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Second extracellular protease mediating maturation of *Vibrio mimicus* hemolysin

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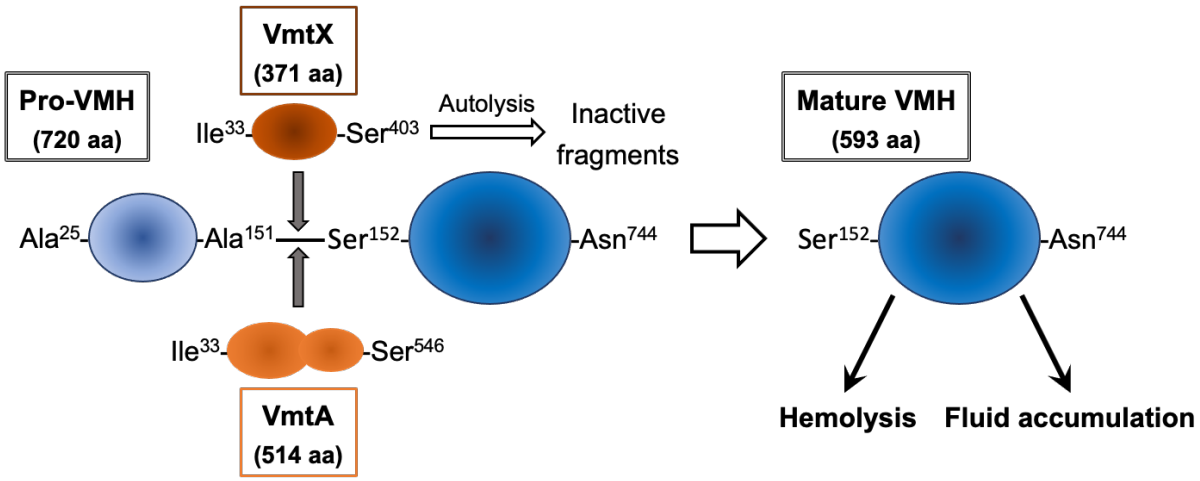
Graphical Abstracts



Abstract

Vibrio mimicus is a bacterium that causes gastroenteritis in humans. This pathogen produces an enterotoxic hemolysin called *V. mimicus* hemolysin (VMH), which is secreted extracellularly as an inactive 80-kDa protoxin and converted to a 66-kDa mature toxin through cleavage between Arg^{151} and Ser^{152}. The 56-kDa serine protease termed *V. mimicus* trypsin-like protease (VmtA) is known to mediate this maturating process. However, some strains including strain ES-20 does not possess the *vmtA* gene. In the present study, the *vmtA*-negative strains were found to have a replaced gene that encodes a 43-kDa (403 aa) precursor of a serine protease designated by VmtX (*V. mimicus* trypsin-like protease X). To examine whether VmtX is also involved in the maturation of VMH, VmtX was isolated from the culture supernatant of *V. mimicus* strain NRE-20, a metalloprotease-negative mutant constructed from strain ES-20. Concretely, the culture supernatant was fractionated with 70% saturated ammonium sulfate and subjected to affinity column chromatography using a HiTrap Benzamidine FF column. The analysis of the N-terminal amino acid sequences of the proteins in the obtained VmtX preparation indicated that the 39-kDa protein was active VmtX consisting of 371 aa (Ile^{33}-Ser^{403}). The VmtX preparation was found to activate pro-VMH through generation of the 66-kDa protein. Additionally, treatment of the VmtX preparation with serine protease inhibitors, such as leupeptin and phenylmethylsulfonyl fluoride, significantly suppressed the activities to hydrolyze the specific peptide substrate and to synthesize the 66-kDa toxin. These findings indicate that VmtX is the second protease that mediats the maturation of VMH.

Keywords: *Vibrio mimicus*, serine protease, hemolysin, maturation

Introduction

Vibrio mimicus is a microorganism ubiquitous in aquatic environments (Janda et al. 1988; Chowdhury et al. 1989) but a human pathogen that causes gastroenteritis through consumption of contaminated raw seafood (Shandera et al. 1983; Janda et al. 1988). Of several toxic factors have been isolated (Spira and Fedorka-Cray 1984; Chowdhury et al. 1987, Yuan et al. 1993), a hemolytic toxin termed *V. mimicus* hemolysin (VMH) is thought to be potentially more harmful (Shi et al. 2000; Shinoda et al. 2004) from the fact that purified VMH induced fluid accumulation in a ligated rabbit ileal loop (Miyoshi et al. 1997), and that the antibody against the purified toxin obviously reduced the enteropathogenicity of living *V. mimicus* cells (Li et al. 2008). In addition, Shinoda et al. (2004) documented that the *vmhA* gene encoding VMH is omnipresent in all *V. mimicus* strains isolated from both clinical and environmental sources.

VMH is secreted extracellularly as the inactive 80-kDa protoxin and is subsequently converted to the 66-kDa mature toxin by removal of the 14-kDa N-terminal pro-peptide (Ala^{25}-Arg^{151}) (Rahman et al. 1996; Sultan et al. 2007). Mizuno et al. (2014) previously isolated the 56-kDa serine protease VmtA (*V. mimicus* trypsin-like protease) from the bacterial culture supernatant, and they revealed that VmtA could mediate the maturation of the protoxin through the limited cleavage between Arg^{151} and Ser^{152}. Our preliminary experiments to detect *vmtA* by polymerase chain reaction (PCR) amplification showed that the gene was widely distributed in *V. mimicus* except some of the strains. On the other hand, the database analysis indicated that, in the *V. mimicus* strain VM603, the genetic locus for *vmtA* was replaced by another gene encoding a 43-kDa (403 aa) serine protease.

In the present study, we analyzed the genetic loci of the *vmtA*-negative strains and found that all the strains tested had the gene designated by *vmtX* (*V. mimicus* trypsin-like protease X) encoding the 43-kDa serine protease. We also isolated the 39-kDa active VmtX from the culture

supernatant of the *vmtA*-negative strain. When incubated with pro-VMH, we found that the isolated VmtX converted the 80-kDa protoxin into the 66-kDa protein, which has significantly high hemolytic activity. These findings indicate that VmtX, as well as VmtA, can mediate the proper maturation of pro-VMH.

Materials and methods

Bacterial strains and cultivation

In the present study, one *vmtA*-positive wild-type strain (ES-37) previously used for purification of VmtA (Mizuno et al. 2014), and four *vmtA*-negative wild-type strains (CS-13, ES-20, ES-26 and ES-44) were used. Among these strains, only strain CS-13 was isolated from a clinical source (a sample of feces from a diarrheal patient).

The metalloprotease gene (*vmp*)-negative mutant was constructed from strain ES-20 by the single crossover homologous recombination method, as previously described by Nishibuchi et al. (1991). Briefly, the 500 bp fragment of *vmp* was amplified by PCR and inserted into a plasmid vector pKTN701. The obtained hybrid plasmid was transformed into *Escherichia coli* strain SY327λ*pir*, and then into *E. coli* strain SM10λ*pir*. Thereafter, the hybrid plasmid was transferred to *V. mimicus* strain ES-20 by conjugation, and the conjugants were cultivated on TCBS agar plates containing 10 μg/mL chloramphenicol. One suitable *vmp*-negative mutant, strain NRE-20, was selected after 48 hr cultivation at 37°C. Disruption of the *vmp* gene was confirmed by PCR using an appropriate primer set described by Mizuno et al. (2014).

V. mimicus was cultivated in peptone broth (0.5% peptone [Becton, Dickinson and Company, Franklin Lakes, NJ, USA], 0.25% yeast extract [Becton, Dickinson and Company], 0.5% NaCl, 10 mM of K_2HPO_4-KH_2PO_4, pH 7.4) and incubated at 26°C for 16 hr with shaking.

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107 **PCR detection of *vmh*, *vmtA* and *vmtX***

108 The oligonucleotide primers for PCR amplification of *vmhA* (vmh-F1 and vmh-R1) and
109 *vmtA* (vmt-F1 and vmt-R1) were prepared as described by Mizuno et al. (2014), while the
110 primers for amplification of *vmtX* (vmx-F1: TACCCTTCAATCCAAACCG [197388 to197406]
111 and vmx-R1: TTTTCGTTCCTCCTGAAGAG [197964 to197983]) were designed based on the
112 nucleotide sequences of the cont356 of *V. mimicus* strain VM603 whole genome (accession
113 number, ACYU01000120).

114 After cultivation, the cells of *V. mimicus* were harvested by centrifugation at 13,000 x g
115 for 5 min and rinsed once with 1.0% NaCl. Thereafter, the cells were suspended into 1.0%
116 NaCl-0.25 mM EDTA and heat-treated at 100°C for 5 min, and then, the supernatant containing
117 DNA was collected by centrifugation. For PCR amplification, the DNA preparation (1 μL), Go
118 Taq Green Master Mix (Promega, Madison, WI, USA) (12.5 μL) and each of the primer set (20
119 pmol in 2 μL) were mixed, and the total volume of the mixture was adjusted to 25 μL with
120 purified water. Thereafter, the PCR mixture prepared was set on a thermal cycler (Astec,
121 Fukuoka, Japan) and heat-treated at 94°C for 2 min, and PCR was carried out for 25 cycles as
122 below: denaturation at 95°C for 15 sec, annealing at an appropriate temperature for 30 sec, and
123 extension at 72°C for 60 sec. After PCR, the products were electrophoresed on a 1.5 % agarose
124 gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) at 100 V and visualized by
125 staining with ethidium bromide. The molecular size of PCR amplicon was determined using the
126 1 Kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA).

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128 **Determination of the nucleotide sequence of *vmtX* of *V. mimicus* strain CS-13**

129 After cultivation, the genomic DNA of strain CS-13 was extracted by using Illustra
130 bacteria genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK),

and the 1,365 bp DNA fragment including the full *vmtX* gene was amplified by PCR. After PCR, the amplicons were electrophoresed on a 1.0% agarose gel, and the target amplicon was extracted by using Geneclean Turbo kit (MP Biomedicals, Santa Ana, CA, USA).

The nucleotide sequence of the *vmtX* gene was determined with ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Specifically, the extracted amplicon (50 ng) was mixed with an appropriate primer set (6.4 pmol in 4.0 μL), BigDye Reaction Mix (1.0 μL), and 5-fold concentrated Dilution Buffer (7.5 μL), and the total volume was adjusted to 40 μL with sterilized pure water. Thereafter, the reaction mixture was set in a thermal cycler and subjected to 25-cycle PCR amplification (denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 4 min). Subsequently, the Dye Terminator was removed from 20 μL of the PCR product by using FastGene Dye Terminator Removal Kit (Nippon Genetics, Tokyo, Japan), and after heat treatment at 96°C for 2 min, the nucleotide sequence of the PCR amplicon was determined with an ABI PRISM 3100-Avant Genetic Analyzer (Thermo Fisher Scientific).

The accession number for the nucleotide sequence data in DDBJ, EMBL-Bank, and GenBank database is LC583889.

Determination of the N-terminal amino acid sequence

The sample was treated with 20% trichloroacetic acid to denature the proteins in the sample, and the denatured proteins were collected by centrifugation, rinsed sufficiently with 100% ethanol, and left 15 min to dry naturally. Thereafter, the proteins were mixed with 10 μL of SDS sample buffer (125 mM Tris-HCI, 2% SDS, 20% glycerol, 0.01% bromophenol blue, pH 6.8) and heat-treated at 100°C for 5 min. Subsequently, the proteins were subjected to SDS-PAGE using Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Hercules, CA, USA). After SDS-PAGE, the gel was stained with 0.5% Coomassie blue R-250 and then destained

with 30% methanol-10% acetic acid. The molecular weight of the protein was determined by using Precision Plus Protein Dual Color standards (10,15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) (Bio-Rad Laboratories).

The proteins separated by SDS-PAGE were transferred to a PVDF membrane by using a Trans-Blot Turbo Transfer Pack (Bio-Rad Laboratories). Thereafter, the membrane was rinsed with 0.3% Tween 20, stained with 0.1% amido black, and destained with 90% methanol-2% acetic acid. Then, small pieces of the membrane containing the target protein were cut off, and the N-terminal amino acid sequences were analyzed with an automatic peptide sequencer PPSQ-31A (Shimadzu, Kyoto, Japan).

Western blot analysis

The sample was subjected to SDS–PAGE, and the separated proteins were transferred to a PVDF membrane. The membrane with the bound proteins was then incubated with the rabbit IgG antibody against VMH, and the antigen–antibody complex was visualized using the antibody against rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a chromogenic substrate (4-methoxy-1-naphthol), and hydrogen peroxide.

Measurement of the proteolytic and hemolytic activity

The proteolytic activity (peptidase activity) of VmtX was measured by the method used for VmtA (Mizuno et al. 2014). The sample (0.1 mL) was mixed with 0.2 mL of 0.1 M Tris-HCI buffer (pH 8.0) containing 4 mM $CaCl_2$ and 1.25 mM *t*-butyloxycarbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) (Peptide Institute, Ibaraki, Osaka, Japan), and the admixture was incubated at 30°C for an appropriate period. Thereafter, the reaction was terminated by the addition of 0.7 mL of 0.1 M citrate buffer (pH 5.0), and the amount of 7-

amino-4-methylcoumarin (AMC) released from the substrate was quantified by measuring the absorbance at 370 nm. One peptidase unit (PU) was defined as the amount of the enzyme releasing 1 nmol of AMC in 1 min.

For measurement of the hemolytic activity, the sample (0.2 mL) prepared in 20 mM Tris-HCI buffer (pH 7.5) containing 0.9% NaCl, 0.01% bovine serum albumin (BSA) and 0.04% NaN_3 was mixed with an equal volume of 1% sheep erythrocyte and incubated at 37°C for 30 min. After incubation, the admixture was centrifuged at 1,000 x g for 5 min. Then, the supernatant containing hemoglobin leaked from the disrupted erythrocytes was decanted, and the amount of hemoglobin was quantified by measuring the absorbance at 540 nm. One hemolysin unit (HU) was defined as the amount of the toxin disrupting 50% of the erythrocytes in the admixture.

The amount of protein in the sample was quantified by the Lowry method using BSA as a standard protein (Lowry et al. 1951).

Purification of recombinant pro-VMH

The recombinant pro-VMH (pro-rVMH) was purified from a periplasmic fraction of an *Escherichia coli* transformant DH5α/pBST67, in which the expression of the *vmhA* gene was under the control of its own promoter (Rahman et al. 1996; Mizuno et al. 2014). More specifically, at the middle logarithmic growth phase, the cells of the transformant were harvested, and the periplasmic fraction was prepared by the osmotic shock method (Neu and Heppel 1965) and fractionated with 70% saturated (472 g/L) ammonium sulfate. The resultant precipitate was collected by centrifugation at 12,000 x g for 10 min at 4°C, dissolved in purified water, and dialyzed overnight at 4°C against 10 mM phosphate buffer (pH 6.5). Thereafter, the obtained preparation was loaded onto a HiLoad 10/10 Phenyl Sepharose HP column (GE Healthcare Life Sciences) equilibrated with 10 mM phosphate buffer (pH 6.5), and pro-rVMH

bound to the column was eluted with 50 mM glycine buffer (pH 9.0).

Purification of VmtX

In human pathogenic vibrios, the production of serine proteases has been reported to be enhanced by disruption of the metalloprotease gene (Miyoshi et al. 2008; Wang et al. 2008). Because *V. mimicus* also secretes a metalloprotease VMP (*V. mimicus* protease) (Chowdhury et al. 1990), in this study, the *vmp*-negative mutant NRE-20 was constructed from strain ES-20 and used for purification of VmtX. After cultivation in 1,200 mL of peptone broth, the culture supernatant was obtained by centrifugation at 8,000 x g for 40 min at 4°C and fractionated with 70% saturated ammonium sulfate. The resultant precipitate was collected by centrifugation, dissolved in 50 mM Tris-HCI buffer (pH 8.0) containing 0.5 M NaCl and 4 mM $CaCl_2$ (buffer A), and dialyzed overnight at 4°C against buffer A.

Thus, the obtained preparation was subjected to affinity chromatography using a HiTrap Benzamidine FF column (GE Healthcare Life Sciences) equilibrated with buffer A. The preparation after the ammonium sulfate fractionation was applied to the column, and the non-bound proteins were washed out. Thereafter, VmtX was eluted in a linear gradient from 0 to 50 mM benzamidine in buffer A, and the peptidase activity of each fraction (1 mL) was measured with Boc-Phe-Ser-Arg-MCA. The fractions showing significant peptidase activity were collected and concentrated with Amicon Ultra-15 10K filter (Merck Millipore, Burlington, MA, USA).

Activation of pro-rVMH with VmtX

The VmtX preparation (50 ng) after the affinity chromatography was allowed to act on pro-rVMH (5 μg) in 15 μL of 0.1 M Tris-HCI buffer (pH 8.0) containing 4 mM $CaCI_2$ at 37°C for 30 min. Thereafter, the hemolytic activity of rVMH maturated by VmtX was measured, and

SDS-PAGE analysis was also performed to detect 66-kDa mature rVMH. In order to examine the effects of serine protease inhibitors, such as leupeptin and phenylmethylsulfonyl fluoride (PMSF), the VmtX preparation was pretreated with each of the inhibitors at 37°C for 30 min.

The experiments were repeated three or more times, and each data was presented as the mean ± SD. The data was analyzed by Student's t test, with the *p* value less than 0.05 being considered to be significantly different.

Results and Discussion

Presence of the *vmtX* gene in the *vmtA*-negative strains

One clinical and three environmental strains of *V. mimicus*, which did not have the *vmtA* gene (1,641 bp) (accession number AB540653), were tested to determine whether the *vmtX* gene was harbored in the genetic locus for *vmtA* as found in strain VM603 (accession number, ACYU01000120). As the results of the PCR amplification using the primer sets vmx-F1 and vmx-R1, the 596 bp amplicon corresponding to *vmtX* was detected in all of the *vmtA*-negative strains (Table 1). However, strain ES-37 (a *vmtA*-positive strain used as the control) showed no production of the amplicon, indicating the absence of the *vmtX* gene. In addition, all the strains used in the experiments were confirmed to be *vmhA*-positive because the 386 bp DNA fragment was commonly amplified (Table 1). Among all the *vmtA*-negative strains found to have the *vmtX* gene, strain CS-13 was used to determine the genetic locus and nucleotide sequence of the *vmtX* gene.

Determination of the nucleotide sequence of the *vmtX* gene of strain CS-13

Several PCR primer sets were prepared based on the nucleotide sequence of strain VM603 whole genome, and the DNA fragment including the full length of the *vmtX* gene (1,212 bp)

was amplified. Then, the nucleotide sequence of the amplicon was determined (accession number, LC583889). The results showed that, as well as strain VM603 (accession number, ACYU01000120), the genetic locus for *vmtA* was replaced by *vmtX* in strain CS-13. Although blastn search against GenBank indicated that the *vmtX* gene was very rare in *V. mimicus*, the orthologous gene *vesB* having 73.0% identity (Gadwal et al. 2014) was widely distributed among *Vibrio cholerae* strains. This finding suggests that the *vmtX* gene was horizontally transferred from *V. cholerae* to *V. mimicus* and then replaced the *vmtA* gene originally harbored in the genetic locus.

The VmtX precursor was found to consist of 403 amino acid residues and to have the molecular weight of 43,261 Da, and the putative catalytic triad was identified as His^{78}, Asp^{125} and Ser^{221} (Fig. 1). The identity of the VmtX precursor with those of two vibrios trypsin-like serine proteases (*V. cholerae* VesB and *V. mimicus* VmtA) was 75.3% and 47.6%, respectively. Therefore, VmtX might be the substitutional trypsin-like serine protease secreted by *vmtA*-negative *V. mimicus* strains. Additionally, it was indicated that the VmtX precursor did not have the C-terminal fragment consisting of 143 amino acid residues.

Maturation of pro-VMH by *vmtA*-negative strains during cultivation

To examine whether active VMH was generated by four *vmtA*-negative strains, the culture supernatants were collected at an early stationary phase and incubated with erythrocytes at 37°C for 30 min. The culture supernatants from two strains (ES-20 and ES-44) showed significant hemolytic activity (higher than10 HU/mL [5.4 HU/mg]), while the supernatants from strain CS-13 and ES-26 showed negligible activity (lower than 1.0 HU/mL [0.5 HU/mg]). Strains ES-20, ES-44 and ES-37 (a *vmtA*-positive strain) were inoculated on a blood-agar plate and cultivated at 37°C for 24 hr. As shown in Fig. 2, all bacterial strains formed clear β-hemolytic zones around the colonies, indicating production of the active extracellular hemolysin.

For expression of sufficient hemolytic activity, significant production of both pro-VMH and a protease that maturates the protoxin is essential. Western blot analysis using the antibody against VMH indicated that culture supernatants from strain ES-20, ES-26, ES-44 and ES-37 contained both 80-kDa and 66-kDa VMH antigens (Fig. 3). However, the culture supernatant of strain CS-13 did not contain the detectable amount of VMH antigen (data not shown). In addition, culture supernatants from strain ES-20 and ES-26 were found to contain another 51-kDa VMH antigen, which is known to be generated through proteolysis by the metalloprotease VMP (Mizuno et al. 2009). We also measured the proteolytic activities (peptidase activities) of the culture supernatants with Boc-Phe-Ser-Arg-MCA (a synthetic substrate for trypsin-like proteases). The culture supernatant of strain ES-20 showed the significant activity (0.17 PU/mL), but those from other strains showed the activity less than 0.1 PU/mL. Therefore, the negligible hemolytic activity of the culture supernatant of strain CS-13 may be due to insufficient production of pro-VMH and/or the protease.

The culture supernatants from strains ES-20 and ES-44 were treated with 20% trichloroacetic acid to denature the proteins, and the denatured proteins were dissolved with SDS and then subjected to SDS-PAGE. Thereafter, the proteins separated were transferred to a PVDF membrane, and a small piece of the membrane containing a 66-kDa protein, a putative active VMH, was cut off. In both strains, the N-terminal amino acid sequence of the 66-kDa protein was determined to be NH_2-Ser-Val-Ser-Ala-Asn, which was identical to the sequence of active VMH maturated by VmtA (Mizuno et al. 2014). The N-terminal amino acid sequence of the 66-kDa protein in the culture supernatant of strain ES-26 was also analyzed. However, its sequence could not be determined. Mizuno et al. (2009) documented that, although the activity is not known, the metalloprotease VMP converted 80-kDa pro-VMH to a 66-kDa intermediate through cleavage between Asn^{157} and Val^{158}. Therefore, the 66-kDa protein in the culture supernatant of strain ES-26 may be the mixture of the mature VMH and the intermediate.

These results suggest that the negligible hemolytic activity of the culture supernatant of strain ES-26 may be due to inactivation of pro-VMH by the metalloprotease VMP.

These findings indicated that the culture supernatants from the *vmtA*-negative strains contained a novel proteolytic enzyme(s) causing the proper maturation of pro-VMH through cleavage between Arg^{151} and Ser^{152}. Therefore, to clarify whether VmtX is the enzyme causing the proper maturation of pro-VMH, VmtX was isolated from the culture supernatant and allowed to act on pro-rVMH.

Isolation of active VmtX

In human pathogenic vibrios, the production of serine proteases has been reported to be enhanced by disruption of the metalloprotease gene (Miyoshi et al. 2008; Wang et al. 2008). Because *V. mimicus* also has the *vmp* gene encoding an extracellular metalloprotease (accession number, AB435238), the *vmp*-negative mutant NRE-20 was first constructed from strain ES-20 to produce an enough amount of VmtX. After cultivation of strain NRE-20 in peptone broth, the culture supernatant was collected and fractionated with 70% saturated ammonium sulfate. The resultant precipitate was dissolved in and dialyzed against buffer A. The crude VmtX preparation thus obtained was subjected to affinity chromatography using a HiTrap Benzamidine FF column, which was suitable for purification of *V. cholerae* VesB (Gadwal et al. 2014). After washing out of the non-bound proteins, VmtX was eluted in a linear gradient from 0 to 50 mM benzamidine in buffer A. The peptidase activity of each fraction (1 mL) was measured with Boc-Phe-Ser-Arg-MCA, a synthetic peptide substrate for trypsin-like serine proteases including VmtA (Mizuno et al. 2014). Then, the fractions showing significant peptidase activity were collected and concentrated with Amicon Ultra-15 10K filter. The VmtX preparation obtained was found to have the specific activity of 5,300 PU/mg protein, which was approximately 4,300 times higher than that of the culture supernatant (Table 2). Even though

the activity recovery was 16.0%, the amount of the protein obtained was as small as 80 μg (Table 2).

However, SDS-PAGE analysis showed that the VmtX preparation after the affinity chromatography contained several proteins (Fig. 4A). Therefore, we analyzed the N-terminal amino acid sequences of the proteins. The results demonstrated only the 39-kDa protein might be produced by the bacterial cells, and the amino acid sequence of the 39-kDa protein (NH_2-Ile-Ile-Asn-Gly-Ser-Asp-Ala-Asn-Ser-Ala) was found to correspond to Ile^{33}-Ala^{42} of the VmtX precursor (Fig. 1). Therefore, the 39-kDa bacterial protein was identified as active VmtX consisting of 371 amino acid residues (Ile^{33}-Ser^{403}) and indicated to have the molecular weight of 39,776 Da. Mizuno et al. (2014) reported that, as with VmtX, the VmtA protein was activated through truncation of the N-terminal 32 amino acid residues. Therefore, the maturation mechanism of VmtX may be quite similar to that of VmtA.

For further purification, the VmtX preparation was applied to the Hiroad 10/1 Phenyl Sepharose HP column (GE Healthcare Life Sciences), as in the case of purification of VmtA described by Mizuno et al. (2014). Some fractions showing significant peptidase activity were collected and concentrated, but no active VmtX was obtained. In addition, no 39-kDa protein band was detected by SDS-PAGE analysis (Fig. 4B), suggesting autolytic inactivation of mature VmtX. We also tried gel filtration and anion exchange column chromatography for purification. However, any active VmtX could not be obtained. Unlike VmtA, VmtX does not have the C-terminal fragment (Fig. 1). The C-terminal fragments of bacterial serine proteases have been reported to possess some important biochemical functions including enhancement of enzyme stability (Gadwal et al. 2014; Huang et al. 2016). Therefore, VmtX is thought to be much less stable due to the absence of the C-terminal fragment in the mature molecule.

On the other hand, the VmtX preparation after the affinity chromatography was found to be considerably stable because the peptidase activity of the preparation was not reduced even

after incubation at 37°C for 30 min (data not shown). This finding suggests that VmtX is protected from autolytic inactivation through a loose association with other factor(s). Similar behavior was documented in the preparation of the partially purified *Vibrio vulnificus* serine protease (Wang et al. 2008).

Therefore, although not purified completely, the VmtX preparation after the affinity chromatography was used in subsequent experiments.

Activation of pro-rVMH with VmtX

First, to confirm the activation of pro-rVMH by VmtX, the VmtX preparation (50 ng) was allowed to act on pro-rVMH (5 μg) at 37°C for 30 min. Then, the maturation of pro-rVMH was assessed by measuring the hemolytic activity. As shown in Fig. 5A, the remarkably high hemolytic activity was detected after incubation with the VmtX preparation. Additionally, SDS-PAGE analysis revealed generation of the 66-kDa protein corresponding to the mature rVMH (Fig. 5B). These results demonstrate that the VmtX preparation has the ability to convert 80-kDa pro-rVMH to active 66-kDa rVMH.

Next, to determine whether the proteolytic activity is essential for activation/maturation of pro-rVMH, the VmtX preparation (50 ng) was pretreated with a suitable serine protease inhibitor at 37°C for 30 min. Thereafter, the residual activities hydrolyzing Boc-Phe-Ser-Arg-MCA and activating pro-rVMH were measured (Table 3). Both leupeptin and PMSF significantly inhibited the peptidase activity of VmtX ($p < 0.05$). In addition, the activity to generate the mature rVMH was also apparently inhibited by these protease inhibitors ($p < 0.05$).

Taken together, it may be concluded that VmtX is a protease that mediates maturation of pro-VMH in the *vmtA*-negative *V. mimicus* strains.

In conclusion, to identify a novel protease capable of mediating the maturation of pro-

VMH in *vmtA*-negative strains, firstly, the genetic loci for *vmtA* were analyzed. The analysis revealed that the *vmtA* gene was replaced by *vmtX* encoding the 43-kDa serine protease precursor. Next, the 39-kDa active VmtX was isolated from the culture supernatant of the *vmtA*-negative strain. The obtained VmtX preparation was found to convert the 80-kDa pro-VMH to the 66-kDa protein, which has significantly high hemolytic activity. On the other hand, the activity to generate mature VMH was lost by treatment with an appropriate protease inhibitor. These findings indicate that, instead of VmtA, VmtX has the ability to mediate the proper maturation of pro-VMH in *vmtA*-negative strains. It should be emphasized that active VmtX isolated was found to be the complex with the protein(s) and that the formation of the complex might prevent from autolytic inactivation of VmtX. Therefore, the presence of the protein(s), which is contained in food and forms the complex with active VmtX, may cause the prolonged activation of pro-VMH and may enhance the pathogenic actions of *V. mimicus*. Further studies on the protein(s) in the active VmtX preparation may be necessary.

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Author Contributions

Conceptualization, SM and TM; funding acquisition, SM; methodology, NT, TD and AN; writing and editing of the manuscript, SM. All authors have read and approved the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Ethical Statement

This study did not contain any human or animal subject.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

References

Chowdhury MAR, Aziz KMS, Kay BA, Rahim Z (1987) Toxin production by *Vibrio mimicus* strains isolated from human and environmental sources in Bangladesh. J Clin Microbiol 25:2200-2203. https://doi.org/10.1128/jcm.25.11.2200-2203.1987

Chowdhury MAR, Yamanaka H, Miyoshi S, Aziz KMS, Shinoda S (1989) Ecology of *Vibrio mimicus* in aquatic environments. Appl Environ Microbiol 55:2073-2078. https://doi.org/10.1128/aem.55.8.2073-2078.1989

Chowdhury MAR, Miyoshi S, Shinoda S (1990) Purification and characterization of a protease produced by *Vibrio mimicus*. Infect Immun 58:4159-4162. https://doi.org/10.1128/iai.58.12.4159-4162.1990

Gadwal S, Korotkov KV, Delarosa JR, Hol WG, Sandkvist M（2014）Functional and structural characterization of *Vibrio cholerae* extracellular serine protease B, VesB. J Biol Chem 289:8288-8298. https://doi.org/10.1074/jbc.M113.525261

Huang J, Wu C, Liu D, Yang X, Wu R, Zhang J, Ma C, He H (2016) C-terminal domains of bacterial proteases: structure, function and the biotechnological application. J Appl Microbiol 122:12-22. https://doi.org/10.1111/jam.13317

Janda JM, Powers C, Bryant RG, Abbott S (1988) Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. Clin Microbiol Rev 1:245-267. https://doi.org/10.1128/CMR.1.3.245

Li T, Kobayashi A, Takata N, Yoshimura T, Maehara Y, Tsuchiya T, Miyoshi S (2008) Role of the enterotoxic hemolysin in pathpgenicity of *Vibrio mimicus*. J Health Sci 54:686-691. https://doi.org/10.1248/jhs.54.686

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275. https://doi.org/10.1016/S0021-9258(19)52451-6

Miyoshi S, Sasahara K, Akamatsu S, Rahman MM, Katsu T, Tomochika K, Shinoda S (1997) Purification and characterization of a hemolysin produced by *Vibrio mimicus*. Infect Immun 65:1830-1835. https://doi.org/10.1128/iai.65.5.1830-1835.1997

Miyoshi S, Nitanda Y, Fujii K, Kawahara K, Li T, Maehara Y, Ramamurthy T, Takeda Y, Shinoda S (2008) Differential gene expression and extracellular secretion of the collagenolytic enzymes by the pathogen *Vibrio parahaemolyticus*. FEMS Microbiol Lett 283:176-181. https://doi.org/10.1111/j.1574-6968.2008.01159.x

Mizuno T, Sultan SZ, Kaneko Y, Yoshimura T, Maehara Y, Nakao H, Tsuchiya T, Shinoda S, Miyoshi S (2009) Modulation of *Vibrio mimicus* hemolysin through limited proteolysis by an endogenous metalloprotease. FEBS J 276:825-834. https://doi.org/10.1111/j.1742-4658.2008.06827.x

Mizuno T, Nanko A, Maehara Y, Shinoda S, Miyoshi S (2014) A novel extracellular protease of *Vibrio mimicus* that mediates maturation of an endogenous hemolysin. Microbiol

457 Immunol 58:503-512. https://doi.org/10.1111/1348-0421.12177
458 Neu HC, Heppel AL (1965) The release of enzymes from *Escherichia coli* by osmotic shock
459 and during the formation of spheroplasts. J Biol Chem 240:3685-3692.
460 https://doi.org/10.1016/S0021-9258(18)97200-5
461 Nishibuchi M, Kumagai K, Kaper JB (1991) Contribution of the *tdh1* gene of Kanagawa
462 phenomenon-positive *Vibrio parahaemolyticus* to production of extracellular
463 thermostable direct hemolysin. Microb Pathog 11:453-460. https://doi.org/10.1016/0882-
464 4010(91)90042-9
465 Rahman MM, Miyoshi S, Tomochika K, Wakae H, Shinoda S (1996) Analysis of the structural
466 gene encoding a hemolysin in *Vibrio mimicus*. Microbiol Immunol 41:169-173.
467 https://doi.org/10.1111/j.1348-0421.1997.tb01183.x
468 Shandera WX, Johnston JM, Davis BR, Blake PA (1983) Disease from infection with *Vibrio*
469 *mimicus*, a newly recognized *Vibrio* species. Ann Intern Med 99:169-171.
470 https://doi.org/10.7326/0003-4819-99-2-169
471 Shi L, Miyoshi S, Bi K, Nakamura M, Hiura M, Tomochika K, Shinoda S (2000) Presence of
472 hemolysin genes（*vmh*, *tdh*, and *hlx*）in isolates of *Vibrio mimicus* determined by
473 polymerase chain reaction. J Health Sci 46:63-65. https://doi.org/10.1248/jhs.46.63
474 Shinoda S, Nakagawa T, Shi L, Bi K, Kanoh Y, Tomochika K, Miyoshi S, Shimada T (2004)
475 Distribution of virulence-associated genes in *Vibrio mimicus* isolated from clinical and
476 environmental origins. Microbiol Immunol 48:547-551. https://doi.org/10.1111/j.1348-
477 0421.2004.tb03551.x
478 Spira WM, Fedorka-Cray PJ (1984) Purification of enterotoxins from *Vibrio mimicus* that
479 appear to be identical to cholera toxin. Infect Immun 45:679-684.
480 https://doi.org/10.1128/iai.45.3.679-684.1984
481 Sultan Z, Mizuno T, Sakurai A, Takata N, Okamoto K, Miyoshi S (2007) Growth phase

dependent activation of the precursor of *Vibrio mimicus* hemolysin (pro-VMH). J Health Sci 53:430-434. https://doi.org/10.1248/jhs.53.430

Yuan P, Ogawa A, Ramamurthy T, Nair GB, Shimada T, Shinoda S, Takeda Y (1993) *Vibrio mimicus* are the reservoirs of the heat-stable enterotoxin (NAG-ST) gene among species of the genus *Vibrio*. World J Microbiol Biotech 10:59-63. https://doi.org/10.1007/BF00357565

Wang J, Sasaki T, Maehara Y, Nakao H, Tsuchiya T, Miyoshi S (2008) Variation of extracellular proteases produced by *Vibrio vulnificus* clinical isolates: genetic diversity of the metalloprotease gene (*vvp*), and serine protease secretion by *vvp*-negative strains. Microb Pathog 44:494-500. https://doi.org/10.1016/j.micpath.2008.01.001

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Table 1. Detection of the genes by PCR

Strain	PCR amplicon		
	vmhA (386 bp)	*vmtA* (413 bp)	*vmtX* (596 bp)
vmtA-negative			
CS-13	+	–	+
ES-20	+	–	+
ES-26	+	–	+
ES-44	+	–	+
vmtA-positive			
ES-37	+	+	–

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Table 2. Isolation of VmtX from the culture supernatant of strain NRE-20

Purification step	Total protein (mg)	Total activity (PU)	Recovery of activity (%)	Specific activity (PU/mg)	Relative activity
Culture supernatant	2,210	2,700	100	1.2	1.0
Ammonium sulfate fraction	38.2	1,510	56.0	39.5	32.2
Affinity column chromatography	0.08	435	16.0	5,310	4,330

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Table 3. Inhibitory effects of serine protease inhibitors on the activity of VmtX

Protease inhibitor added	Hemolytic activity of rVMH generated (HU/tube)	Peptidase activity of VmtX (x 10^{-2} PU/tube)
No addition	870 ± 290	35.3 ± 5.1
Leupeptin (20 μM)	587 ± 98	24.5 ± 3.3
PMSF (1 mM)	418 ± 103	17.9 ± 3.8
PMSF (10 mM)	133 ± 74	< 10

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Figure legends

Fig. 1. The amino acid sequence of the VmtX precursor. The amino acid residues shown in the italic letters indicate the N-terminal propeptide removed during the maturation process, and the underlined amino acid residues indicate the N-terminal amino acid sequence of the mature enzyme. The putative catalytic triad (His^{78}, Asp^{125} and Ser^{221}) is also shown in bold shaded letters.

Fig. 2. Extracellular production of an active hemolysin by *V. mimicus* strain ES-20, ES-44 and ES-37. The bacterial strains were inoculated on a blood-agar plate and cultivated at 37°C for 24 hr.

Fig. 3. Western blot analysis of the culture supernatants. The culture supernatant from each strain was subjected to SDS–PAGE, and the separated proteins were transferred to a PVDF membrane. The membrane with the bound proteins was then incubated with the rabbit IgG antibody against VMH, and the antigen–antibody complex was visualized using the antibody against rabbit IgG conjugated with horseradish peroxidase, a chromogenic substrate, and hydrogen peroxide.

Fig. 4. SDS-PAGE protein profiles of the VmtX preparation after benzamidine affinity chromatography (A) and Phenyl Sepharose column chromatography (B). The VmtX preparation (200 μL) was treated with 20% trichloroacetic acid to denature the proteins, and the denatured proteins were mixed with 10 μL of SDS sample buffer and heat-treated at 100°C for 5 min. Subsequently, the proteins were subjected to SDS-PAGE. After SDS-PAGE, the gel was stained with 0.5% Coomassie blue R-250. Lane M: molecular weight marker proteins

527 (10,15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), and lane S: the VmtX preparation.

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529 **Fig. 5.** Activation of pro-rVMH with VmtX. The VmtX preparation (50 ng) and pro-
530 rVMH (5 μg) were mixed and incubated at 37°C for 30 min. After the incubation, the hemolytic
531 activity of rVMH maturated by VmtX was measured (A), and SDS-PAGE analysis was
532 performed to detect 66-kDa mature rVMH (B).

533

Figure 1 (Miyoshi et al.)

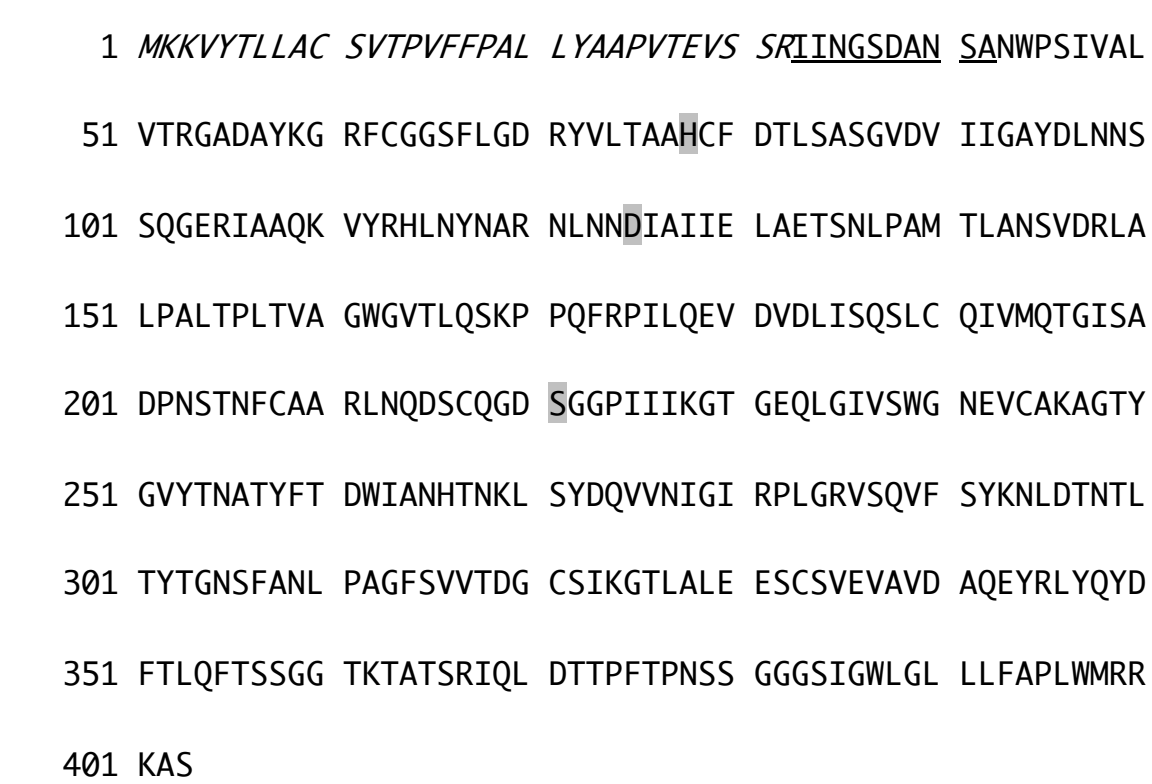

Figure 2 (Miyoshi et al.)

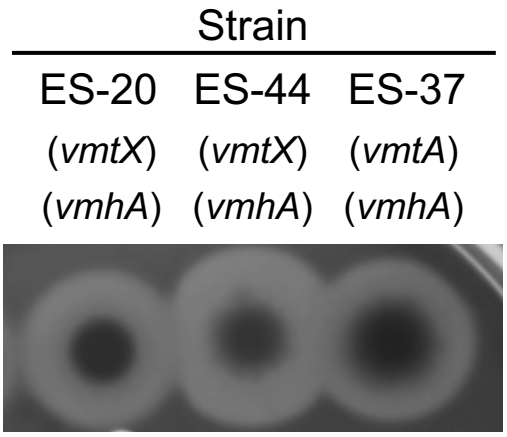

Figure 3 (Miyoshi et al.)

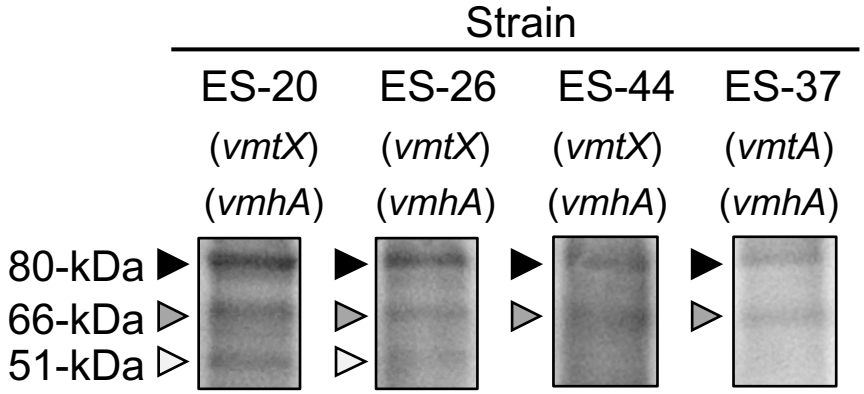

Figure 4 (Miyoshi et al.)

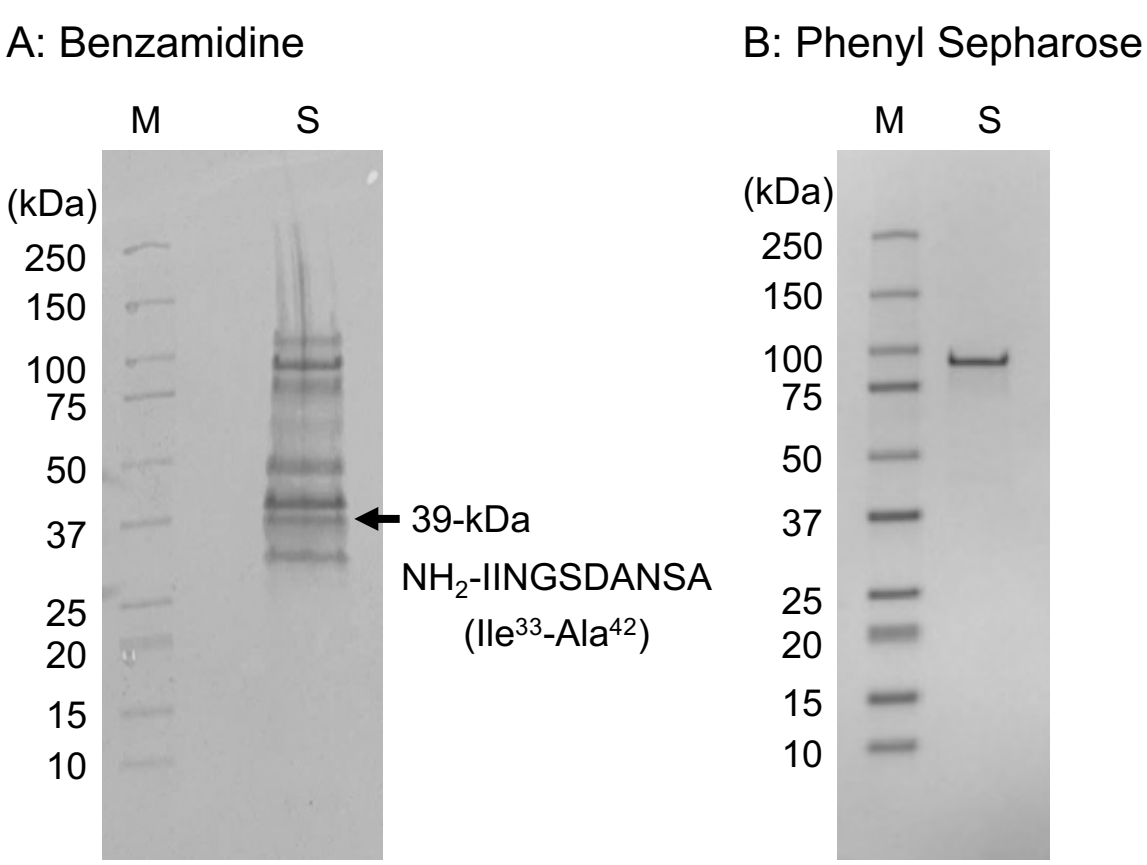

Figure 5 (Miyoshi et al.)