Growth Phase Dependant Activation of the Precursor of *Vibrio mimicus* Hemolysin (Pro-VMH)

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(Received March 12, 2007; Accepted May 7, 2007)

Vibrio mimicus (*V. mimicus*), a causative agent of gastroenteritis and food poisoning, secretes a 63-kDa enterotoxic hemolysin as the most potent virulence factor. The *vmhA* gene encoding an 83-kDa precursor of the hemolysin was expressed from the early to late log phase of the bacterial growth, and the 79-kDa inactive protoxin was detected from the culture supernatant in the same growth phase. The N-terminal amino acid sequence of the protoxin was determined to be NH_2-Asn-Ile-Ser-Asp-Pro-Val indicating cleavage of the Ala^{25}-Asn^{26} bond by a signal peptidase. In contrast, the hemolytic activity and the mature hemolysin in the culture supernatant were detected only at the late log phase. The maturation of the hemolysin, therefore, is suggested to be achieved by two-step processing, cleavage of the signal peptide followed by the growth phase-dependent removal of the 16-kDa propeptide.

Key words —— *Vibrio mimicus*, hemolysin, precursor, processing

INTRODUCTION

Vibrio mimicus (*V. mimicus*), a human pathogen closely related to *Vibrio cholerae* (*V. cholerae*) causing epidemic cholera, is an etiological agent of sporadic watery diarrhea and food poisoning after consumption of raw fish and/or shelfish.[1–3] Although a number of extracellular toxic factors are elaborated by the bacterium,[4–7] *V. mimicus* hemolysin (VMH) possessing both hemolytic and enterotoxic activities[5, 8, 9] is the most common factor of the species.[10] The *vmhA* gene encoding a precursor of VMH shows 76% homology to the *hlyA* gene in *V. cholerae* biovar El Tor, which encodes a precursor of an enterotoxic hemolysin (HlyA).[11] The HlyA has been documented to contribute to gastroenteritis caused by *V. cholerae* strains lacking the cholera toxin gene.[12] The molecular weight of the HlyA precursor is 82-kDa but that of mature HlyA isolated from the culture supernatant is 65-kDa.[13] It is thus considered that the activation and maturation of HlyA may occur through two-step processes as following: the 82-kDa preprotoxin synthesized in the bacterial cell is secreted to the culture medium as the 79-kDa inactive protoxin after removal of the signal peptide, and the 79-kDa protoxin is then further processed to the 65-kDa active toxin by release of the N-terminal 15-kDa propeptide.[13]

In *V. mimicus*, the *vmhA* gene encodes an 83-kDa protein[11] but active VMH purified from the culture supernatant is 63-kDa.[5] Therefore, it can be assumed that activation and maturation of VMH are also achieved by the similar two-step manner. In the present study, we investigated expression of *vmhA* gene and production of active VMH at different growth stages of *V. mimicus*.

MATERIALS AND METHODS

Bacterial Strains and Cultivation —— *V. mimicus* strain CS-5, a clinical isolate produces a high level of VMH, and strain TKT-2 (*vmhA*::*cat*), a *vmhA* mutant constructed by single crossover homologous recombination[14] from another clinical isolate (strain CS-20), were used. The bacteria were grown in heart infusion broth (HIB) at 37°C with shaking (120 cycles/min) until the culture reached to the desired optical density at 600 nm (OD_{600}).

Reverse Transcription PCR (RT-PCR) —— The RT-PCR was carried out to compare the expression level of *vmhA* gene with that of a house keeping

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Table 1. Primers Used for the RT-PCR

Primer	Direction	Sequence (5′–3′)	Amplicon
VMH-2	Forward	ggtagccatcagtcttatcacg	390 bp from *vmhA* gene
VMH-3	Reverse	atcgtgtcccaatacttcaccg	
recA F	Forward	ggacgtatcgttgagatcttcg	260 bp from *recA* gene
recA R	Reverse	gagtcgacaacaatcacatctac	

gene, *recA*, encoding RecA protein (GenBank accession no. AF301036). Total RNA was prepared from 1 ml culture of strain CS-5 using Sepasol-RNAI Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instruction. RT-PCR was performed using Ready-To-Go RT-PCR kit (GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.) and oligonucleotide primers shown in Table 1. The RT for generation of cDNA from mRNA was carried out at 42°C for 30 min, and the PCR was performed with the program consisting of 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 53°C and extension of 1 min at 72°C.

Assay of the Hemolytic Activity —— The culture supernatants were collected from different growth stages by centrifugation (12000 × g for 5 min at 4°C) followed by filtration through a 0.2 µm-pore-size membrane. The hemolytic activity was measured with 1% horse erythrocytes as described previously.[15] Briefly, the culture supernatant was diluted serially with 10 mM Tris-HCl buffer (pH 7.5) containing 0.9% NaCl. Each of the samples (1 ml) was allowed to act on horse erythrocytes (1 ml) at 37°C for 1 hr. Thereafter, each reaction mixture was centrifuged at 1000 × g for 5 min, the supernatant was withdrawn, and the amount of hemoglobin released from disrupted erythrocytes was determined by measuring absorbance at 540 nm. One hemolysin unit (HU) was defined as the amount of hemolysin eliciting 50% hemolysis.

Western Blot Analysis —— Western blot analysis was carried out with the rabbit IgG antibody against purified VMH.[5] The culture supernatants were treated with 2% SDS (sodium dodecyl sulfate) at 100°C for 3 min and subjected to SDS-PAGE (polyacrylamide gel electrophoresis) on the PhastSystem using a PhastGel Gradient 10–15 (GE Healthcare Bio-Sciences). After SDS-PAGE, the proteins separated were electrophoretically transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences), and pro- and mature VMH were detected with the antibody.

Isolation and Sequencing of Pro-VMH —— The pro-VMH was isolated from the concentrated culture supernatant of strain CS-5 grown in 250 ml of HIB until OD_{600} of 0.7. In order to prevent from proteolytic degradation by a metalloprotease from the same bacterium,[4,5] 10 mM EDTA and 10 mM tetraethylenepentamine (TEP) were added to the culture supernatant. Thereafter, proteins in the culture supernatant were precipitated by the addition of ammonium sulfate to 70% saturation (472 g/l). The resulting precipitates were collected by centrifugation, dissolved in 10 ml of 6.7 mM phosphate buffer (pH 7.4) containing 0.9% NaCl, 10 mM EDTA and 10 mM TEP and dialyzed against the same buffer. The pro-VMH was specifically isolated from the concentrated preparation by the immunomagnetic beads technique. Namely, Dynabeads M-280 (Dynal Biotech, Oslo, Norway) were coated with the rabbit IgG antibody against purified VMH and allowed to incubate with the concentrated preparation containing pro-VMH with shaking (20 cycles/min). The pro-VMH formed the complex with the beads was precipitated by centrifugation, and the immunocomplex collected was treated with 2% SDS at 100°C for 3 min and subjected to SDS-PAGE on the PhastSystem using a PhastGel Gradient 10–15. After SDS-PAGE, the proteins separated were transferred to a PVDF membrane and stained with Coomasie brilliant blue R-250. The 79-kDa protein band corresponding to pro-VMH was cut off from the membrane, and the N-terminal amino acid sequence was determined by Edman degradation with an automatic protein sequencer model 473A (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS

Expression of *vmhA* Gene

Expression of the hemolysin gene in different growth stages was studied by RT-PCR (Fig. 1). It was shown that a detectable amount of *vmhA* mRNA was present in the bacterial cells at the early log phase, of which OD_{600} was as low as 0.3, and that, at the middle log phase (OD_{600} = 0.5), the amount of *vmhA* mRNA reached the comparable level to that

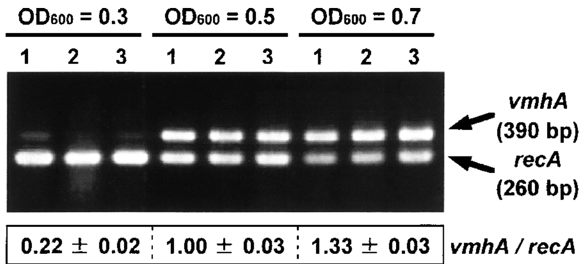

Fig. 1. Expression of *vmhA* Gene at Different Growth Stages of *V. mimicus* Strain CS-5

Total RNA was extracted from three individual cultures at growth stages indicated. RT-PCR was carried out with the total RNA extracted, and an aliquot of the product (10 µl) was run in each lane of 2% agarose gel.

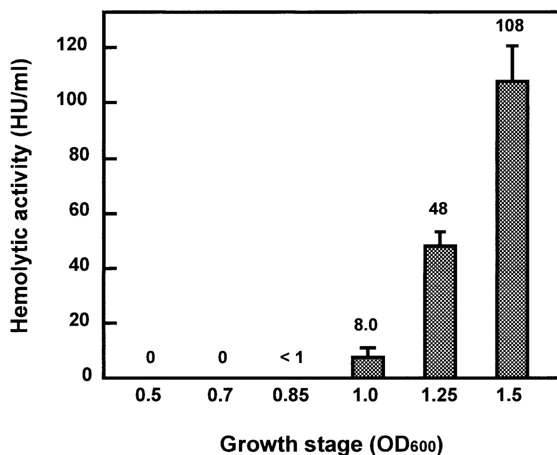

Fig. 2. The Hemolytic Activities of the Culture Supernatants of *V. mimicus* Strain CS-5

The culture supernatants were collected at growth stages indicated, and the hemolytic activities (HU/ml) were determined using 1% horse erythrocytes. The data presented are mean and S.D. of three individual experiments.

of *recA* mRNA. Sufficient expression of *vmhA* gene continued on the early stationary growth phase (data not shown).

Hemolytic Activity

The culture supernatants of strain CS-5 were examined if the active hemolysin was present or not (Fig. 2). Although expression of *vmhA* gene started at the early log phase ($OD_{600} < 0.5$), the significant hemolytic activity was not detected until the late log phase ($OD_{600} = 1.0$). Once the active hemolysin was generated, the level of the hemolytic activity was increased rapidly. The mean hemolytic activity in the culture supernatants from OD_{600} of 1.0, 1.25 and 1.5 were 8.0, 48 and 108 HU/ml, respectively.

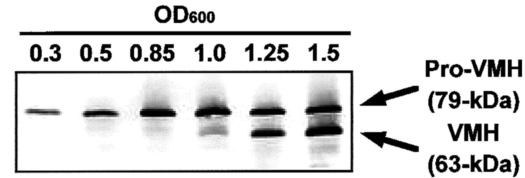

Fig. 3. Western Blot Analysis of the Culture Supernatants

The culture supernatants collected at growth stages indicated were subjected to SDS-PAGE. Thereafter, the proteins were transferred to a PVDF membrane, and pro- and mature VMH were detected with the antibody against mature VMH.

Generation of Active VMH in the Non-hemolytic Culture Supernatant through Growth of a VMH Mutant

A *vmhA* disruptant, strain TKT-2, was inoculated into the culture supernatant from strain CS-5 with OD_{600} of 0.7, which contained no detectable hemolytic activity, and the disruptant was cultivated at 37°C until OD_{600} of 1.0. When the hemolytic activity of the culture was measured, the considerable level of the activity (5.5 HU/ml) was detected. In contrast, when strain TKT-2 was grown in the own culture supernatant, no hemolytic activity was detected. These results indicate that the culture supernatant from strain CS-5 may contain the protoxin, which is activated by a factor(s) from strain TKT-2.

Western Blot Analysis of the Culture Supernatants

Western blot analysis was done to detect pro- and mature VMH in the culture supernatants from different growth stages of strain CS-5 (Fig. 3). Only the 79-kDa protoxin could be detected in the culture supernatants at the early and middle log phase ($OD_{600} < 0.85$). On the other hand, the 63-kDa mature toxin was contained in the culture supernatants at the late log ($OD_{600} = 1.0$) or later growth phase. Also, the amount of mature VMH seemed to increase gradually with the progress in the bacterial growth. It is thus evident that cleavage of pro-VMH to generate mature VMH with the hemolytic activity is started only when the culture has reached an enough cell density.

N-terminal Amino Acid Sequence of Pro-VMH

The N-terminal amino acid sequence of 63-kDa mature VMH was determined previously by Rahman *et al.*,[11] and they reported that the maturation was achieved by cleavage between Arg^{151} and Ser^{152}. Therefore, we were interested to know the N-terminal amino acid sequence of the 79-kDa pro-

toxin. Pro-VMH was isolated by the immunological technique using the magnetic beads coated with the antibody against mature VMH. The amino acid sequence of the protoxin isolated was determined to be NH_2-Asn-Ile-Ser-Asp-Pro-Val. This finding demonstrates that 79-kDa pro-VMH is generates through cleavage of the Ala^{25}-Asn^{26} bond by a signal peptidase.

DISCUSSION

VMH is a potential virulence factor of *V. mimicus*, and several investigations have demonstrated the enterotoxic potential of VMH.[5, 9, 10] However, no study on the mechanism of secretion and activation of the hemolysin has been done. When we measured the hemolytic activity at different growth stages of *V. mimicus*, although *vmhA* gene was expressed even when OD_{600} was as low as 0.3, significant hemolytic activity was not detected until OD_{600} of 1.0. It was thus hypothesized that pro-VMH had been secreted from the early log phase, while conversion of pro-VMH to mature VMH took place afterwards. In order to confirm this hypothesis, the VMH null mutant was grown in the non-hemolytic spent culture supernatant, and appearance of the active hemolysin was tested. As it thought, the considerable level of the hemolytic activity was produced. On the other hand, western blot analysis remarkably showed the presence of 79-kDa pro-VMH in the culture supernatants of the early growth phase (OD_{600} < 0.85); however, 63-kDa mature VMH was detected only in the later phases (OD_{600} > 1.0). Taken together, it may be concluded that pro-VMH secreted in the early growth phase remains to be inactive until the bacterial cell density reaches to a threshold level.

The 79-kDa pro-VMH was isolated from the concentrated culture supernatant by using the immunomagnetic beads. The analysis of the N-terminal amino acid sequence revealed that cleavage of the 83-kDa precursor took place between Ala^{25} and Asn^{26} before extracellular secretion. This finding indicates that pro-VMH is secreted extracellularly through the system depend on a signal peptidase. The factor responsible for activation of pro-VMH is not identified yet. However, the activating factor is probably expressed in the late growth phase. In the case of pro-HlyA from *V. cholerae*, when the activation experiment was carried out *in vitro*, the protoxin was activated by incubation with an endogenous metalloprotease, as well as exogenous proteolytic enzymes including trypsin.[16] The work to clarify the activation mechanism of pro-VMH is going on in our laboratory.

In conclusion, VMH may be activated through the similar manner to that of HlyA; however, cleavage of pro-VMH results in conversion to active VMH may occur after the culture reaches a sufficient cell density.

Acknowledgements This study was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Sciences.

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