Purification and Characterization of the Vascular Permeability Factor Produced by *Bacillus cereus*

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(Received 21 August 1990/ Accepted 17 December 1990)

ABSTRACT. Purification of an extracellular protein exhibiting the vascular permeability activity produced by *Bacillus cereus* was performed by ammonium sulfate precipitation followed by chromatography on DE-32 cellulose, Sephadex G-100, and Sephadex G-75. The purified protein was found to be electrophoretically and antigenically almost homogeneous although it contained a trace of contaminant. The molecular weight of the protein was calculated to be 45,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The purified protein showed vascular permeability activity and mouse lethal toxicity, and caused fluid accumulation in ligated mouse intestinal loops, whereas it did not show any hemolytic and lecithinase activities. From these findings, the purified protein is suggested to be an enterotoxin (or a diarrheagenic toxin) responsible for diarrhea caused by *B. cereus* in a diarrheal-type food poisoning.—KEY WORDS: *Bacillus cereus*, purification, vascular permeability factor.

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Two types of food-borne illness caused by *Bacillus cereus* are demonstrated, one causing diarrhea and the other causing emesis [7, 8, 23]. Like enterotoxins (ETs) produced by *Vibrio cholerae* [1, 3], enterotoxigenic *Escherichia coli* [5, 16], and *Clostridium perfringens* [4, 17], culture filtrates of *B. cereus* cause fluid accumulation in rabbit ileal loop (RIL) [21] and mouse ileal loop (MIL) assays [20], and increase vascular permeability (VP) in rabbit skin [10]. The VP activity of *B. cereus* culture filtrate correlated with the RIL activity [10, 24] although hemolysins and proteases in culture also caused weak VP activities [23]. Thus, the ability of *B. cereus* to cause diarrhea is suggested to be attributable to the production of an ET or a diarrheagenic toxin [10, 11, 19, 22]. Although attempts to purify an extracellular proteineous substances with the VP activity have been made in different laboratories [6, 21, 23, 25], it is not yet concluded at the present time whether the substance is composed of a single entity or more than one component. Thus, attempts were made to purify the vascular permeability factor (VPF) produced by *B. cereus* and to characterize some properties of purified VPF to see what substance(s) is responsible for the VP and fluid accumulation caused by *B. cereus* culture filtrate.

MATERIALS AND METHODS

**Bacterial strain and production of VPF:** *B. cereus* strain FM-1, originally isolated from a diarrheal-type food poisoning outbreak, was used to prepare the VPF. For VPF production, a fresh brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich. U.S.A.) agar slant culture, incubated for 12 hr at 32°C, was used to inoculate 500-ml Sakaguchi-flask containing 50 ml of BHI broth. After shaking (100 cycle/min) for 14–16 hr at 32°C, 10 ml of the seed culture was transferred to 1,000 ml of BHI broth containing 10 g glucose (BHIG) in 3-liter Sakaguchi-flask. The culture was incubated with shaking (80 cycle/min) for 5–6 hr at 32°C. Bacterial cells were removed by centrifugation at 8,000 rpm for 20 min at 4°C; the supernatant fluid was used as the starting material for purification of the VPF.

**Purification of VPF:** Solid ammonium sulfate was added to the culture supernatant to a final concentration of 70% (532 g/1,000 ml). One hour later, the precipitate formed at 4°C was obtained by centrifugation at 8,000 rpm for 20 min at 4°C. The precipitate was dissolved in 50 ml of distilled water and the suspension was extensively dialyzed at 4°C against 5 mM Tris-HCl, pH 8.6. The insoluble substances formed during dialysis were removed by centrifugation. The supernatant fluid was subjected
to a DE-32 cellulose column (1.5 × 20 cm) equilibrated with the same buffer. After unadsorbed proteins were completely washed out with the same buffer, adsorbed proteins were eluted in a linear gradient with NaCl from 0 to 0.3 M in the same buffer. The fractions containing the VP activity were collected and concentrated by lyophilization. The lyophilized material was dissolved in 2 ml of distilled water and then fractionated by gel filtration on Sephadex G-100 (2.5 × 95 cm) equilibrated with 5 mM Tris-HCl, pH 8.6. The fractions containing the VP activity were again collected and further fractionated by gel filtration on Sephadex G-75 superfine (1.5 × 95 cm) equilibrated with the same buffer. The fractions containing VPF were pooled and used as purified VPF for the following experiments.

**Biological assays:** The VP activity was determined by the methods of Glatz et al. [10]. One hundred microliters and 50 μl of the sample were subcutaneously injected into rabbits and ICR mice, respectively. After 3 hr, each rabbit or mouse was intravenously injected with 2% solution of Evans blue. Reactions were read after additional one hour. Bluing zones of larger than 9.0 mm in diameter for rabbits and larger than 7.0 mm in diameter for mice were considered to be positive response.

Mouse-lethal toxin (MLT) activity was determined by intravenous injection of 0.2 ml of each sample into ICR mice according to the methods of Johnson and Bonventre [12]. Death within one hour was considered to be positive response.

Intestinal loop assays were carried out by the procedure of Spira and Goepfert [21], Punyashtihi and Finkelstein [18], and Yamamoto et al. [27]. Rabbits and mice were anesthetized by intraperitoneal injection with sodium pentobarbital (200 mg/kg of rabbit and 600 μg/mouse, respectively) and the abdomen was opened along the midline. Ligations were made at 7–8 cm from the jejunum and spaced to yield 6–7 test loops per rabbit: the test loops were separated by interloops of approximately 3 cm in length. In mice, on the other hand, ligations were made at the upper part of the jejunum to yield one loop per mouse. Two ml and 0.1 ml of the test sample were injected into each loop of rabbits and mice, respectively. Animals were held for 6-8 hr postsurgery before they were sacrificed. Loops containing fluid were withdrawn to measure the fluid and then measured to determine the exact length. A positive response was determined as a volume/length ratio greater than or equal to 0.01.

Hemolytic (HL) and lecithinase (LC) activities were determined by the methods of Johson and Bonventre [12] and Glaz and Goepfert [9], respectively. The titers were expressed as the reciprocal of the highest dilutions giving these activities.

**Preparation of antiserum and immunological methods:** Six hundred microliters of purified VPF were emulsified with an equal volume of Freund’s complete adjuvant (Difco Laboratories, Detroit, Mich. U.S.A.) and injected intraperitoneally into rabbits. Injections of 0.5, 2, 4, 10, 20, 40, 50, 100 and 160 μg of purified VPF were made on days 0, 3, 6, 9, 12, 15, 18, 21, and 24. Two weeks later, the rabbits were boosted with 200 μg of purified VPF. The antigenicity of the VPF was determined by microslide double gel diffusion. The antigenic titers were expressed as the reciprocal of the highest dilutions to form the precipitin line(s). For neutralization studies, 0.5 ml of 20-fold concentrated culture supernatant was mixed with 0.5 ml of antiserum and incubated at 37°C for 1 hr. After incubation, the mixture was analyzed for VP, MLT, HL, and LC.

**Other methods:** Polyacrylamide gel electrophoresis [2, 14] and determination of protein content [15] were performed by the methods described previously. The molecular weight of the purified VPF was calculated by Sodium dodecyl sulfate-polyacrylamide electrophoresis [26] using molecular marker such as phosphorylase B (MW: 97,400), bovine serum albumin (MW: 67,000), egg albumin (MW: 45,000), carbonic hydrolase (MW: 29,000), and trypsin inhibitor (MW: 20,100).

**RESULTS**

**Purification of VPF:** The VP, MLT, HL, and LC activities were detected in culture supernatant of *B. cereus*. About 41% of VP activity and 20% of HL and LC activities were recovered by ammonium sulfate precipitation of culture supernatant at 70% saturation. After dialysis of the concentrate against 5 mM Tris-HCl, pH 8.6, all of these activities in the concentrate were adsorbed to DE-32 cellulose equilibrated with the same buffer. Adsorbed proteins were eluted by a linear gradient of NaCl from 0 to 0.3 M in the same buffer (Fig. 1). The VP activity was found in fractions (No.86–No.128), whereas the MLT activity in fractions (No.105–No.114) (Fig. 1). The LC activity was detected in fractions (No.88–No.108). On the other hand, the HL activi-
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Fig. 1. Chromatography of concentrated culture supernatant (300 ml) on DE-32 cellulose (1.5x30 cm) equilibrated with 5 mM Tris-HCl, pH 8.6 in linear gradient increase in NaCl concentration. Abbreviations: VPF, vascular permeability factor; MLT, mouse lethal toxin; LC, lecithinase; HL, hemolysin.

Fig. 2. Gel filtration of the vascular permeability factor purified by Sephadex G-100 on Sephadex G-75 superfine (1.5x95 cm) equilibrated with 5 mM Tris-HCl, pH 8.6. Abbreviations used were the same as those in Fig. 1.

Fig. 3. Gel filtration of the vascular permeability factor purified by Sephadex G-100 on Sephadex G-75 superfine (1.5x95 cm) equilibrated with 5 mM Tris-HCl, pH 8.6. Abbreviations used were the same as those in Fig. 1.

After exposure of these two fractions to 90°C for 2 min, the HL activity was still found in the earlier eluted fractions but not in the retarded eluted ones. For further purification, the fractions (No. 86–No.128) containing the VP activity were collected and concentrated by lyophilization. The concentrate was fractionated by gel filtration on Sephadex G-100 equilibrated with 5 mM Tris-HCl, pH 8.6 (Fig. 2). The MLT activity was found in fractions (No. 120–No.130), whereas the VP activity in fractions (No.116–No.140) (Fig. 2). The HL activity was eluted in fractions (No.132–No.157). The LC activity was detected in fractions (No.150–No.170). Fractions (No.115–No.132) were collected and similarly concentrated by lyophilization. The concentrate was further purified by gel filtration on Sephadex G-75 equilibrated with the same buffer. Two protein peaks were eluted as shown in Fig. 3. Both VP and MLT activities were found in the first peak. These fractions were collected and used as purified VPF for the following

Table 1. Some biological activities of purified vascular permeability factor produced by B. cereus

<table>
<thead>
<tr>
<th>Biological Activity</th>
<th>Activity</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular permeability (VP) reaction</td>
<td>+</td>
<td>(0.05 μg/rabbit)(^a)</td>
</tr>
<tr>
<td>Mouse lethal activity</td>
<td>+</td>
<td>(0.2 μg/mouse)(^b)</td>
</tr>
<tr>
<td>Mouse ileal loop (MIL) activity</td>
<td>+</td>
<td>(12 μg/mouse)</td>
</tr>
<tr>
<td>Rabbit ileal loop activity</td>
<td>+</td>
<td>(30-50 μg/loop)</td>
</tr>
<tr>
<td>Hemolytic activity</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lecithinase activity</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>VP and MIL activity neutralized with anti-VPF serum</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Numbers in parentheses are minimum doses of purified VPF to induced biological activities. +, positive; -, negative
experiments. Approximately 120–150 μg of VPF was obtained from 1,000 ml of culture supernatant.

Some properties of the purified VPF: Biological activities of purified VPF are summarized in Table 1. Minimum amounts of purified VPF to give the VP activity were 50 ng for rabbits and 200 ng for mice, respectively (Table 1). Intravenous injection of 12 μg of purified VPF into mice resulted in death within 10 min (Table 1). The minimum amounts of purified VPF to cause fluid accumulation in ligated intestinal loops were 30-50 μg for mice and 500 μg–1 mg for rabbits, respectively (Table 1). However, neither hemolytic (HL) nor lecithinase (LC) activities were found in the purified VPF preparation at the highest concentration used.

In polyacrylamide gel electrophoresis with purified VPF, one major and one minor protein bands were observed (Fig. 4). Each protein was separately eluted from the gel and assayed for their VP activity. The VP activity was found in the major protein but not in the minor one. The molecular weight of the major protein band was calculated to be 45,000 by SDS-PAGE.

In double immunogel diffusion, one major and one minor precipitin lines formed with rabbit antiserum to purified VPF (Fig. 5). These two precipitin lines were also observed with concentrated culture supernatant. The minor precipitin line fused with the precipitin line of concentrated BHI broth (Fig. 5). With rabbit antiserum treated with BHI broth, however, the minor precipitin line did not form (data not shown), suggesting that the minor precipitin line may be component(s) of BHI broth. Both VP and MLT activities of purified VPF were neutralized by mixing rabbit anti-VPF serum with purified VPF, whereas they remained by mixing preimmune rabbit serum with purified VPF (Table 1).

DISCUSSION

The present study demonstrated that almost
homogeneous VPF was isolated from culture filtrate of *B. cereus* by ammonium sulfate precipitation followed by different chromatography on DE-32 cellulose, Sephadex G-100, and Sephadex G-75. The purified VPF was found to be composed of a single entity with a molecular weight of 45,000. It showed vascular permeability activity and mouse lethal toxicity, and caused fluid accumulation in ligated intestinal loops of rabbits and mice, whereas it did not show any hemolytic (HL) and lecithinase (LC) activities. These findings are consistent with previous findings [7, 13, 21, 23-25] that an ET produced by *B. cereus* had a molecular weight of 38,000 to 46,000 by SDS-PAGE or 50,000 to 57,000 by gel filtration on Sephadex G-100. Thus, the present findings confirmed that the VPF produced by *B. cereus* is an enterotoxin (or a diarrheagenic toxin) responsible for diarrhea caused by *B. cereus*.

On the other hand, Thompson et al. [23] have recently reported that two major components and one minor component, when combined, were responsible for enterotoxic activity, were isolated from *B. cereus* culture filtrates by a combination of different chromatography steps. These two components were nontoxic when tested individually, whereas enterotoxigenic activities appeared when they were combined. It is difficult to explain these results in light of the fact that the single protein purified in this study produced the same biological activities as did the two proteins obtained in the previous study [23]. At the present time monoclonal antibodies to ET are being prepared to be used in purification of ET by affinity chromatography. The results from this study should definitely show whether only one protein is needed for the biological activities attributed to ET.

To detect an ET produced by *B. cereus*, enteropathogenicity of the culture filtrate has been usually determined by biological assays such as VP tests, MLT activity, or RIL assays since immunological methods are not available. By preparing antisera against homogeneous VPF, however, immunological methods will be developed to antigenically detect crude or pure VPF (or ET) of *B. cereus*. To prepare highly specific antisera, improvement of the methods for purification of VPF will be also needed in terms of purity and recovery.

**ACKNOWLEDGEMENTS.** The authors would like to express gratitude to Dr. M. S. Bergdoll at University of Wisconsin for his valuable advice in preparing the manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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