**Human Serum Albumin Enhances the Hemolytic Activity of Vibrio vulnificus**

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**Vibrio vulnificus** hemolysin (VvhA) is inactivated in the late growth phase by its oligomerization. Albumin is known to affect the activities of many bacterial toxins. In this study, we investigated the effects of human or bovine serum albumin (HSA or BSA) on the production and activity of VvhA. HSA did not affect **V. vulnificus** growth and vvhA transcription. However, VvhA hemolytic activity in culture supernatants was significantly higher in the presence of HSA than in the absence of HSA. By Western blot analysis, the oligomerization of VvhA was inhibited and the remaining active VvhA monomer was increased in culture supernatants containing HSA. BSA produced similar results. These findings indicate that both HSA and BSA stabilize VvhA and delay VvhA inactivation by oligomerization, and thus enhance VvhA activity.

**Key words** Vibrio vulnificus; hemolysin; human serum albumin; bovine serum albumin

**V. vulnificus** is a halophilic estuarine bacterium which causes fatal septicemia and necrotizing wound infections, especially in patients with hepatic diseases, heavy alcohol drinking habits and hemochromatosis. **V. vulnificus** septicemia is characterized by a rapid and fulminant progression, and results in a mortality rate exceeding 50%.

Several bacterial components have been suggested to be the virulence factors of **V. vulnificus**. Of these, an extracellular hemolysin or cytolsin (named **VvhA**), the most potent exotoxin, kills mice and shows a variety of biological activities including hemolysis or cytolsis, apoptosis, and vasodilatation. In animal studies, the injection of purified **VvhA** reproduces the pathological manifestations of septicemia caused by injecting live bacteria. However, the pathogenetic significance of **VvhA** is being seriously doubted due to the reported effects of **VvhA**-deficient mutants on mouse-lethality. The inactivation of the **vvhA** gene does not affect mouse-lethality, which raises the possibility that only small amounts of **VvhA** are produced in vivo, and that this **VvhA** is rapidly inactivated by host factors like cholesterol and bacterial factors such as proteases. Therefore, in order to better characterize the pathogenetic roles of **VvhA**, further detailed studies regarding the in vitro and in vivo production and inactivation of **VvhA** are required.

In laboratory common media, **VvhA** is produced in the early growth phase and becomes abruptly inactivated in the late growth phase with a concomitant production of proteases, which results in the belief that the inactivation of **VvhA** is due to its destruction by proteases. However, our recent study demonstrated that the inactivation of **VvhA** was due to a novel oligomerization of **VvhA** by an unknown mechanism, but not to its destruction by proteases. In the present study, we found that bovine serum albumin (BSA) and human serum albumin (HSA) can inhibit or delay **VvhA** oligomerization, and thus enhance its hemolytic activity.

**MATERIALS AND METHODS**

**Bacterial Strains, Media, and Growth Conditions**

**V. vulnificus** MO6-24/O strain and its chromosomal **PvvhA::lacZ** reporter strain (named CMM2105) were used in this study. 2.5% NaCl-Heart Infusion (HI; Difco) was used to cultivate the bacteria. The bacteria grown in this medium at 37°C for 12 h were inoculated into the fresh medium to a concentration of 1×10⁶ cfu/ml, and cultured with vigorous shaking (220 rpm) at 37°C for 24 h.

**β-Galactosidase Assay and Hemolysin Assay**

During culture, culture aliquots were taken at appropriate times to monitor bacterial growth, and to measure β-galactosidase activity. Bacterial growth was monitored by measuring OD600 (DU530, Beckman Coulter). β-Galactosidase activity was measured as described by Miller. Culture supernatants were obtained by centrifuging (10000 rpm, 5 min) culture aliquots obtained at the same times. Hemolytic activity was measured using fresh human RBC as described by Hor et al.

**SDS-PAGE, Native-PAGE, and Western Blotting**

For SDS-PAGE, same volumes (20 μl) of culture supernatants were mixed with SDS-sample buffer, allowed to react at 37°C for 5 min, electrophoresed without heating, and transferred to nitrocellulose membranes. Details of native-PAGE were described in our previous study. The membranes were then sequentially reacted with rabbit polyclonal anti-VvhA antibody and goat anti-rabbit-IgG (Fc)-body conjugated with horseradish peroxidase, and finally visualized with diaminobenzidine and H₂O₂.

**Hemolytic Activity According to the Addition of HSA or BSA**

Various concentrations of HSA or BSA (0.05 to 0.5 mg/ml) were added to the culture supernatant which was obtained from a 6-h culture in 2.5% NaCl HI broth not containing HSA or BSA, and the mixtures were allowed to react for 60 min at 37°C. Hemolytic activity in the mixtures was measured using fresh human RBC.

**RESULTS AND DISCUSSION**

The growth profile of **V. vulnificus** MO6-24/O strain in 2% NaCl HI broth with or without 0.5 mg/ml of HSA is shown in...
Fig. 1. Effect of Human Serum Albumin (HSA) on *Vibrio vulnificus* Growth, *vvhA* Transcription, and VvhA Hemolytic Activity

*V. vulnificus* MO6-24/O wild type strain (A and C) and its chromosomal P*vvhA*::lacZ reporter strain (CMM2105: B) were inoculated into 2.5% NaCl-HI broth containing PBS or HSA (0.5 mg/ml) to 1x10⁶ cfu/ml, and cultured with vigorous shaking at 37 °C for 24 h. Culture aliquots and supernatants were removed at the indicated times. Bacterial growth (A) was monitored by measuring OD₆₀₀, *VvhA* transcription (B) was monitored by measuring β-galactosidase activity (Miller unit). Hemolytic activity (C) in culture supernatants was measured using 1% human RBC solution and is expressed as % hemolysis.

Fig. 1A. The chromosomal P*vvhA*::lacZ reporter strain (CMM2105) showed a similar growth kinetic to that of MO6-24/O strain. HSA did not affect the growth of the two *V. vulnificus* stains. The transcription of *vvhA* in the CMM2105 strain started 3 h after culture initiation and dramatically increased during the exponential growth phase (Fig. 1B). HSA did not affect the transcription of *vvhA*. In MO6-24/O strain, hemolytic activity in culture supernatants was observed from 3 h after culture initiation, peaked at 6 h, and then abruptly declined (Fig. 1C). Noticeably hemolytic activity in culture supernatants was much higher in the presence of HSA than in its absence. BSA produced similar results (data not shown). These results indicate that both HSA and BSA enhanced the hemolytic activity of *VvhA* already produced, but that they have no influence on the growth or *VvhA* expression in *V. vulnificus*. Considering that *VvhA* is inactivated by oligomerization,¹⁹ these results suggested that both HSA and BSA can inhibit or delay the inactivation of *VvhA* by oligomerization.

Therefore, in order to confirm this possibility, we performed the following two experiments. First, when various concentrations of HSA were added into the culture supernatant obtained at 6 h in 2.5% NaCl HI broth not containing HSA, hemolytic activity in the culture supernatant was enhanced in proportion to the amount of HSA added (Fig. 2A). Moreover, when native PAGE and Western blot were performed in order to visualize the inhibition of *VvhA* oligomerization by HSA, the amount of *VvhA* monomer was increased, or alternatively, the intermediate form (about 100 kDa) of *VvhA* oligomer was decreased when 0.5 mg/ml of HSA was added into the culture supernatant (Fig. 2B). BSA produced similar results (data not shown). These results indicate that both HSA and BSA enhance the hemolytic activity of *VvhA* already produced via the inhibition of *VvhA* oligomerization.

Secondly, we performed SDS-PAGE without heating of samples and Western blot by using the culture supernatants obtained from the cultures in Fig. 1 (Fig. 3). The oligomerization of *VvhA* was inhibited or delayed in the presence of HSA, or alternatively, active *VvhA* monomer was stabilized in the presence of HSA. BSA showed similar results (data not shown). These results indicate that both HSA and BSA inhibit or delay the inactivation of *VvhA* by oligomerization even though neither HSA nor BSA could completely block the oligomerization of *VvhA*.

We detected only *VvhA* by using *VvhA*-specific antibody in this study. It is known that *V. vulnificus* also produces other hemolysins,²² but we think that the hemolytic activity in Fig. 1C is due to the *VvhA* production because a *VvhA*-deficient mutant exhibited no hemolytic activity under the same conditions as those used in this study.¹⁹ A minute discrepancy between the total hemolytic activity in Fig. 1C and the Western blot result in Fig. 3 may be due to SDS-PAGE. In our previous study,¹⁹ when SDS-PAGE was performed with heating of samples, the discrepancy was noticeable because a considerable amount of *VvhA* oligomer was dissociated into *VvhA*.
monomer. However, when SDS-PAGE was performed without heating of samples in this study, the discrepancy decreased considerably but still remained.

It is well known that BSA affects the activities of several bacterial toxins, and thus, BSA has been used as a stabilizer of functional proteins in variety of biological buffers.\textsuperscript{5,24} \textit{Escherichia coli} $\alpha$-hemolysin is known to exist as a high molecular weight oligomer that can be dissociated by chaotropic agents or Triton X-100, which decreases its molecular weight. The molecular weight oligomer that can be dissociated by chaotropic agents or BSA.\textsuperscript{24) Like other bacterial toxins, the activity of \textit{V. haemolyticus} hemolysin is known to exist as a high molecular weight oligomer, and its leukotoxin activity can also be enhanced by its dissociation by exposure to chaotropic agents or BSA.\textsuperscript{24)}

Our previous study demonstrated that \textit{V. vulnificus} hemolysin (\textit{VvhA}) is also inactivated by oligomerization especially during the late growth phase, and that a considerable amount of \textit{VvhA} oligomer is dissociated to monomers by SDS-PAGE.\textsuperscript{19)} Like other bacterial toxins, the activity of \textit{VvhA} is also affected by BSA. Kreger \textit{et al}. reported that \textit{VvhA} activity is lost from buffers unless crystalline BSA (1 mg/ml) was added.\textsuperscript{5)} The present study shows that both HSA and BSA stabilized active \textit{VvhA} monomer to delay \textit{VvhA} inactivation by oligomerization (Fig. 3); moreover, both HSA and BSA were found to dissociate the intermediate form of \textit{VvhA} oligomer and thus enhance the hemolytic activity of \textit{VvhA} monomers (Fig. 2).

Considering that HSA is a major proteinaceous component in human body fluids, our results give a new significant information that HSA may contribute to \textit{VvhA}-induced pathophysiological changes by enhancing \textit{VvhA} activity. There is an example that a major host protein can contribute to pathophysiological changes by enhancing activities of bacterial toxins. Actin is one of the most abundant cytosolic proteins in mammalian host cells, and can be released into the extracellular spaces in some pathological conditions. The extra-cellular actin is known to enhance the activities of \textit{E. coli} hemolysin and \textit{P. haemolytica} leukotoxin and to be implicated in the pathogenesis of \textit{E. coli} and \textit{P. haemolytica} infections.\textsuperscript{25,26)