Purification and Characterization of Enterotoxigenic El Tor-Like Hemolysin Produced by *Vibrio fluvialis*

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The halophilic bacterium *Vibrio fluvialis* is an enteric pathogen that produces an extracellular hemolysin. This hemolysin was purified to homogeneity by using sequential hydrophobic-interaction chromatography with phenyl-Sepharose CL-4B and gel filtration with Sephacryl S-200. It has a molecular weight of 63,000 and an isoelectric point of 4.6, and its hemolytic activity is sensitive to heat, proteases, and preincubation with zinc ions. The hemolysin lyzes erythrocytes of the eight different animal species that we tested, is cytotoxic against Chinese hamster ovary cells in tissue culture, and elicits fluid accumulation in suckling mice. Lysis of erythrocytes occurs by a temperature-dependent binding step followed by a temperature- and pH-dependent lytic step. Fourteen of the first 20 N-terminal amino acid residues (Val-Ser-Gly-Glu-Ala-Asn-Thr-Leu-Pro-His-Val-Ala-Phe-Tyr-Ile-Asn-Val-Asn-Arg) are identical to those of the El Tor hemolysin of *Vibrio cholerae* and the heat-labile hemolysin of *Vibrio mimicus*. This homology was further confirmed by PCR analysis using a 5′ primer derived from the amino-terminal sequence of the hemolysin and a 3′ primer derived from the El Tor hemolysin structural gene. The hemolysin also reacts with antibodies to the El Tor-like hemolysin of non-O1 *V. cholerae*.

*MATERIALS AND METHODS*

Bacterium and seed culture preparation. *V. fluvialis* (strain 807-77) was obtained from Stephen H. Richardson (Wake Forest University, Winston-Salem, N.C.). The bacterium was stored at −70°C in trypticase soy broth (BBL, Cockeysville, Md.) supplemented with 1% NaCl and 25% glycerol. The frozen culture was rapidly thawed and inoculated onto six plates containing trypticase soy agar (BBL) supplemented with 1% NaCl (TS-S). The plates were incubated at 37°C for 7 h, and the bacteria were harvested in 30 ml of CYE broth (3% Casamino Acids, 0.4% yeast extract, 0.05% K₂HPO₄ [pH 7.4]).

Hemolysin production. The ability of *V. fluvialis* to produce extracellular hemolysin was examined by inoculating CYE broth in 2-liter flasks with a 7-h-old inoculum harvested from TS-S and assaying the culture supernatant fluids after the cultures had been incubated for 17 h at 35°C on a shaker at 100 rpm. The effect of Na⁺ ion concentration was studied by inoculating the broth containing 1 and 2% NaCl with 10⁸ CFU of seed culture. The effect of inoculum concentration was examined by inoculating 100 ml of broth with 10⁶, 10⁷, and 10⁸ CFU of the seed culture. The effect of aeration was studied by inoculating 50, 100, and 200 ml of broth with 5 × 10⁶, 10⁷, and 2 × 10⁸ CFU, respectively, of seed culture.

Purification of VFH. Unless otherwise noted, all steps were carried out at 4°C.

Stage 1: culture supernatant fluids. Twenty 2-liter flasks containing 100 ml of CYE broth each were inoculated with 10¹⁰ CFU of the seed culture suspension. The culture was incubated for 17 h at 35°C on a rotary shaker at 100 rpm. Culture supernatant fluids (stage 1) were recovered by centrifugation at 16,000 × g (20 min).

Stage 2: hydrophobic-interaction chromatography. Na₂HPO₄ and NaCl were dissolved in the stage 1 preparation to a final molarity of 0.067 and 0.077 M, respectively, and the pH was adjusted to 7.0 with concentrated HCl. This preparation was then applied to a column (1.6 by 30 cm) of phenyl-Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, N.J.) equilibrated with phosphate-

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buffered saline (PBS; 0.067 M Na₂HPO₄–0.077 M NaCl [pH 7.0]). The column was washed with PBS and PBS diluted (1:10) with water, and toxin was eluted by washing the column with 50% ethylene glycol in diluted PBS. Fractions were monitored for absorbance at 280 nm and for hemolytic activity against sheep erythrocytes, and peak fractions having activity were pooled (stage 2).

Stage 3: gel filtration chromatography. The stage 2 preparation was applied to a column (2.6 x 94 cm) of Sephacryl S-200 (Amersham Pharmacia Biotech) equilibrated with 0.05 M ammonium bicarbonate. Fractions were monitored for absorbance at 280 nm and for hemolytic activity. Peak fractions having activity were pooled (stage 3).

Concentration of VFH. For various studies, the purified hemolysin was concentrated by using Centricon (Amicon, Bedford, Mass) units that were treated with bovine serum albumin (BSA). Briefly, the units were filled with PBS containing BSA (1 mg per ml), incubated at 4°C for 16 to 18 h, and rinsed thoroughly with PBS prior to use.

Hemolytic assay. Hemolytic activity against erythrocytes was determined by the method of Bernheimer and Schwartz (2). Blood samples of sheep, goat, rabbit, calf, gosling, chicken, horse, and guinea pig were used for the preparation of erythrocyte suspensions that were obtained from Colorado Serum Company (Denver, Colo.). All studies, except those for the hemolytic spectrum, were performed with sheep erythrocytes. Briefly, VFH was diluted to 0.5 ml with PBS and added to 0.5 ml of PBS containing 1 mg of BSA (Sigma, St. Louis, Mo.) per ml (PBS-BSA). This mixture, 1 ml of washed erythrocyte suspension in PBS (0.7%, vol/vol) was added, and the tube was incubated at 37°C for 30 min. Unlysed erythrocytes were pelleted by centrifugation, and the absorbance of the supernatant at 545 nm was measured. One hemolytic unit (HU) is defined as that amount which causes the release of 50% of the hemoglobin in the standardized erythrocyte suspension.

CHO cell assay. The stage 3 preparation was assayed for activity against CHO cells in tissue culture by using a method (16) previously described for assaying the activity of a CHO cell enlargement factor. CHO cells were grown in Eagle’s minimum essential medium (Sigma) supplemented with 10% fetal calf serum and 10% trypsin phosphate broth. Briefly, for the assay, the cells were grown to confluence in the growth medium and were harvested with trypsin-EDTA. The cells were removed by centrifugation and resuspended at a concentration of 10,000 cells per ml of the Eagle’s minimum essential medium containing 1% fetal calf serum. A 100-μl aliquot (1,000 cells) of the cell suspension was added to each well in a 96-well microtiter plate. VFH was diluted 10- to 1,280-fold in the assay medium without the fetal calf serum, and 10 μl of the dilution was added to each well. The cells were incubated at 37°C in an incubator with 5% CO₂ and checked periodically for cytotoxic effects. The minimum amount of toxin that exhibited cytotoxic activity towards ca. 50% of the cells was determined.

Sucking mouse assay. Purified VFH was tested for its ability to cause fluid accumulation in sucking mice as previously described (16). Pregnant ICR mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.), and studies were carried out in accordance with Institutional Animal Care and Use Committee approval. Pregnant mice (number 413) were allowed to deliver 2.5 to 4.0 g were starved for 6 h and were orally fed either 100 μl of 0.05 M ammonium bicarbonate buffer or 100 μl of the same buffer containing purified VFH. Mice were sacrificed at 2, 3, or 4 h postchallenge for the optimization study and at 4 h postchallenge for the dose-response study. The fluid accumulation ratio (FA ratio) was expressed as 1.000 times the ratio of the weight of the stomach plus intestine to the remaining body weight. The FA ratios were analyzed by using paired Student’s t test as previously described (28).

Protein determination. Protein was estimated by the method of Bradford (3). The standard (BSA) and the reagent were obtained from Bio-Rad Laboratories (Richmond, Calif.).

Isoelectric focusing. Duplicate samples of purified VFH were analyzed by thin-layer isoelectric focusing, using pH 3 to 9 gels in the PhastSystem (Amersham Pharmacia Biotech). The gel was divided into two parts, with one part being stained with Coomassie brilliant blue R, and the isoelectric point (pl) of VFH was determined by comparing its relative mobility in the gel to the mobility of pl standards obtained from Amersham Pharmacia Biotech. The other part of the gel was soaked in PBS containing BSA (0.25 mg per ml) for 5 min and examined by a zymogram technique with an overlay of 0.35% (vol/vol) sheep erythrocyte suspension in PBS containing BSA (0.25 mg per ml) and 1% agar.

Hemolytic activity was detected after incubation at 37°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 8 to 25% gradient gels in the PhastSystem. The molecular weight of the denatured, reduced VFH was estimated by the relative mobility method of Laemmli (11).

Western blotting. The protein samples were subjected to SDS-PAGE and then blotted onto either ProBlott membranes (Applied Biosystems, Foster City, Calif.) for sequencing or onto nitrocellulose membranes (Bio-Rad) for immunological reactions in a transfer buffer (10 mM 3-[cyclohexylamino]-1-propane sulfonic acid containing 10% methanol [pH 11.0]). Proteins on the ProBlott membrane were visualized by staining with Coomassie brilliant blue R.

Amino acid sequencing. The VFH preparation was concentrated by using a ProSpin cartridge obtained from Applied Biosystems. The N-terminal amino acid sequence of the excised ProSpin membrane or the Coomassie brilliant blue R-stained band from a Western blot was determined by Edman degradation using a model 477A protein sequencer (Applied Biosystems).

Immunoblotting. VFH and culture supernatant fluids (from overnight growth in CYE broth containing 1% NaCl of V. cholerae O1 biotype El Tor (strain 1074-78) and non-O1 V. cholerae strain 2194c were subjected to SDS-PAGE and Western blotting using nitrocellulose membranes. The blot was washed with 20 mM Tris–500 mM sodium chloride (pH 7.5) containing 0.05% Tween 20 (TTBS) and blocked with 5% skim milk in TTBS. The blot was then incubated with antiserum raised against purified hemolysin of non-O1 V. cholerae strain 2194c (21) for 2 h, and positive reactions were detected by using goat anti-rabbit alkaline phosphatase conjugate and a substrate kit purchased from Bio-Rad.

Inactivation studies. The VFH was tested for its sensitivity to heat at 25, 37, 56, and 100°C for 30 min in 0.05 M ammonium bicarbonate. The effect of pH on the activity of VFH was studied by preincubating the preparation in the absence and presence of BSA (0.10 to 0.1 mg per ml) and dissolving the preparation in 0.05 M ammonium bicarbonate prior to the assay. The sensitivities of VFH to four proteases (Sigma) were examined by incubating VFH with each enzyme (100 μg each of trypsin, chymotrypsin, and papain, and 1, 10, and 100 μg of subtilisin) in 0.05 M ammonium bicarbonate at 37°C for 30 min and assaying for residual activity. Sensitivities to chelating agents (EGTA and EDTA), dithiothreitol, cholesterol, and mixed gangliosides were determined by preincubating VFH with the reagents for 30 min at 27°C and then assaying for residual activity. The organic solvents in which the cholesterol and mixed gangliosides were dissolved were evaporated with a stream of nitrogen, and the reagents were suspended in 0.5 ml of 0.05 M ammonium bicarbonate and sonicated for 20 s using a sonicator equipped with a microtip (Tekmar Co., Cincinnati, Ohio). VFH (0.5 ml) was then added to the sonicated suspension, and the mixture was incubated at 27°C for 30 min. The effect of divalent cations (Ca²⁺, Mg²⁺, and Zn²⁺) was examined by assaying VFH in the presence of the reagents. However, instead of using PBS and PBS-BSA, the assays were performed with 0.02 M Tris-hydrochloride–0.15 M NaCl (pH 7.0) supplemented with BSA.

Variables influencing erythrocyte lysis. A standardized sheep erythrocyte suspension (final concentration of 0.35% [vol/vol] in the reaction mixture) was used in experiments to examine the effects of temperature and pH on erythrocyte lysis. Three replicates of temperature (27, 32, 37, 42, and 47°C) and pH (7, 8, 9, and 10) were examined by using 2 HU of VFH. The effect of temperature concentration on lysis was examined by incubating 2 HU with different amounts (0.175 to 3.5%) of residual activity. The organic solvents in which the cholesterol and mixed gangliosides were dissolved were evaporated with a stream of nitrogen, and the reagents were suspended in 0.5 ml of 0.05 M ammonium bicarbonate and sonicated for 20 s using a sonicator equipped with a microtip (Tekmar Co., Cincinnati, Ohio). VFH (0.5 ml) was then added to the sonicated suspension, and the mixture was incubated at 27°C for 30 min. The effect of divalent cations (Ca²⁺, Mg²⁺, and Zn²⁺) was examined by assaying VFH in the presence of the reagents. However, instead of using PBS and PBS-BSA, the assays were performed with 0.02 M Tris-hydrochloride–0.15 M NaCl (pH 7.0) supplemented with BSA.

Screening of V. fluvialis for gene sequences matching those of the El Tor hemolysin. PCRs using Platinum PCR Supermix (Invitrogen Life Technologies, Chicago, Ill.) were performed in 50-μl solutions containing 5 μl of bacterial cell lysate. The amplification cycle was as follows: 94°C for 3 min, followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min 30 s, with a final extension at 72°C for 7 min to complete the cycle. Primers were derived from sequence data for the V. cholerae El Tor hemolysin (accession number X51746). The 5’ primer was derived from the amino-terminal sequence of the VFH that was identical to the El Tor hemolysin.

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TABLE 1. Primers used to test for homology between V. fluvialis hemolysin and the V. cholerae El Tor hemolysin

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Position within El Tor gene (nucleotide no.)</th>
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<tbody>
<tr>
<td>Bam10</td>
<td>AATACCTGGCGGATGTGCC</td>
<td>885–906</td>
</tr>
<tr>
<td>Eth6</td>
<td>CAATAAGCGCAGATCTCTG</td>
<td>1263–1281</td>
</tr>
<tr>
<td>Eth2</td>
<td>GCGGCAATACGTGCCAGATG</td>
<td>1921–1940</td>
</tr>
<tr>
<td>Eth4</td>
<td>GCGGTCGTCCCGTACAGATC</td>
<td>2311–2330</td>
</tr>
<tr>
<td>Eth8</td>
<td>AGAGACCTGCGGCAATAGCCA</td>
<td>2631–2650</td>
</tr>
<tr>
<td>Eth7</td>
<td>CTATTATGTTGTCACAGAATT</td>
<td>2777–2798</td>
</tr>
</tbody>
</table>

* Sequence is derived from the N-terminal amino acid sequence of V. fluvialis hemolysin.

RESULTS

Production and purification of VFH. The maximal amount of VFH was produced in 100 ml of broth, which was inoculated with 10^10 CFU of an inoculum prepared from a 7-h-old culture (Table 2). Use of a 24-h-old culture as the inoculum resulted in significant variations (0 to 100 U per ml) in the amounts produced in different flasks (results not shown). The addition of 1 and 2% NaCl to the CYE medium drastically reduced the amounts of VFH produced, by 90 and 99%, respectively. Results obtained by using different volumes of the medium indicated that excessive (50 ml per flask) and insufficient (200 ml per flask) aeration of the culture reduces the amount of VFH produced. The behavior of VFH during hydrophobic-interaction chromatography and gel filtration is shown in Fig. 1, and the quantitative results of the purification are summarized in Table 3. The stage 3 preparation was homogeneous by both SDS-PAGE and thin-layer isoelectric focusing (Fig. 2). Zymogram analysis (Fig. 2B) using an overlay of 0.35% erythrocyte suspension in PBS-BSA showed that hemolytic activity corresponded to the single band observed on the stained gel. N-terminal amino acid sequencing of the stage 3 preparation yielded only one sequence, thereby confirming the homogeneity of the purified preparation. About 58% of the VFH was recovered in a purified state. The amount of protein in this preparation was 628 μg, and the specific activity was approximately 93,000 HU per mg of protein.

Molecular weight and pI. The molecular weight of the denatured and reduced VFH was estimated to be 63,000 (Fig. 2A). VFH has a pI of 4.6 (Fig. 2B).

Inactivation of hemolytic activity. VFH lost 0, 50, 100, and 100% of its activity when it was incubated at temperatures of 25, 37, 56, and 100°C, respectively, for 30 min (Table 4). However, the loss of activity at 37°C was prevented by the addition of 0.25 mg of BSA per ml to the purified hemolysin. Thus, the hemolysin assay was routinely carried out at 37°C for 30 min in the presence of BSA. VFH lost all its activity when it was lyophilized; however, the addition of 0.025, 0.05, and 0.1 mg of BSA per ml to the purified preparation prior to lyophilization helped in retaining 25, 50, and 100% of its activity, respectively. VFH was sensitive to digestion with trypsin, chymotrypsin, papain, and subtilisin; subtilisin was the most effective enzyme, with the hemolysin losing 75% of its activity when incubated with 1 μg of the enzyme. The addition of 100 μg of cholesterol, 100 μg of mixed gangliosides, 1 mM concentrations of divalent cations Ca^{2+} and Mg^{2+}, and 1 mM EGTA
and 1 mM EDTA to the reaction mixture did not affect the hemolysin’s activity. However, the activity was inhibited when the cation Zn^{2+} was added to the reaction mixture. Preincubation of VFH with a reducing agent (10 mM dithiothreitol) did not result in any loss of activity.

**Hemolytic and cytotoxic activity.** In addition to its activity against the sheep erythrocytes, VFH was active against erythrocytes from seven other animal species tested (Table 5). Erythrocytes from guinea pig were the most sensitive, while those from chicken were the least sensitive. In addition to lysing erythrocytes, VFH was cytotoxic towards CHO cells in tissue culture. The activity was visible within 4 h of incubation of the cells with the hemolysin, and the minimum cytotoxic dose was 0.003 HU (0.03 ng of protein).

**Variables influencing erythrocyte lysis.** The rate of hemolysis was temperature and pH dependent (Fig. 3). Optimal hemolysis was observed at temperatures of 37 to 47°C; however, the rate of hemolysis (in the linear portion of the curves) was maximal at the highest temperature. The optimal pH for hemolysis was 7; there was very little lysis at pH 10. Buffers with pH values lower than 7 were not examined because of the instability of the sheep erythrocytes at low pH. An increase in the number of erythrocytes (0.175 to 3.5%) resulted in an increase of the total absorbance caused by the lysis (Fig. 4), but the percentage of hemolysis (lysed erythrocytes as a proportion of the total number of erythrocytes) decreased with increased erythrocyte concentration.

The results of binding studies indicate that VFH binds to erythrocytes much more rapidly at 37°C than at 4°C. At 4°C, there was no significant difference between the amounts of hemolysin bound at 1 and 8 min. In contrast, prolonged (up to 8 min) incubation at 37°C resulted in more of the hemolysin binding to the erythrocytes. For example, when 4 HU of VFH was incubated with erythrocytes at 4°C for 1, 2, 4, and 8 min, the absorbance (expressed as percentage of control) associated with the pellets (i.e., bound VFH) was only 7, 8, 6, and 6%, respectively. In contrast, when the same amount of VFH was incubated with erythrocytes at 37°C for 1, 2, 4, and 8 min, the absorbance associated with the pellets was 5, 27, 67, and 83%, respectively. These results suggest that binding of VFH to erythrocytes is a temperature-dependent step.

**Amino acid sequence.** The first 20 N-terminal amino acids of VFH are shown in Table 6. Identical sequences were obtained from both the native protein and from the denatured and

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**TABLE 3. Purification of *V. fluvialis* hemolysin**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Vol (ml)</th>
<th>Protein</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Conc (µg/ml)</td>
<td>Total (µg)</td>
</tr>
<tr>
<td>1 (culture supernatant fluids)</td>
<td>2,000</td>
<td>16.5</td>
<td>33,000</td>
</tr>
<tr>
<td>2 (hydrophobic-interaction chromatography)</td>
<td>22</td>
<td>87.5</td>
<td>1,925</td>
</tr>
<tr>
<td>3 (gel filtration chromatography)</td>
<td>146</td>
<td>4.3</td>
<td>628</td>
</tr>
</tbody>
</table>

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**FIG. 2.** SDS-PAGE and isoelectric focusing of *V. fluvialis* hemolysin preparations. (A) SDS-PAGE. Lanes: 1, molecular mass markers (values at left are in kilodaltons); 2, stage 1 (1.5 µg); 3, stage 2 (0.05 µg); 4, stage 3 (0.2 µg). The gel was stained with Coomassie brilliant blue R. (B) Analytical thin-layer isoelectric focusing. Stained gel is shown at left (a). Lanes: 1, pl markers; 2, stage 3 (0.4 µg). The gel was stained with Coomassie brilliant blue R. Shown at right is a zymogram analysis of an isoelectric focusing gel using an overlay of 0.35% erythrocyte suspension in PBS containing BSA (0.25 mg per ml) and 1% Noble agar (b).
reduced protein band excised from a Western blot of an SDS-PAGE gel.

**Immunological cross-reactivity.** VFH reacted with antibodies to El Tor-like hemolysin of non-O1 *V. cholerae* in a Western blot (Fig. 5). The reactive band, however, has a slightly higher molecular weight (63,000) than do the major reactive bands of the other two hemolysins of *V. cholerae*.

**Fluid accumulation induced by VFH.** When 100 HU (1.07 μg) was fed to the mice, fluid began to accumulate within 2 h postchallenge, reaching an FA ratio at 4 h which was greater than 100 (Table 7). The minimum amount of VFH required for inducing fluid was determined to be 6.25 HU, which corresponds to 0.067 μg of protein per mouse. The amount of fluid induced gradually increased with increased amounts of the hemolysin (Fig. 6). The FA ratios induced by 0.067 to 1.07 μg of VFH were significantly higher than those induced by buffer alone, with *P* values of ≤0.001.

**PCR analysis.** Only the combination of the Bam 10 (derived from the N-terminal amino acid sequence of VFH) and Eth6 primers (Table 1) produced a product, using *V. fluvialis* lysate as the template. Both positive controls, non-O1 *V. cholerae* strain 2194c and *V. cholerae* O1 biotype El Tor, and the *V. fluvialis* strain produced a 395-bp product. The other four 3′ primers (Table 1) in combination with Bam 10 did not produce any PCR product, using *V. fluvialis* lysate as the template.

**TABLE 5.** Sensitivities of erythrocytes to *V. fluvialis* hemolysin

<table>
<thead>
<tr>
<th>Animal</th>
<th>Hemolytic activity (HU/ml)</th>
<th>Relative sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>670</td>
<td>335</td>
</tr>
<tr>
<td>Rabbit</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>Calf</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Goat</td>
<td>125</td>
<td>63</td>
</tr>
<tr>
<td>Horse</td>
<td>110</td>
<td>55</td>
</tr>
<tr>
<td>Goose</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Chicken</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

*Compared to activity against sheep erythrocytes. Final concentration of erythrocytes was 0.35% (vol/vol).*

**DISCUSSION**

**Production and purification of VFH.** Production of VFH was inhibited by the addition of 1 to 2% NaCl to the medium. In an earlier study, Rahim and Aziz (26) also reported that the addition of 0.5 to 5% NaCl reduced the production of hemolysin by *V. fluvialis*. Insufficient (200 ml per flask) and excessive (50 ml per flask) aeration of the medium resulted in lower yields of VFH. Surprisingly, use of a 24-h-old plate culture as the inoculum resulted in inconsistent amounts of VFH in different flasks. Based on results obtained during these studies, VFH was routinely produced by inoculating 100 ml of CYE broth in a 2-liter flask with 10²⁰ CFU of the inoculum that was prepared from a 7-h-old culture and incubation on a rotary shaker (100 rpm) at 37°C for 17 h.

Purification of the hemolysin was achieved by using two steps. During the first step, the strong binding of the protein to phenyl-Sepharose CL-4B in the absence of ammonium sulfate
during hydrophobic-interaction chromatography and the requirement of 50% ethylene glycol for the its subsequent elution suggest that VFH is extremely hydrophobic (Fig. 1A). The hydrophobic nature of the protein was also apparent during the second step (gel filtration chromatography) of the purification process (Fig. 1B); VFH eluted from the column in a very broad peak, after the bed volume of 500 ml, with an apparent molecular weight of less than 10,000 (results not shown). This elution pattern is suggestive of an interaction between the gel and the protein molecule. Similar interactions between chromatographic gel matrices and proteins have been reported for hemolysins produced by *V. cholerae* O1 and non-O1 strains, *Vibrio vulnificus*, *Vibrio mimicus*, and *Vibrio tubiashii* (6, 7, 11, 14, 20, 22, 31). The extremely hydrophobic nature of the protein may also have been responsible for the loss of activity that was observed for the various purification methods (ammonium sulfate precipitation, ultrafiltration, dialysis, and lyophilization) we employed to isolate and concentrate VFH from the culture supernatant fluids. It was also observed that the purified preparation lost all its activity when stored in plastic containers; this loss was accompanied by the disappearance of the protein (presumably because of binding to the plastic). To minimize the loss during concentration of the hemolysin with Centricon concentrators, the units were filled with PBS containing BSA (1 mg per ml), incubated at 4°C for 16 to 18 h, and rinsed thoroughly with PBS prior to use. By use of this procedure, ca. 30% of the VFH was recovered in a concentrated form.

**Properties of VFH.** The molecular weight (63,000) of VFH is the same as that reported for the *V. mimicus* hemolysin (22) but is slightly higher than the 60,000 of the El Tor and El Tor-like hemolysins of *V. cholerae* (20, 31) and the 48,000 and 52,000 of the aberrant El Tor *V. cholerae* O1 strain (8, 11). The molecular weight of the denatured and reduced VFH is much higher than the apparent molecular weight of the native protein (<10,000 [data not shown]) as determined by gel filtration using the method described by Andrews (1). Similar observations have been made for the El Tor and El Tor-like hemolysins of *V. cholerae* O1 and non-O1 strains that also exhibit a very low molecular weight by gel filtration but have a much higher molecular weight by SDS-PAGE (11, 14, 20, 31). The pI of VFH is the same as that of the aberrant El Tor-like hemolysin of non-O1 *V. cholerae* (11) but is different from the values of 5.3 and 5.7 reported for the other El Tor-like hemolysins of *V. cholerae* non-O1 (11, 20). The results of immunoblot analysis (Fig. 5) also showed that the VFH reactive band has a slightly higher molecular weight (63,000) than do the major reactive bands of the El Tor and El Tor-like hemolysins, which have a molecular weight of 60,000 (20, 31). VFH is heat labile.

**Figure 4.** Effect of erythrocyte concentrations on hemolysin-induced lysis of erythrocytes. The reaction mixture contained 2 HU of hemolysin and different amounts of erythrocytes. Erythrocyte samples (1 ml) were incubated with the hemolysin, and unlysed erythrocytes were sedimented by centrifugation. The absorbance of the supernatant fluids at 545 nm was measured and compared with that of the control (saponin-lysed 0.175 to 3.5% [vol/vol] erythrocyte suspensions).

**Figure 5.** Western blot (immunoblot) analysis of *V. fluvialis* hemolysin. Denatured and reduced samples were subjected to SDS-PAGE and were electrophoretically transferred to nitrocellulose membranes, and bands were visualized by sequential probing with a 1:200 dilution of rabbit anti-hemolysin of *V. cholerae* non-O1 strain 2194c, goat antirabbit alkaline phosphatase conjugate, and the phosphatase substrate. Lane 1, prestained molecular weight markers (values at left are in kilodaltons); lane 2, culture supernatant fluids of *V. cholerae* non-O1 strain 2194c (0.6 HU); lane 3, culture supernatant fluids of *V. cholerae* O1 biotype El Tor strain 1074-78 (0.3 HU); lane 4, purified *V. fluvialis* hemolysin (1.5 HU).

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**Table 6.** N-terminal amino acid sequences of *V. fluvialis*, *V. cholerae*, and *V. mimicus* hemolysins

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. fluvialis</em></td>
<td>Val-Ser-Gly-Gly-Glu-Ala-Asn-Thr-Leu-Pro-His-Val-Ala-Phe-Tyr-Ile-Asn-Val-Asn-Arg</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>Asn-Ser-Gly-Thr-Asn-Thr-Leu-Pro-His-Val-Ala-Phe-Tyr-Ile-Asn-Val-Asn-Arg</td>
</tr>
<tr>
<td><em>V. mimicus</em></td>
<td>Asn-Ser-Gly-Thr-Asn-Thr-Leu-Pro-His-Val-Ala-Phe-Tyr-Ile-Asn-Val-Asn-Arg</td>
</tr>
</tbody>
</table>

* Underlined amino acids are identical to those present in the *V. cholerae* and *V. mimicus* hemolysins.
TABLE 7. Effect of incubation time on FA ratios induced by V. fluvialis hemolysin

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>FA ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>59.0 ± 2.45</td>
<td>78.3 ± 4.99</td>
</tr>
<tr>
<td>3</td>
<td>57.0 ± 2.45</td>
<td>85.7 ± 3.69</td>
</tr>
<tr>
<td>4</td>
<td>59.0 ± 5.1</td>
<td>105 ± 9.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> FA ratios were determined for four mice per sample; values represent means ± standard deviations.

<sup>b</sup> 100 HU (1.1 μg).

<sup>c</sup> Paired Student’s t test.

and sensitive to proteases and is inhibited by the divalent cation Zn<sup>2+</sup>, which has been reported to inhibit the pore-forming stage of the V. mimicus hemolysin (22). The inability of cholesterol and mixed gangliosides to inhibit the hemolytic activity of VFH suggests that these biologically active molecules may not be target host cell receptors for the hemolysin.

The hemolytic spectrum of VFH is slightly different from those reported for the El Tor-like hemolysin of non-O1 V. cholerae (30) and the variant hemolysin of non-O1 V. cholerae (20). Erythrocytes from rabbit and guinea pig are the most sensitive and those from goose and chicken are the least sensitive to both the El Tor-like hemolysin of non-O1 V. cholerae and VFH. Erythrocytes from goat and horse are as sensitive as sheep erythrocytes to the El Tor-like hemolysin but are less sensitive than sheep erythrocytes to VFH. Calf erythrocytes are less sensitive than sheep erythrocytes to the El Tor-like hemolysin but exhibit the same sensitivity as sheep erythrocytes to VFH. Studies with sheep erythrocytes showed that binding of the hemolysin occurs at 37°C but not at 4°C. Results of studies aimed at examining the effect of erythrocyte concentration on hemolysin-induced lysis indicate that in the presence of an excess of erythrocytes, the amount of VFH binding to each erythrocyte is reduced to levels insufficient to cause its lysis (Fig. 4). These results also demonstrate that like various other cytolsins and hemolysins (6, 10, 12–15, 19, 24), VFH lyse an erythrocyte by a multihit process whereby more than one molecule of the protein is required to lyse each erythrocyte. In addition to lysing erythrocytes, VFH is cytotoxic towards CHO cells in tissue culture. This is not surprising; cytotoxic activity towards different cells in tissue culture is exhibited by various bacterial hemolysins. VFH induces fluid accumulation in suckling mice; amounts as low as ca. 0.07 μg cause significant fluid accumulation within 4 h after oral inoculation. Ichinose et al. (9) obtained similar results during their studies with the El Tor-like hemolysin of non-O1 V. cholerae. However, they reported that the fluid accumulation peaked at 3 h and then decreased by 4 h.

The N-terminal amino acid sequence of VFH (Table 6) shares significant homology to that of the El Tor hemolysin of V. cholerae (4) and the thermolabile hemolysin of V. mimicus (27). The first four amino acids of VFH are different, but 14 of the remaining 16 amino acids are identical to those of the V. cholerae and V. mimicus hemolysins. This homology was confirmed by PCR analysis. However, a PCR-positive result with only one primer pair suggests that the area of homology between the two hemolysins may not be extensive. All of the other 3′ primers tested gave negative results, suggesting that the hemolysin gene does not match the El Tor hemolysin gene closely enough in those areas (Table 1). These results do not rule out some degree of homology and/or homologous sequences in areas of the gene not covered by these primers.

Our results show that V. fluvialis produces an extracellular hemolysin that is heat labile, sensitive to proteases, and has many other physicochemical and biological properties that are similar to those of the El Tor and El Tor-like hemolysins of V. cholerae and V. mimicus. PCR data indicate that the VFH DNA sequence is not identical to, but has a region of DNA homology to, the sequences of the El Tor and El Tor-like hemolysins. VFH lyses a wide variety of erythrocytes, is cytotoxic towards CHO cells, is immunologically related to the El Tor hemolysin, and is enterotoxigenic in suckling mice. The presence of all these properties suggests that VFH may play an important role in diarrheal diseases caused by V. fluvialis. Among the Vibrio spp., the role of hemolysins in the pathogenesis of gastrointestinal diseases is not very clear, but evidence from their enterotoxigenic activity in various animal models suggests that in the absence of other known virulence factors, hemolysins may play a significant role in the disease process.

REFERENCES


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