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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.

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 et 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

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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

Vibrio vulnificus strain CDC B3547 isolated from human leg ulcer was used in the present study. The bacterium was cultivated overnight at 37 °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 °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 °C, 1 min annealing at an appropriate temperature, 2–3 min extension at 68 °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 *Escherichia coli* Linear Template Generation Set and His_6-tag, and the admixture was subjected to the second PCR. After denaturation at 94 °C for 4 min, the reaction was carried out for 30 cycles as following: 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 2–3 min extension at 68 °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 °C for 6 h 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 °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 °C. At 2 h-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 × 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 (1–10 μg in 1 μl of ethanol) in a total of 0.1 ml of TBS for 30 min at 0 or 37 °C. Thereafter, the cholesterol particles were collected by centrifugation (12000 × g for 5 min), rinsed twice with TBS and dissolved in an equal volume of SDS-ME by incubation at 37 °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 °C for 2 h. Thereafter, the resealed ghosts were collected, and the toxin associated with the ghost was detected by SDS-PAGE followed by Western blotting.