sample thus prepared was subjected to SDS-PAGE on the PhastSystem using a PhastGel Gradient 10-15, and the toxin was detected by Western blotting.

2.5. Binding to erythrocyte membranes

The resealed ghosts of sheep erythrocytes were prepared as reported by Funder and Wieth

(1976) and suspended into KRT buffer (128 mM NaCl, 5.1 mM KCl, 1.34 mM $MgSO_4$, 2.7 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) at a concentration of 8 %. The resealed ghosts (50 μl) thus prepared was mixed with the toxin preparation (50 μl) and incubated at 37 ^oC for 2 hr. Thereafter, the resealed ghosts were collected, and the toxin associated with the ghost was detected by SDS-PAGE followed by Western blotting.

3. **Results**

3-1. Hemolytic activities of mutated toxins

Senoh et al. (2008) documented the sufficient hemolytic activity of the wild type toxin (sVVH) produced by using the cell-free translation system. In the present study, we prepared the cytolysin domain (Gln^1 to Ala^{318}) by using the same system and measured its hemolytic activity in the presence or absence of the lectin-like domain (His^{319} to Leu^{415}). However, no hemolytic activity was observed even in the presence of the lectin-like domain, suggesting that the toxin must be produced as a single polypeptide to show the hemolytic activity. Next, we tested effect of the addition of amino acid residues to the N-terminus of the lectin-like domain. The hemolytic activity was reduced to 30 % by insertion of glycine-alanine between Ala^{318} and His^{319} and abolished completely by insertion of six glycine residues. Theses findings indicate that the direct connection of two domains is crucial to cause hemolysis.

The tertiary structure model reveled that the C-terminus of the lectin-like domain (Leu^{451}) was located close to the joint of two domains. Therefore, it was considered that the mutation of the C-terminus might affect the hemolytic activity. As shown in Table 1, the activity was reduced to less than 20 % by deletion of the C-terminal leucine (CΔ1). The preparation of the CΔ2 mutant was found to have no hemolytic activity; however, the Western blotting analysis indicated that this preparation contained the undetectable amount of the mutated toxin (data

not shown). Next, we tested effect of the addition of a residue to the C-terminus (Table 1). Although addition of leucine (VVH-L) or phenylalanine (VVH-F) gave a little impact in the hemolytic activity, addition of aspartic acid (VVH-D) or lysin (VVH-K) caused significant reduction of the activity. In addition, we examined whether substitution of Leu^{451} also causes inactivation of the toxin (Table 1). The toxin substituted to isoleucine (L451I) showed the partial hemolytic activity; however, substitution to aspartic acid (L451D) or lysin (L451K) resulted in drastic decrease in the activity. In contrast, change of Lys^{450} elicited less effect on the hemolytic activity (Table 1).

The wild type and mutated toxins were partially purified by using a Phenyl-Sepharose HP 5/5 column (Senoh et al., 2008) and subjected to SDS-PAGE followed by Western blotting. The results demonstrated that any mutation did not affect the translation process because the comparative amounts of the mutated toxins could be detected (data not shown). Therefore, it is concluded that the C-terminus leucine in the lectin-like domain is important for the hemolytic activity.

3-2. Binding of mutated toxins to cholesterol

In the first step of hemolysis, VVH binds to cholesterol in a temperature-independent manner (Shinoda et al., 1985). Since the lectin-like domain alone can mediate this first step (Senoh et al., 2008), it was speculated that the mutation of the C-terminus did not affect the ability to bind to cholesterol. The wild type or mutated toxin was allowed to act at 0 or 37 ^{o}C on cholesterol particles (10 μg at 0 ^{o}C, 1 or 5 μg at 37 ^{o}C), and the toxin associated with cholesterol particles was detected by Western blotting. When incubated at 37 ^{o}C, both the wild type and mutated toxins revealed the sufficient binding to the cholesterol particles (data not shown). Additionally, two inactive mutants (CΔ1 and L451K), as well as an active one (VVH-L), could associate with cholesterol particles even when the reaction was carried out at

0 ^{o}C (Fig. 1A). We also examined the binding of the mutated toxin, L451K, to erythrocyte membranes. The Western blotting analysis of the resealed erythrocyte ghosts incubated with sVVH or L451K demonstrated the comparative binding of the toxin to the membranes (Fig. 1B). These findings indicate that the mutated toxins have the sufficient ability to bind to cholesterol. Therefore, the second process for hemolysis, assembly of the toxin molecules, may be blocked in the mutated toxins. However, the follow-up experiments to determine dissociation constants or to compare the affinity of the toxins to cholesterol.

3-3. Hemolysis in the presence of the mutated toxin

The native PAGE is a useful method to detect the toxin oligomer formed by auto-assembly on erythrocyte membrane or cholesterol particles (Miyoshi et al., 2004). However, when the cholesterol particles incubated with sVVH were subjected to native PAGE, no protein band corresponding to the toxin oligomer could be detected (data not shown). Next, we carried out modified SDS-PAGE of cholesterol particles incubated with sVVH, in which the heat-treatment of the sample with SDS-ME was omitted to protect dissociation of the toxin oligomer to the monomer. However, the oligomer of sVVH could not be detected in the modified SDS-PAGE analysis (data not shown). Although it is not known why the toxin oligomer was not detected, the N-terminal His_6-Tag in sVVH might disturb the mobility of the toxin oligomer in native PAGE or modified SDS-PAGE.

We also tested whether the presence of the excess amounts of the mutated toxin could interfere the hemolysis elicited by the wild type toxin. The sVVH was mixed with CΔ1 or L451K at the molar ratio of 1:0 to 1:5, and the hemolytic activity of each mixture was compared. As shown in Fig. 2, either mutated toxin showed no effect on the hemolytic activity. This suggests that the mutated toxin could not interact with sVVH on the erythrocyte membrane.

4. Discussion

VVH is a single polypeptide toxin consisting of two functional domains, the cytolysin domain (Gln^{1} to Arg^{318}) and lectin-like domain (His^{319} to Leu^{451}) (Olson and Gouaux, 2005). This toxin is known to cause hemolysis through several steps (Miyoshi et al., 1993). Namely, in the first step, the lectin-like domain binds specifically to cholesterol in a temperature-independent manner, and in the second step, the toxin forms a hollow oligomer through autoassembly in a temperature-dependent manner. Senoh et al. (2008) recently demonstrated that Thr^{438} in the lectin-like domain was related to the binding to cholesterol. On the other hand, Kashimoto et al. (2010) reported that mutation of Phe^{314}, which is in the cytolysin domain but is located near to the joint of two domains, elicited elimination of the ability to form the toxin oligomer. The present study showed that mutation of Leu^{451}, of which location is also close to the joint of the domains, caused inactivation of VVH without significant reduction of the ability to bind to cholesterol particles and sheep erythrocyte membranes. In addition, VVH had lost the hemolytic activity by insertion of residues to the N-terminus of the lectin-like domain. Although significant change in the tertiary structure model was not observed, these mutations might result in blockage of formation of the toxin oligomer through autoassembly.

V. vulnificus is a ubiquitous microorganism in the estuarine and marine environments (Janda et al., 1988; Chakraborty et al., 1997) whereas an etiological agent causing fatal septicemia in humans by eating contaminated seafood (Strom and Paranjpye, 2000; Miyoshi 2006). VVH is commonly produced by *V. vulnificus* as a major exocellular toxin (Shinoda et al., 1985; Miyoshi et al., 1993), and its structure gene has been used as a characteristic gene to identify the bacterium (Hill et al., 1991; Senoh et al., 2005). In our knowledge, VVH shows

no immunological cross-reactivity to other bacterial hemolytic/cytolytic toxins including those produced by human pathogenic *Vibrio* species. Therefore, the development of the immunological assay method targeting VVH is useful for the clinical diagnosis of *V. vulnificus* infection and screening of the seafood or environmental samples contaminated with the bacterium. The mutated toxins prepared in this study may be strong candidates for the non-toxic and safety antigens/toxoids to produce the specific antibody to the toxin.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1. Hemolytic activities of toxins produced by using the cell-free translation system

Toxin		Hemolytic activity (%)
Wild type toxin	sVVH	100
Mutated toxin		
Deletion	CΔ1	16.7 ± 3.3
	CΔ2	ND*
Addition	VVH-L	126 ± 6.7
	VVH-F	118 ± 11
	VVH-D	23.7 ± 3.9
	VVH-K	24.1 ± 4.4
Substitution (Leu^{451})	L451I	60.4 ± 5.1
	L451F	16.6 ± 4.7
	L451D	3.2 ± 1.1
	L451K	0.9 ± 0.7
Substitution (Lys^{450})	K450R	102 ± 2.5
	K450H	90.2 ± 4.5
	K450E	62.2 ± 6.4
	K450L	45.6 ± 5.0

* The activity could not be determined because of the sufficient amount of the toxin was not produced.

Figure legends

Fig. 1. The cholesterol-binding abilities of the wild type and mutated toxins. (A) Each of the toxin preparation (10 μL) was allowed to act on cholesterol (10 μg) in a total of 0.1 ml of TBS at 0 °C for 30 min. Thereafter, the toxin bound to cholesterol particles was collected and detected by Western blotting. (B) The resealed ghosts (50 μl) was mixed with the toxin preparation (50 μl) and incubated at 37 °C for 2 hr. Thereafter, the resealed ghosts were collected, and the toxin associated with the ghost was detected by Western blotting. The data indicated are representative results of three experiments.

Fig. 2. Effect of the mutated toxin on the hemolysis caused by the wild-type toxin. The wild toxin was mixed with the mutated toxin (CΔ1 or L451K) at the molar ratio 1:0 to 1:5, and the hemolytic activity of each mixture was measured with sheep erythrocytes at 37 °C for 2 hr. The data indicated are the means and standard deviations of three experiments.

Fig. 1

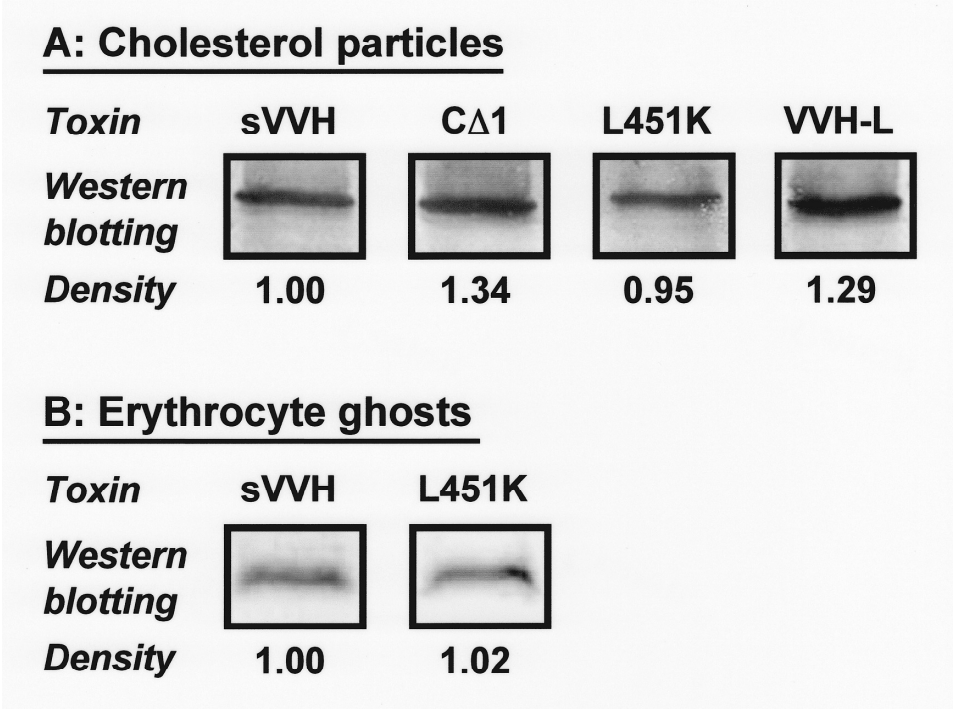
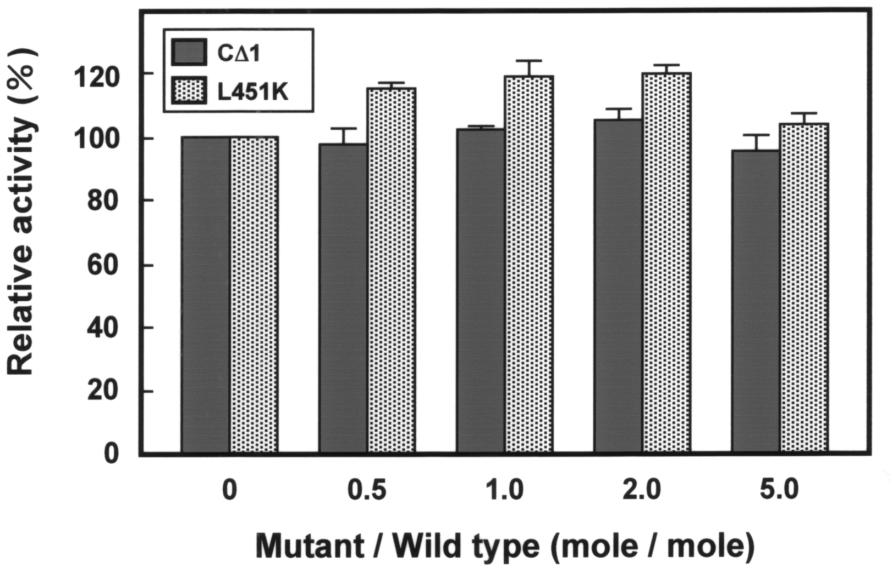

A: Cholesterol particles
Toxin
sVVH
CΔ1
L451K
VVH-L
Western blotting
Density
1.00
1.34
0.95
1.29
B: Erythrocyte ghosts
Toxin
sVVH
L451K
Western blotting
Density
1.00
1.02

Fig. 2


CΔ1
L451K
Relative activity (%)
120
100
80
60
40
20
0
0
0.5
1.0
2.0
5.0
Mutant / Wild type (mole / mole)