Functional and Structural Characterization of Vibrio cholerae Extracellular Serine Protease B, VesB*

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The chymotrypsin subfamily A of serine proteases consists primarily of eukaryotic proteases, including only a few proteases of bacterial origin. VesB, a newly identified serine protease that is secreted by the type II secretion system in Vibrio cholerae, belongs to this subfamily. VesB is likely produced as a zymogen because sequence alignment with trypsinogen identified a putative cleavage site for activation and a catalytic triad, His-Asp-Ser. Using synthetic peptides, VesB efficiently cleaved a trypsin substrate, but not chymotrypsin and elastase substrates. The reversible serine protease inhibitor, benzamidine, inhibited VesB as an immobilized ligand for VesB affinity purification, further indicating its relationship with trypsin-like enzymes. Consistent with this family of serine proteases, N-terminal sequencing implied that the propeptide is removed in the secreted form of VesB. Separate mutagenesis of the activation site and catalytic serine rendered VesB inactive, confirming the importance of these features for activity, but not for secretion. Similar to trypsin but, in contrast to thrombin and other coagulation factors, Na+ did not stimulate the activity of VesB, despite containing the Tyr250 signature. The crystal structure of catalytically inactive pro-VesB revealed that the protease domain is structurally similar to trypsinogen. The C-terminal domain of VesB was found to adopt an immunoglobulin (Ig)-fold that is structurally homologous to Ig-folds of other extracellular proteins. Possible roles of the Ig-fold domain in stability, substrate specificity, cell surface association, and type II secretion of VesB, the first bacterial multidomain trypsin-like protease with known structure, are discussed.

Many bacteria require secretion of a variety of proteins for optimal adaptation to the diverse environments they encounter. One of the several bacterial protein secretion machineries is the type II secretion (T2S)5 system. This large two-membrane spanning assembly is remarkable in that it translocates multidomain and sometimes multimeric proteins in folded form across the outer membrane of Gram-negative bacteria (1–3). In Vibrio cholerae, the T2S system is responsible for the secretion of cholera toxin, the major virulence factor, and a large number of enzymes, including proteases into the extracellular milieu (4, 5).

Proteases are naturally occurring enzymes that are found in all domains of life. One of the largest classes of proteases, serine proteases, contains over 80 families. The S1, or chymotrypsin family consists of structurally homologous endoproteases with conserved His, Asp, and Ser residues that form the catalytic triad of the active site. Most members of this family are secreted as inactive precursors, or zymogens, with an N-terminal propeptide. The propeptide is proteolytically cleaved at a conserved activation site, which induces a conformational change that results in an active enzyme (6, 7).

Although structurally very similar, members of the chymotrypsin family are further subdivided into three groups based on substrate specificity. Trypsin-like proteases cleave at an arginine or lysine residue, chymotrypsin-like proteases act on tryptophan, tyrosine, or phenylalanine, and elastase-like proteases target small, uncharged residues (7). Eukaryotic proteases of the chymotrypsin family are important for many cellular processes. Functionally, they aid in digestion, blood coagulation, complement activation, inflammation, and development (7). Although the digestive enzymes, trypsin and chymotrypsin, are single domain proteases, proteins like tissue plasminogen activator, plasmin, and the complement component C1r contain multiple regulatory domains that are N-terminal to the protease domain (6, 8, 9). Furthermore, some chymotrypsin family proteases are anchored to membranes by either glycosylphosphatidylinositol, a single-pass transmembrane domain at the C terminus, or a signal anchor at the N terminus (10).

The S1A, or chymotrypsin A subfamily, consists primarily of eukaryotic proteases; however, a few bacterial serine proteases belong to this subfamily according to the peptidase database.

The extracellular Vibrio cholerae protease, VesB, is expressed in vitro and in stools of cholera patients.

Results: VesB is homologous to trypsin and thrombin and the C-terminal domain has an immunoglobulin-fold.

Conclusion: In contrast to thrombin, but similar to trypsin, VesB is not activated by sodium.

Significance: This is the first structure of a bacterial modular trypsin-like protease.
Function and Structure of VesB

MESOPs (11). To date, proteases that are structurally related to trypsin have been identified in Streptomyces species (12, 13). These were shown to contain a single protease domain that has collagenolytic activity (12–14). In addition, we recently identified three related V. cholerae extracellular serine proteases, VesA, VesB, and VesC, in a proteomic screen designed to detect proteins secreted via the T2S system (5). Consistent with their reliance on the T2S system for extracellular secretion, when overexpressed, measurable activity of all three proteases has been detected in culture supernatants of V. cholerae (5, 15). Furthermore, these proteases have a predicted N-terminal signal peptide that allows them to enter the periplasm via the Sec pathway prior to engaging with the T2S system (16). In addition, they all consist of an N-terminal protease domain containing a putative activation site, residues that comprise the catalytic triad, and a C-terminal domain that varies in length and includes a Gly-Gly-CTERM extension of unknown function (Fig. 1) (17). The domain organization of VesA, VesB, and VesC differs from that of most bacterial and eukaryotic trypsin-like proteases in that the additional non-protease domain is positioned at the C terminus.

VesB, the focus of this study, shares ~30% sequence identity with trypsin and other members of the SIA family of serine proteases and displays a similar positioning of its catalytic residues. Although the biological role of VesB has yet to be determined, VesB is produced both in vitro and in vivo. Besides its detection in laboratory-grown cultures, VesB has also been detected in V. cholerae that was isolated from stools of patients with clinical cholera, inferring that VesB may contribute to intestinal growth or pathogenesis (5, 18, 19). Also, the intestinal vesB gene expression was detected in V. cholerae cultivated in a rabbit ileum loop, an experimental model for cholera, further suggesting a possible role in survival or disease (20). Although VesB may contribute to intestinal growth of V. cholerae, it is not the only factor required for intestinal survival as vesB inactivation had no negative effect on infant mice colonization (5). Finally, VesB is capable of cleaving the A subunit of cholera toxin, a process important for cholera toxin activation (5, 21).

The rarity of characterized bacterial serine proteases in the chymotrypsin A subfamily, the unique domain arrangement, and the possible role of VesB in intestinal growth or pathogenesis led us to study VesB in detail. Here, we focus on the activity and structure of VesB. We show that the protease domain of pro-VesB contains a trypsin-like fold with an incomplete oxyanion hole, and an additional C-terminal domain with an Ig-fold containing seven β-strands and two short α-helices. Furthermore, we show that purified VesB has activity against synthetic peptides, cleaves after Arg independently of Na+ ions, can be inhibited by serine protease inhibitors, and is produced with a propeptide that is critical for activity but not for extracellular secretion by V. cholerae.

EXPERIMENTAL PROCEDURES

Growth Conditions—All strains were grown on Luria-Bertani (LB) agar (Fisher). Single colonies were inoculated in Luria-Bertani (LB) broth (Fisher) with 100 μg/ml of carbenicillin and 1 or 100 μM isopropyl β-D-thiogalactopyranoside (Sigma) and grown for 16 h at 37 °C.

Bacterial Strains and Plasmids—The V. cholerae strain N16961, an El Tor O1 biotype, and the isogenic ΔvesABC strain (5) were used for all experiments. VesB was expressed from pMMB67EH-vesB (22), a low copy vector with an isopropyl β-D-thiogalactopyranoside-inducible promoter and ampicillin cassette, and pMMB67EH served as a control plasmid or a cloning vector for additional constructs. The QuikChange Site-directed Mutagenesis Kit (Agilent) was used to create vesB-S221A and vesB-R32E with the following primers 5′-TCATGTCAGGGGAGATGCTGGTGCCCAATTGTA-3′ (Fwd), 5′-TACATTGGGCCACACATCTCCTTGGACATGA-3′ (Rev) and 5′-TCCACACGAGATTTTCTCGTAATATTATATATGTTGCAATGCA-3′ (Fwd), 5′-TGCATTGAACATTATATTTCAGATGAAATATCTTGCTGTGGAGA-3′ (Rev), respectively. Primers were synthesized at IDT Technologies. Restriction enzymes, buffers, and T4 DNA ligase were purchased from New England Biolabs. Plasmid constructs were transformed into Escherichia coli MC1061 and a triparental conjugation protocol using the helper strain, MM294, was used to transfer the plasmids into N16961 and ΔvesABC strains (23).

Native VesB Purification for Protease Assays—VesB was overexpressed in V. cholerae and culture supernatant was isolated and precipitated with 60% ammonium sulfate for 1 h at 4 °C. The sample was centrifuged at 10,000 × g for 35 min at 4 °C. The pellet was washed in 20 ml of 50 mM Tris-HCl, pH 8.0, 450 mM NaCl (Buffer A) and residual ammonium sulfate was removed through dialysis against Buffer A. The dialyzed sample was subjected to affinity chromatography on benzamidine-Sepharose (GE Healthcare). The flow-through fraction was collected and the column was washed with Buffer A. Then, VesB was eluted using 10 ml of 100 mM benzamidine, 50 mM Tris-HCl, pH 8.0, Buffer. Fractions were pooled and the buffer was exchanged to 50 mM Tris-HCl, pH 8.0, using a PD10 column (GE Healthcare). VesB was concentrated by ultrafiltration (30 kDa cut-off; Millipore). Protein concentration was determined with the Bradford assay (Bio-Rad) using bovine serum albumin as a reference. Polyclonal antiserum against VesB was generated by Covance Inc.

SDS-PAGE and Immunoblotting—Samples were prepared and analyzed via SDS-PAGE and immunoblotting as described previously (23) with the following modifications. VesB antiserum was incubated in culture supernatants from the ΔvesABC
strain for 1 h to pre-absorb cross-reactive antibodies prior to incubating with the membrane for 2 h (1:5,000 dilution) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) was used at 1:20,000 dilution. Membranes were developed using ECL 2 Western blotting reagent (Thermo Scientific) and protein was visualized using a Typhoon Trio variable mode imager system and ImageQuant software. SDS-PAGE gels were stained with GelCode Blue (Thermo Scientific) or Silver Stain Kit reagents (Invitrogen).  

Protease Assay—Protease activity in V. cholerae culture supernatants was measured using Boc-Gln-Ala-Arg-7-amino-4-methylcoumarin as described previously (24). In other assays, supernatants were preincubated with benzamidine (Thermo Scientific) at the various concentrations listed for 10 min at 37 °C prior to measuring protease activity. Purified VesB was measured for protease activity with various methylcoumarin-conjugated peptides (Peptides International) listed in Table 1 in a final reaction volume of 100 μl. The peptides, Suc-Ala-Ala-Ala-AMC, Suc-(OMe)-Ala-Ala-Pro-Val-AMC, Z-Leu-Arg-Gly-Gly-AMC, Leu-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Boc-Glu-Lys-Lys-AMC, and Boc-Gln-Ala-Arg-AMC are substrates that have been successfully used for elastase, pancreatic elastase, isopeptidase, aminopeptidase, chymotrypsin, plasmin, and trypsin, respectively. All assays were done with 50 mM Tris-HCl, pH 8.0. Where stated, assays were performed in the presence of different concentrations of NaCl. Inhibition assays were done with purified VesB that was preincubated for 10 min at 37 °C with 50 μM leupeptin (Thermo Scientific), 10 mM EDTA, or 1 mM benzamidine. A change in fluorescence per minute was converted to moles of liberated 7-amino-4-methylcoumarin (AMC) per minute via a standard curve with known amounts of AMC. The results were then normalized by the amount of purified VesB or A600 of the bacterial cultures. Standard errors were generated from three independent experiments each performed in three technical replicates.  

N-terminal Sequencing—Purified VesB secreted by V. cholerae was subjected to SDS-PAGE and transferred to a PVDF membrane using 10 mM CAPS, 10% methanol, pH 11. The membrane was stained with 0.025% Coomassie Brilliant Blue R-250, 40% methanol, 5% acetic acid solution, destained using 40% methanol, 5% acetic acid, washed with water, and then air-dried. The VesB band was cut and subjected to automated Edman degradation at the protein facility core at University of Michigan, Ann Arbor, MI.  

Statistical Analysis—Student’s t tests were done on samples as indicated. Results yielding a p value of <0.05 were considered statistically significant.  

Protein Expression and Purification for Crystallization—A DNA fragment corresponding to residues 24–373 of VesB was PCR amplified and cloned into a modified pCDF-Duet-1 vector (EMD Millipore). The construct contains an N-terminal pelB signal sequence and a C-terminal tobacco etch virus protease cleavage site followed by a His6 tag. To overcome possible difficulties when overexpressing WT pro-VesB in E. coli, the Ser221 residue was replaced with Ala using QuikChange mutagenesis protocol (Stratagene). The S221A variant of pro-VesB was expressed in E. coli Rosetta2(DE3) cells at 18 °C for 4 h after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside. The cells were harvested by centrifugation and resuspended in buffer containing 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM PMSF, 1 mM benzamidine, and 15 mM imidazole. Pro-VesB was purified via a nickel-nitrilotriacetic acid-agarose (Qiagen) column followed by His6 tag cleavage with tobacco etch virus protease and a second nickel-nitrilotriacetic acid-agarose chromatography step. The yield of pro-VesB was improved from <0.5 to ~1.5 mg/liter of culture after addition of 2 mM Fe2+ ions to the clarified cell lysate (25). The final size-exclusion chromatography was performed using a Superose 6 column (GE Healthcare) in buffer containing 20 mM Tris-HCl, pH 7.4, and 0.5 M NaCl. Pro-VesB was concentrated to 3 mg ml−1 and flash frozen in liquid nitrogen (26).  

Crystallization, Data Collection, and Structure Solution—Screening for crystallization conditions was performed using Index (Hampton Research) and Wizard I and II (Emerald Bio) screens. Crystallization trials were set-up in vapor diffusion sitting drop format (0.2 μl of protein + 0.2 μl of crystallization solution) using a Phoenix crystallization robot (Art Robbins Instruments). The initial crystals grew with 0.1 M HEPES, pH 7.5, 3.0 M NaCl. The optimized crystals were obtained using a crystallization solution containing 0.1 M Tris-HCl, pH 9.0, 3.0 M NaCl (1.0 μl of protein + 1.0 μl of crystallization solution + 0.5 μl of water). Crystals were cryoprotected in crystallization solution supplemented with 20% glycerol and flash-cooled in liquid nitrogen. A 2.4-Å native dataset was collected at the Advanced Light Source beamline 8.2.2. In addition, a derivative dataset was obtained in-house using a Saturn 94 CCD detector on a Rigaku Micromax HF-7 rotating anode from a crystal incubated in 0.1 M Tris-HCl, pH 9.0, 2.0 M NaCl, 1.0 M NaI. Data were processed using HKL2000 and XDS (27, 28). The pro-VesB structure was solved by a combination of molecular replacement and single wavelength diffraction methods as implemented in Phaser (29). The initial molecular replacement solution was found by using the Balser server (30) and the structure of MT-SPI/matriptase (PDB code 1EAX) as a search model for the protease domain of pro-VesB (30, 31). 12 iodide sites were found by Phaser during the first round of phasing. After density modification using Parrot, model building using Buccaneer, and iterative model improvement using Coot (32–34), the preliminary model was submitted for the second round of phasing, which led to identification of 5 additional (17 total) iodide sites. Subsequent iterative density modification and automated model building led to an almost complete model that included the second domain of VesB. The model was improved using Coot and refined using REFMAC5 and 9 translation/libration/screw (TLS) groups identified by the TLSMD server (35, 36). The quality of the model was assessed using Coot and the Molprobity server (37). Residues 32–372 are included in the final model, whereas residues 166–172, 214–217, and 244–247 had poorly defined density and were not modeled. The final model includes 93 water molecules and has good geometry with 97.5% residues in the favorable areas of the Ramachandran plot (Table 2). The atomic coordinates and structure factors for pro-VesB (PDB code 4LK4) have been deposited in the Protein Data Bank.
**RESULTS**

*Purification of Secreted VesB*—To determine whether benzamidine-Sepharose could be utilized for affinity purification of native VesB, the reversible serine protease inhibitor benzamidine was analyzed for its ability to inhibit VesB activity. Specifically, VesB was overexpressed from pMMB67EH-vesB in ΔvesABC, a *V. cholerae* strain lacking all three serine protease genes vesA, vesB, and vesC (5). Culture supernatant was isolated and concentrated, and applied to benzamidine-Sepharose chromatography. VesB was eluted with benzamidine and all of the fractions were analyzed (Fig. 2, A), whereas there was no additional loss of VesB during washing (lanes 5–10). Flow-through fraction was discarded (lane 4), whereas there was no additional loss of VesB during washing (lanes 5–10). Bound VesB eluted efficiently with 100 mM benzamidine (lanes 11–14) and once benzamidine was removed the activity of VesB could be measured (Fig. 3). Antibodies were then raised against the purified material and the saved fractions were analyzed by SDS-PAGE and immunoblotting with anti-VesB antiserum (Fig. 2D).

*Characterization of Purified Secreted VesB*—Previously, we have shown that Boc-Gln-Ala-Arg-7-amino-4-methylcoumarin is cleaved when incubated with supernatant from *V. cholerae* overexpressing VesB and that this activity is inhibited by common serine protease inhibitors (5) (Fig. 2A). To further determine the substrate specificity and verify that purified VesB is inhibited by serine protease inhibitors, we added purified VesB to different commercially available synthetic peptides that are conjugated to AMC (Table 1 and Fig. 3A) and preincubated VesB with different inhibitors (Fig. 3B). VesB efficiently cleaved the trypsin substrate Boc-Gln-Ala-Arg-AMC, whereas the plasmin and aminopeptidase substrates, Boc-Glu-Lys-Lys-AMC and Leu-AMC, respectively, were cleaved with greatly reduced efficiency, and no cleavage was observed for the chymotrypsin or elastase substrates (Table 1).

![FIGURE 2. VesB purification by benzamidine-Sepharose affinity chromatography. A, the supernatants of ΔvesABC strains with pMMB67EH (p) or pVesB were incubated for 10 min at 37 °C with different concentrations of the serine protease inhibitor, benzamidine, and the protease activity was measured (experiments were done in triplicates and S.E. bars are shown). B–D, the supernatant from ΔvesABC overexpressing VesB (lane 1) was added to 60% saturation of ammonium sulfate and the precipitated material (lane 2) and supernatant (lane 3) were separated by centrifugation. The pellet was resuspended and dialyzed in 50 mM Tris-HCl, pH 8.0, 450 mM NaCl. The sample was used for affinity chromatography using a benzamidine-Sepharose column. Flow-through fraction was discarded (lane 4) and the column was washed with 50 mM Tris-HCl, pH 8.0, 450 mM NaCl (lanes 5–10). VesB was eluted using 100 mM benzamidine (lanes 11–14). The samples were analyzed by SDS-PAGE and GelCode blue staining (B), silver staining (C), or Western blotting with anti-VesB antibodies (D).](image)

![FIGURE 3. Protease activity of purified VesB. A, VesB activity was measured with different concentrations of Boc-Gln-Ala-Arg-7-amino-4-AMC in 5 mM HEPES, pH 7.5, at 37 °C. B, purified VesB (0.08 μg/ml) was incubated with 50 μM leupeptin, 1 mM benzamidine, or 10 mM EDTA for 10 min at 37 °C. The Boc-Gln-Ala-Arg-7-amino-4-AMC (0.05 mM final concentration) was added and VesB activity was measured. All experiments were done in triplicates and S.E. bars are shown.](image)

| Peptide sequence | VesB activitya | Boc-Gln-Ala-Arg-AMC | Boc-Gln-Ala-Arg-AMC + 200 mM NaCl | Boc-Gln-Ala-Arg-AMC + 400 mM NaCl | Boc-Gln-Ala-Arg-AMC + 600 mM NaCl | Boc-Gln-Ala-Arg-AMC + 800 mM NaCl |
|------------------|----------------|---------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Suc-Ala-Ala-AMC   | 0              | 0                   | 0.142                         | 0.124                         | 0.106                         | 0.060                         |
| Suc (OMe)-Ala-Ala-Pro-Val-AMC | 0 | 0 | 0.150 ± 0.124 | 0.137 ± 0.030 | 0.127 ± 0.0025 | 0.122 ± 0.014 |
| Leu-AMC           | 0.518 ± 0.124  | 0                   | 0.449 ± 0.060                 | 0.431 ± 0.368                 | 0.431 ± 0.368                 | 0.431 ± 0.368                 |
| Suc-Ala-Ala-Pro-Phe-AMC | 0 | 0 | 0.124 | 0.122 | 0.122 | 0.122 |
| Boc-Glu-Lys-Lys-AMC | 0.449 ± 0.060 | 0.431 ± 0.368 | 0.431 ± 0.368 | 0.431 ± 0.368 | 0.431 ± 0.368 | 0.431 ± 0.368 |
| Boc-Gln-Ala-Arg-AMC | 286 ± 34.0   | 0                   | 0.122 ± 0.214                 | 0.137 ± 0.137                 | 0.137 ± 0.313                 | 0.137 ± 0.313                 |
| Boc-Gln-Ala-Arg-AMC + 200 mM NaCl | 122 ± 21.9f | 0 | 0.122 | 0.137 | 0.137 | 0.137 |
| Boc-Gln-Ala-Arg-AMC + 400 mM NaCl | 137 ± 31.3f | 0.122 | 0.137 | 0.137 | 0.137 | 0.137 |
| Boc-Gln-Ala-Arg-AMC + 600 mM NaCl | 110 ± 18.9f | 0.122 | 0.137 | 0.137 | 0.137 | 0.137 |
| Boc-Gln-Ala-Arg-AMC + 800 mM NaCl | 97.2 ± 21.6c | 0.122 | 0.137 | 0.137 | 0.137 | 0.137 |

a Peptide sequence of the substrates all conjugated to AMC.

b Substrate concentration was 0.05 mM when Boc-Gln-Ala-Arg-AMC was analyzed and 7.9 μg/ml for all other substrates. Experiments were done in triplicates and the mean ± S.E. are shown.

p < 0.01: all compared to the sample with no NaCl.

Characterization of Purified Secreted VesB—Previously, we have shown that Boc-Gln-Ala-Arg-7-amino-4-methylcoumarin is cleaved when incubated with supernatant from *V. cholerae* overexpressing VesB and that this activity is inhibited by common serine protease inhibitors (5) (Fig. 2A). To further determine the substrate specificity and verify that purified VesB is inhibited by serine protease inhibitors, we added purified VesB to different commercially available synthetic peptides that are conjugated to AMC (Table 1 and Fig. 3A) and preincubated VesB with different inhibitors (Fig. 3B). VesB efficiently cleaved the trypsin substrate Boc-Gln-Ala-Arg-AMC, whereas the plasmin and aminopeptidase substrates, Boc-Glu-Lys-Lys-AMC and Leu-AMC, respectively, were cleaved with greatly reduced efficiency, and no cleavage was observed for the chymotrypsin or elastase substrates (Table 1). Measurement of kinetic parameters yielded $V_{\text{max}}$ and $K_m$ values of 0.137 ± 0.0030 nmol/min and 0.0327 ± 0.0025 mm, respectively, for Boc-Gln-Ala-Arg-AMC (Fig. 3A). We were unable to determine $V_{\text{max}}$ and $K_m$ for Boc-Glu-Lys-Lys-AMC and Boc-Leu-AMC as the reactions never approached maximum rates due to the lack of solubility of the substrates at higher concentrations. In addition, we observed inhibition of purified VesB activity by the serine protease inhibitors benzamidine and leupeptin, whereas VesB was not affected by the presence of the metal.
Function and Structure of VesB

VesB is produced as a zymogen—The alignment of trypsin, thrombin, and the protease domain of VesB revealed two important features of the chymotrypsin family of serine proteases: the catalytic triad and an activation site, with the latter corresponding to residues Arg32–Ile33 in pro-VesB (Fig. 1). To verify cleavage at the predicted activation site, purified VesB was subjected to N-terminal sequencing by Edman degradation. The sequence of the first five residues, IINGS, indicated that the propeptide had been removed from VesB and that VesB is cleaved at Arg32–Ile33 similar to other trypsin-like proteases. To determine whether cleavage of the propeptide is necessary for VesB activity, we mutated the putative catalytic residue Ser221 to Ala. The mutant proteins were expressed in E. coli and subjected to SDS-PAGE and Western blot analysis using anti-VesB antibodies.

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the N-terminal amino group of Ile$^{33}$ in active VesB (compare Fig. 7, A and B). These structural features probably contribute to the disorder of three loops in the vicinity of the active site, which include residues 166–172, 214–217, and 244–247 (Fig. 5A). Consistent with VesB being a trypsin-like protease, the disordered loops in pro-VesB are reminiscent of the disordered loops in the crystal structure of bovine trypsinogen (39).

In thrombin, residues Tyr$^{184}$, Arg$^{221}$, and Lys$^{224}$ were shown to coordinate a Na$^+$ ion that binds to and allosterically enhances thrombin activity (41, 42). Although the catalytic activity of several other coagulation and complement factors is also enhanced by sodium, trypsin is not. In the Na$^+$-activated members of the trypsin-like family, Tyr$^{225}$ (thrombin numbering) plays a critical role. A proline residue at this position changes the orientation of the nearby Lys$^{224}$ such that its carbonyl O atom can no longer coordinate Na$^+$ (41). When thrombin residue Tyr$^{225}$ was replaced with a Pro, as occurs in several trypsin-like proteases that are not Na$^+$-sensitive, there was a loss in Na$^+$ activation (41). The sequence alignment of VesB with thrombin shows that the residue corresponding to Tyr$^{225}$ in thrombin is Tyr$^{250}$ in VesB (Fig. 6B), i.e., a residue that is compatible with Na$^+$ activation. The structural superposition of pro-VesB and thrombin is shown in Fig. 7C, specifically highlighting the position of Tyr$^{225}$ and surrounding residues in thrombin and the corresponding residues in pro-VesB. However, no sodium ion is apparent in the pro-VesB electron density (Fig. 7D). The absence of sodium may be due to the inability of pro-VesB to coordinate sodium. Similarly, sodium is not present in the zymogen forms of thrombin (43). Thus, the structure of pro-VesB does not provide insight into the presence of a putative Na$^+$ binding site in active VesB. To establish whether active VesB can be stimulated by Na$^+$, we determined the activity of purified VesB in the absence and presence of increasing concentrations of NaCl using Boc-Gln-Ala-Arg-AMC (Table 1). The presence of Na$^+$ did not stimulate VesB, instead we observed a decrease in activity (Table 1). When a different substrate with Arg at the P1 position ($Z$-Gly-Gly-Arg-AMC) was tested, a similar negative effect on the VesB activity was observed (not shown). In addition, we isolated culture supernatant from a *V. cholerae* culture grown in a medium without NaCl and determined the activity of VesB in the absence or presence of 200 mM NaCl. There was no change in VesB activity when 200 mM NaCl was included (data not shown). Taken together, VesB does not appear to be activated by Na$^+$ in a similar fashion to thrombin.

The crystal structure of the C-terminal domain of VesB revealed a domain rich in β-strands that adopts an Ig-fold (Fig. 5). A structural similarity search with the Dali server identified a number of distant homologs with Ig-folds (Table 3). Although the best hit was a domain from a *Porphyromonas gingivalis* protein of unknown function (*Z* factor = 9.3 and 12% amino acid sequence identity), homologs of possibly greater interest
are domains from bacterial PapD-like chaperones such as SfaE (Z factor = 8.2) and subtilisin-like proteases like cucumisin (Z factor = 7.7). Superpositions of the C-terminal domain of VesB with SfaE and cucumisin domains are shown in Fig. 8. Interestingly, VesB contains two additional short α-helices compared with the other proteins, and two extra β-strands compared with SfaE. We also observed a degree of structural homology, albeit less closely than with the previous three homologs, between the Ig domain of VesB and domains from several extracellular Vibrio proteins, including chitin-binding protein (GbpA), chitinase (ChiA), rugose and biofilm structure modifier (RbmA), and another protease of V. cholerae (PrtV) (Table 3). The Ig domain of GbpA has been reported to support its V. cholerae surface association (44), whereas the Ig domain of ChiA is a carbohydrate binding module (45). Although we were unable to detect binding of purified VesB to chitin, suggesting that VesB is not a chitin-binding protein (data not shown), we have observed that a large fraction of extracellular VesB is surface
We have not yet confirmed a role for the Ig domain in this interaction as the removal of the Ig domain of VesB results in an unstable protein that cannot be detected when expressed in *V. cholerae* (data not shown).

**Interface between the Protease and C-terminal Domains**—In the crystals, the two domains of pro-VesB interact with each other with a buried surface area of 1220 Å² (Fig. 5B). This interface is classified by the PISA server as insignificant (46), although physiologically relevant interfaces with the same extent of buried surface area have been reported (47). Hence, it is uncertain whether the two domains of pro-VesB have a different or flexible relative orientation with respect to each other in solution or during secretion via the T2S apparatus.

### DISCUSSION

In this study, we show that the protease domain of VesB is related to trypsin in sequence, structure, and substrate specificity. Upon sequence alignment of trypsin and VesB, the catalytic triad and the activation site of VesB became apparent (Fig. 6B).

**Table 3: Structural homologs of the C-terminal domain of VesB**

| Protein   | Z-score | Root mean square deviation (Å) | Aligned aa | Percentage Sequence identity (%) | PDB ID | Reference |
|-----------|---------|--------------------------------|------------|----------------------------------|--------|-----------|
| Unknown   | 9.3     | 2.5                            | 94         | 12                               | 2QSV   | NA³       |
| SfaE      | 8.2     | 2.7                            | 92         | 5                                | 1L4I   | 65        |
| Cucumisin | 7.7     | 2.7                            | 91         | 11                               | 3VTA   | 57        |
| RbmA      | 7.0     | 2.6                            | 88         | 10                               | 4KEQ   | 64        |
| GbpA      | 5.8     | 3.4                            | 84         | 11                               | 2XXW   | 44        |
| ChiA      | 4.5     | 3.7                            | 73         | 11                               | 3B8S   | 45        |
| PrtV      | 3.7     | 2.8                            | 63         | 17                               | 419D   | 60        |
| KP-43     | 2.3     | 2.9                            | 60         | 7                                | 1WMD   | 59        |
| ASP       | 2.0     | 2.9                            | 59         | 10                               | 3HHR   | 58        |

³ Z-score from the Dali server.

NA, not applicable.

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**Figure 7. Close-up views of the activation site and the Tyr²⁵⁰ region of pro-VesB.**

A, stereo view of the activation site of VesB. Selected residues including Ile³¹⁵ are shown in stick representation and labeled. σ₆-Weighted 2Fo − Fc map is shown as *gray mesh* (contoured at 1σ). B, stereo view of the equivalent region in trypsin is shown (PDB code 2PTC). Note that the N-terminal Ile¹⁶ residue occupies the hydrophobic pocket formed by Ile¹₃₈ and Ile¹₅₈, and coordinates Asp¹₉₄. C, stereo view of the superposition of the Na⁺ binding loop of thrombin (magenta) and the equivalent loop of pro-VesB (green). Na⁺ ion is shown as a *purple sphere*. D, the same view as in C showing the σ₆-Weighted 2Fo − Fc map as *gray mesh* (contoured at 1σ) of pro-VesB.

**Figure 8. Structural homologs of the C-terminal domain of VesB.**

A, stereo view of superposition of the C-terminal domain of VesB (green and purple) and the N-terminal domain of S pilus chaperone (blue) (PDB code 1L4I) (65). The N and C termini of the C-terminal domain of VesB are labeled. Note that the C-terminal domain of VesB has two additional β-strands and two additional short helices (purple). B, stereo view of the superposition of the C-terminal domains of VesB (green and purple) and cucumisin (orange) (PDB 3VTA) (57). Pro-VesB contains two additional short helices (purple).
**Function and Structure of VesB**

Mutating these motifs rendered VesB inactive, suggesting that the catalytic triad and the activation site are essential for activity, but have no impact on extracellular secretion of VesB (Fig. 4). Our enzymatic studies suggested that VesB may preferentially cleave peptides that have an Arg at the P1 position and showed that VesB is inhibited by the serine protease inhibitors, leupeptin and benzamidine (Figs. 2 and 3, Table 1). Last, the crystal structure revealed that the N-terminal protease domain of pro-VesB is structurally homologous to mammalian trypsin of the S1A subfamily and the C-terminal domain contains an Ig-fold (Figs. 5–8).

Bacterial serine proteases belonging to the S1A subfamily were once considered rare and only thought to exist in certain *Streptomyces* species (48, 49). With the rapidly growing number of sequenced genomes, however, it is becoming increasingly clear that additional bacterial species also carry genes for trypsin-like enzymes. Besides the *V. cholerae* vesB, vesA, and vesC, genes encoding proteases of the chymotrypsin family are also found in other marine Gram-negative bacteria, many belonging to the *Vibrionaceae* and *Shewanellaceae* families. Previously, a study was carried out to determine the nature of acquiring trypsin-encoding genes in bacterial genomes. A phylogenetic tree based on the sequence and structure of trypsins from various mammalian species and *Streptomyces griseus* revealed that these proteases originate from a common ancestor (50). A later study performed a similar analysis looking at a larger selection of trypsin-like proteases from a variety of species, but only including a single *Streptomyces* protease as bacterial representative (48). Based on the data presented in the latter study it is difficult to determine the evolutionary relationship between the trypsin-like proteases. It is not clear whether trypsin encoding genes originated in bacterial cells, and were maintained during subsequent evolutionary steps in eukaryotic species but lost in many bacterial species, or if the genes have been transferred horizontally between species.

A way to distinguish and further analyze the relationship between the serine proteases of the chymotrypsin family is by determining how the activity of these proteases is regulated. For several trypsin-like proteases, which function in blood coagulation or complement formation, activation by Na⁺ plays a well-established role (41, 51). It has been shown that in these proteases the residue equivalent to Tyr²²⁵ in thrombin plays a key role in the proper formation of the Na⁺-binding site (41). In VesB, Tyr²⁵⁰ is equivalent in position to Tyr²²⁵ of thrombin (Figs. 6B and 7C) and hence it would be possible that VesB is activated by sodium ions. However, addition of NaCl does not appear to increase purified VesB activity. In the crystal structure of pro-VesB no density representing a sodium ion at the expected position is evident, although it should be noted that thezymogen form of thrombin also lacks sodium (Fig. 7D) (43). These results are not too surprising for two reasons.

First, elegant protein engineering studies on *S. griseus* trypsin, which contains a Pro at the equivalent position of Tyr²²⁵ in thrombin and is not activated by Na⁺, have shown that up to 19 residues need to be altered, in addition to the Pro to Tyr change near the Na⁺ activation site, to obtain a *S. griseus* trypsin variant that is fully Na⁺-sensitive (52). Hence, the presence of Tyr at the position equivalent to Tyr²²⁵ in thrombin is a necessary but not sufficient requirement for a member of the trypsin-like family to become activated by Na⁺, which is also supported by studies on complement mannann-binding lectin-associated serine protease 2 (53).

Second, VesB is secreted and needs to function in the extracellular milieu like the gut and/or aquatic habitats, sites with highly variable Na⁺ ion concentrations. For example, at aquatic environmental sites where *V. cholerae* is frequently isolated, the NaCl concentration varies between 0.2 and 3.0% (54). These conditions are quite different from that of blood, where the sodium ion concentration is precisely controlled. Hence, Tyr²⁵⁰ plays most likely a structural role and is not involved in Na⁺ activation of this *V. cholerae* protease. Taken together, our findings suggest that a tyrosine in trypsin-like proteases equivalent to thrombin Tyr²²⁵ cannot be used as the sole indicator of Na⁺-mediated activation.

The C-terminal domain of VesB is structurally similar to Ig-folds of PapD-like chaperones, subtilisin-like proteases, and several extracellular proteins from *Vibrio*. The role of the Ig-fold in VesB is currently unknown; however, understanding the role of Ig-folds in other proteins may shed light on a possible function of the VesB Ig-fold. The PapD chaperones reside in the periplasm of Gram-negative bacteria and bind to type 1 pilin subunits via a donor-strand complementation mechanism to target them to the Usher protein for outer membrane translocation and pilus assembly on the cell surface (55). The chaperone-mediated stabilization of the pilin subunits is evident in a *papD*-deficient strain, in which DegP degrades the pilin subunits (55, 56). The subtilisin-like serine protease cucumisin contains a C-terminal Ig-fold domain that interacts with its catalytic domain (57) and in the *Aeromonas sobria* subtilisin-like protein ASP, the Ig-fold domain is located close to the active site; therefore, it has been proposed that this domain is involved in substrate specificity (58). The Ig-fold domain of yet another subtilisin-like protease, KP-43 from *Bacillus*, is suggested to provide stability, as KP-43 without its Ig-fold domain could not be expressed (59). Although the Ig-fold domain may similarly provide stability to VesB, as we are unable to detect VesB when expressed without its C-terminal domain in *V. cholerae*, the Ig-fold may alternatively, or in addition, provide substrate specificity although it is located relatively far (~20 Å) from the active site (Fig. 5B).

Other possible roles of the Ig-fold domain may relate to the extracellular secretion or surface association of VesB. VesB, GbpA, ChiA, PrtV, and RbmA are extracellular *Vibrio* proteins that contain Ig-fold domains (44, 45, 60, 61). Some of these proteins (VesB, GbpA, and ChiA) have been shown to utilize the T2S system for outer membrane translocation (5, 62, 63). Although the Ig-fold domains may be involved in the transport of VesB and other extracellular proteins via this translocation system, it is also possible that they assist in surface association of these proteins similar to the Ig-fold domain of GbpA, which binds to the bacterial cell surface (44). Surface localization of VesB may optimally allow for the immediate uptake and intracellular delivery of peptides generated by VesB-mediated proteolysis.

In summary, we have shown that VesB has a structure and specificity profile resembling that of eukaryotic trypsin-like...
proteases. Additionally, the structure of the C-terminal domain of VesB revealed an Ig-fold domain that may be involved in one or more different functions such as stabilizing the protease domain, co-defining substrate specificity, binding to the bacterial surface, and being part of a yet undefined secretion motif of the T2S system (2).

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