In Vitro Stability in Biological Activity and Antigenicity of the Vascular Permeability Factor Produced by Bacillus cerus

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In food-borne illness caused by Bacillus cerus, two types of clinical symptoms are recognized, one causing diarrhea and the other causing emesis [8, 9, 18]. Like enterotoxins (ETs) produced by Vibrio cholerae [3, 4], enterotoxigenic Escherichia coli [6, 11], and Clostridium perfringens [5, 12], culture filtrates of B. cerus cause fluid accumulation in rabbit ileal loop (RLL) assays [14] and increase vascular permeability (VP) in rabbit skin [10]. The VP activity of B. cerus culture filtrate correlated with the RLL activity [9, 14]. Cytotoxicity to cultured cells [1] and lethality to mice after intravenous injection [2, 17] have been also shown as other biological activities of B. cerus culture filtrates. From these findings, the ability of B. cerus to cause diarrhea and to induce these biological activities is suggested to attribute to the VP factor (VPF) produced by B. cerus [9, 10, 13, 15]. Although attempts to isolate the VP factor for diarrhea have been made in different laboratories [7, 15, 17, 18], it is not yet concluded whether or not the VPF is composed of a single entity. It is also unknown whether or not the VPF is easily degraded by proteolytic enzyme(s) during purification process although in vitro biological stability of the VPF in culture filtrate has been studied [7, 9, 15–18]. Thus, this study was undertaken to characterize stability in biological and antigenic activities of partially purified VPF of B. cerus isolated from a diarrheal-type food poisoning since lecithinase, hemolysins, and proteases in culture filtrate of B. cerus have been shown to also give VP activities [15].

B. cerus strain FM-1, originally isolated from a diarrheal-type food poisoning outbreak, was used to prepare VPF. For VPF production, a fresh brain heart infusion (BHI) agar slant (Difco Laboratories, Detroit, Mich., U.S.A.) 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 cultured was transferred to 1,000 ml of BHI broth supplemented with 10 g glucose (BHG) contained in 3-liter Sakaguchi-flask. The culture was incubated with shaking (80 cycle/min) for 5 hr at 32°C. Bacterial cells were removed by centrifugation at 8,000 rpm for 20 min at 4°C. According to the methods of Spira and Goepfert [15], solid ammonium sulfate was added to the supernatant to 70%. The precipitate formed was obtained by centrifugation and dissolved in 50 ml of 5 mM Tris-HCl, pH 8.6. The suspension was extensively dialyzed against the same buffer at 4°C. The clear supernatant obtained by centrifugation was fractionated by gel filtration on Sephadex G-100 (Pharmacia, Uppsala, Sweden) (2.5 × 95 cm) equilibrated with the same buffer. The fractions containing the VP activity were collected and used as partially purified VPF. The partially purified VPF was about 50-fold purified from culture supernatant on a protein basis.

The VP activity was determined by the methods of Glatz et al. [10]. One hundred microliters of each sample (diluted to 1:10) was subcutaneously injected into rabbits. At 3 hr after injection, each rabbit was intravenously injected with 2% solution of Evans blue (2 mg/kg). The reaction was read after additional one hour. A bluing zone of larger than 9.0 mm in diameter was considered to be positive response. The activity was expressed as percentage to the bluing area (or zone) induced by partially purified VPF.

For preparation of rabbit antiserum against VPF, 0.6 ml (5–100 μg) of partially purified VPF was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) and injected subcutaneously into rabbits. Injections were made on days 0, 7, 14, and 21. Two weeks later, the rabbits were boosted with partially purified VPF (100 μg) emulsified in Freund's incomplete adjuvant. The antigenicity of each sample was detected in microslide double gel diffusion. One immunodiffusion unit (1 IDU) was defined as the reciprocal of the highest dilution giving precipitin line(s).

For tests for stability at different pH, 0.1 ml of partially purified VPF (620 μg/ml) was incubated at 35°C for 30 min in 0.9 ml of various buffers at pH from 3 to 12. The buffers used were 0.1 M citrate-phosphate buffer (pH 3.1, 4.5, and 6), 0.1 M phosphate buffer (pH 7 and 8), and 0.1 M glycine-NaOH (pH 9.2, 10, 11, and 12). For tests for stability in different temperature and in storage for different periods, 0.1 ml of partially purified (320 μg/ml) was incubated at 45, 50, and 56°C for 5 or 10 min in 3.9 ml of 0.1 M phosphate buffer, pH 7.0. One hundred microliters of partially purified VPF was stored at 4°C and −20°C for 1, 2, 4, 6, and 8 weeks in 0.9 ml of 5, 10, and 50 mM Tris-HCl, pH 7.0 and 8.0.

For digestion of partially purified VPF with trypsin, 0.1 ml of partially purified VPF was incubated with 25, 50, 100, 200, and 400 μg/ml of trypsin (1:250, Difco Laboratories, Detroit, Mich., U.S.A.) for 30 min at 35°C.

The VP activity and antigenicity of partially purified VPF were determined after exposure to different pH. As
shown in Fig. 1, the VP activity of partially purified VPF was found to be stable at pH between 6 and 8, whereas it was less or much less active at pH lower than 5 or higher than 9. Similar findings were obtained in antigenicity of partially purified VPF (Fig. 1). The VP activity and antigenicity of partially purified VPF were affected by trypsin digestion (Fig. 2). Both activities were found to decrease by digestion with increasing amount of trypsin. With 400 μg/ml of trypsin, both activities almost completely disappeared (Fig. 2). Similar findings were also observed with pepsin at 100 μg/ml (data not shown).

The VP activity of partially purified VPF was inactivated by exposure to 56°C for 5 min (Table 1). Almost all of the VP activity remained of incubating at 45°C for 10 min, whereas 54% of the VP activity was lost of incubating at 50°C for 5 min (Table 1). Similar findings were also obtained in antigenicity of partially purified VPF (Table 1).

![Graph showing VP activity and antigenic activity vs pH](image)

**Fig. 1.** Resistance of partially purified vascular permeability factor to different pH. Symbols: ---, vascular permeability activity; ---, antigenic activity. IDU was the same as that described in legend of Table 1.

![Graph showing VP activity vs trypsin concentration](image)

**Fig. 2.** Resistance of partially purified vascular permeability factor to trypsin digestion. Symbols: ---, vascular permeability activity; ---, antigenic activity. IDU was the same as that described in legend of Table 1.

![Graph showing VP activity vs incubation time](image)

**Fig. 3.** Vascular permeability activity of partially purified vascular permeability factor after storage at 4°C and −20°C for different periods. Symbols: ---X---, 50 mM Tris-HCl, pH 7.0 at 4°C; ---, 50 mM Tris-HCl, pH 8.0 at 4°C; ---X---, 50 mM Tris-HCl, pH 7.0 at −20°C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% in vascular permeability activity</th>
<th>% in antigenic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100</td>
<td>4 IDU*</td>
</tr>
<tr>
<td>45°C, 5 min</td>
<td>100</td>
<td>4 IDU</td>
</tr>
<tr>
<td>10 min</td>
<td>100</td>
<td>4 IDU</td>
</tr>
<tr>
<td>50°C, 5 min</td>
<td>46</td>
<td>2 IDU</td>
</tr>
<tr>
<td>10 min</td>
<td>37</td>
<td>1 IDU</td>
</tr>
<tr>
<td>56°C, 5 min</td>
<td>0</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

* IDU (immunodiffusion unit) was defined as the reciprocal of the highest dilution to form the precipitin line(s) against rabbit antiserum reactive with partially purified vascular permeability factor at 6 μg/ml.
By storage at 4°C for longer than 2 weeks, the VP activity of partially purified VP was found to decrease (Fig. 3). After storage for 6-8 weeks, the activity was almost disappeared. On the other hand, almost all of the activity was still detected after storage at −20°C for 8 weeks. No significant difference in the remaining activities of partially purified VP was found in storage of various buffers of different ionic strength and neutral pH.

From the present results, both biological and antigenic activities of partially purified VPF of B. cereus are found to be similarly heat-labile, susceptible to trypsin and pepsin, and stable at pH between 6 and 8 although in vitro stability in biological activity of crude VPF of B. cereus has been reported [7, 9, 15-18]. The present results suggest that the VPF may be extracellular proteaceous substance(s). Furthermore, they suggest that antigenicity of the VPF will be a useful indicator to determine VPF produced by B. cereus.

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REFERENCES