An improved method for detecting cytostatic toxin (emetic toxin) of *Bacillus cereus* and its application to food samples

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Abstract: We developed an improved HEp-2 cell assay method for the detection of *Bacillus cereus* toxin, which affects the proliferation of HEp-2 cells. The cytostatic toxin was stable upon exposure to heat, pH 2, pH 11 and trypsin, which suggests it is an emetic. Using the HEp-2 cell assay, we examined the distribution and contamination of *B. cereus* strains that produced an emetic toxin in various foods. Although there were 228 enterotoxin producers among 310 *B. cereus* strains obtained from foods, 16 of them produced the cytostatic type (emetic toxin). All of the strains that produced the cytostatic toxin were of the H.1 serotype.

Key words: *Bacillus cereus*; Heat-stable toxin; Enterotoxin; HEp-2 cell assay; Emetic toxin

Introduction

*Bacillus cereus* is an aerobic or facultatively anaerobic spore bacillus found in soil, water and plants which causes food poisoning [1]. The symptoms of *B. cereus* food poisoning have been classified into two types; (i) emesis caused by an emetic toxin; and (ii) diarrhea caused by an enterotoxin [2]. A diagnostic method of detecting the latter type is commercially available. However, the emetic toxin has not been fully examined. The emetic toxin lowers antigenicity, and its detection by immunochemical methods is difficult. Hughes et al. [3] have reported that vacuoles are produced in HEp-2 cells by the emetic toxin, and they explained the relationship between these vacuoles and the toxin. Szabo et al. [4] reported that the toxin affected the HEp-2 cells by changing the vacuoles, and by acid production, cell rounding, cell granule promoting, and cytostatic activity [4].

In this study, we tried to develop an improved method for the detection of *B. cereus* emetic toxin which affects proliferation of HEp-2 cells, and to apply it to isolates from food samples.
Materials and Methods

Bacterial strain and preparation of heat-stable toxin

*B. cereus* strain No. 27, originally isolated from an emetic type food poisoning outbreak, was used to prepare heat-stable toxin. The medium was 10% skim milk autoclaved at 121°C for 20 min. *B. cereus* was transferred from brain heart infusion (BHI, Difco Laboratories, Detroit, MI) liquid medium to the milk medium and incubated for 12 h at 32°C with agitation (150 rpm). Thereafter, the culture was autoclaved at 121°C for 20 min, then centrifuged at 17000 × g for 20 min. The supernatant was filtered through a membrane (0.45 μm, Steradisc; Kurabou Industry Ltd., Osaka, Japan) and used as the cytostatic toxin preparation.

HEp-2 cell assay

HEp-2 (human carcinoma of the larynx) cells were maintained in Basal Eagle's minimum essential medium (BME, Sigma Chemical Co., MO) with Earle's Salts containing 2 mM L-glutamine, 10 mM sodium bicarbonate, 100 units/ml penicillin G, 100 μg/ml streptomycin and 10% (v/v) foetal calf serum (FBS; Hazleton, PA), at 37°C in a 5% CO₂ incubator. HEp-2 cells (3 × 10⁴ cells/well) in 96-well micro-test plates (Becton Dickson and Co., Oxnard, CA) were incubated for 24 h. Then the medium was gently aspirated and replaced with 100 μl of BME medium without FBS. Aliquots (10 μl) of the toxin (or filtrates from culture supernatants of *B. cereus*), 2-fold serially diluted with BME medium, were added and incubated for 24 h. Changes in the color of the medium caused by the production of acid were measured at 545 nm using an Immuno Reader NJ-2000 (Intermed Co., Tokyo, Japan).

Growth inhibition of HEp-2 cells by the toxin

HEp-2 cells 1 × 10⁵ cells/well in 24-well test plates (Becton Dickinson and Co.) were incubated for 24 h. The medium was then aspirated and replaced with 1 ml of BME medium without FBS. Thereafter, 100 μl of toxin or medium was added and incubated for 24 and 48 h. The number and the viability of the cells were assayed by Trypan blue dye exclusion using a hemocytometer.

Stability of the toxin

Heat stability of the toxin. The toxin (3.0 ml) was heated at 121°C for 20 min, cooled, then toxin activity was measured.

Acid stability at pH 2.0. 1 ml of toxin was diluted with 9.0 ml of Clark and Lubs solution (pH 2.0) and incubated at room temperature for 2 h. The pH was adjusted to 7.6 with 0.1 N NaOH, and the toxin activity was measured.

Alkaline stability at pH 11.0. 1 ml of toxin was diluted with 9.0 ml of phosphate buffer (pH 11.0) and incubated at room temperature for 2 h. The pH was adjusted to 7.6 with 0.1 N HCl, and the toxin activity was measured.

Trypsin digestion. 1 ml of toxin was added to trypsin (Sigma Chemical Co.) 0.02 g in 9.0 ml of Tris(hydroxymethyl)aminomethane buffer (pH 8.0) and incubated at 37°C for 24 h. After boiling for 15 min, the pH was adjusted to 7.6 with 1 N HCl, and the toxin activity was measured.

Hemolytic activity

Sheep red blood cells were adjusted to a density of 5 × 10⁶ per well of micro test plates, mixed with toxin and incubated at 37°C for 24 h. Hemolytic activity was measured by the absorption at 640 nm.

Origins of B. cereus


Separation of B. cereus

Foods were homogenized in sterile saline (90 ml). Aliquots (1 ml) of the homogenate were inoculated into liquid medium (dry beef extract, 30 g; maltose, 1 g; 0.2% Phenol red, 12 ml;
polymyxin B, 10,000 units, distilled water, 1000 ml) and incubated at 32°C for 24 h. One loop of the medium was then spread over NaCl glycine Kim Goefert agar medium (NGKG: Nissui Seiyaku Co., Ltd., Tokyo, Japan) and incubated at 37°C for 24 h. A positive lecithinase reaction and irregular circumferences of colonies were regarded as B. cereus. 310 strains of B. cereus were isolated and incubated with BHI medium.

**Serotyping of B. cereus**

The serotype was assayed according to a previous report using anti-H-antibody (Society for Food-Poisoning Research of the Tohoku District, Yamagata Prefectural Public Health, Yamagata, Japan) [5].

**Enterotoxin (diarrheal toxin)-producing activity**

The cells were incubated with BHI broth at 32°C for 6 h, agitated (110 rpm/min), then centrifuged at 3000 × g for 20 min. The toxin in the supernatant was measured using a commercial kit for the detection of enterotoxin (CRET-RPLA: Denka Seiken Co., Ltd., Tokyo, Japan).

**Results**

**Growth inhibition of HEp-2 cells by the toxin**

The influence of the heat stable toxin upon the growth of HEp-2 cells was examined after 24 and 48 h. The number and viability of HEp-2 cells were estimated by means of hemocytometer and Trypan blue staining, respectively. As shown in Table 1, the growth of HEp-2 cells incubated with the toxin was inhibited by about 61 and 57% at 24 and 48 h, respectively, compared with the control. The viability of the toxin-treated cells did not differ from that of the control. These results indicated that this toxin is cytostatic.

**Heat stability of the cytostatic toxin**

Emetic toxins are characterized by their heat stability [1]. We examined the stability of the toxins by autoclaving them at 121°C for 20 min. The toxic activity was not affected (Fig. 1). These results suggested that the cytostatic toxin is an emetic.

**Acid and alkaline stability of cytostatic toxin**

The stability of the toxin against acidity and alkalinity was tested. The toxin was adjusted to pH 2 or 11, then incubated at room temperature for 2 h. The cytostatic toxicity against HEp-2 cells did not decrease (Fig. 2). The stability of the toxin after trypsin digestion for 2 h at 37°C was tested in HEp-2 cells. The toxin was not influenced by this procedure (data not shown).

**Hemolytic activity**

Toxin and sheep red blood cells were mixed in phosphate-buffered saline without calcium and...
magnesium (pH 7.2) and incubated at 37°C for 24 h. There was no hemolytic activity compared with that of the saline-treated group.

Table 2

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Number of isolates</th>
<th>Cytostatic toxin</th>
<th>Enterotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bean paste (Miso)</td>
<td>60</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td>Prunus persica</td>
<td>20</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Malus pumila</td>
<td>70</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>Pyrus pyrifolia</td>
<td>15</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Pyrus communis</td>
<td>35</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>Diospyros kaki</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Glycine max</td>
<td>30</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Cucumis sativus</td>
<td>15</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Spinacia oleracea</td>
<td>15</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Brassica campestris</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Raphanus sativus</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Prunus pauciflora</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Oryza sativa (rice)</td>
<td>15</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Vitis vinifera</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Total number</td>
<td>310</td>
<td>16</td>
<td>228</td>
</tr>
</tbody>
</table>

Heat-stable toxin-producing strains, 16 strains (5.2%); enterotoxin-producing strains, 228 strains (73.5%).

Cytostatic toxin producing activity of B. cereus against HEp-2 cells

The inhibition of HEp-2 cells by the cytostatic toxin obtained from 310 strains of B. cereus isolated from various foods is shown in Table 2. The cytostatic toxins were produced by 11 of the 60 strains of B. cereus obtained from bean paste (18.5%), and five of 20 strains from Prunus persica (25%). Only 16 strains (5.2%) had cytostatic toxin activity, at an 800–6400-fold increase compared with strain cultivated in the milk medium. On the other hand 228 of 310 strains (73.5%) of B. cereus produced enterotoxin.

The other biological properties of cytostatic toxin-producing strains were examined. These strains did not produce enterotoxin. The flagellar serotypes were all H.1 and starch hydrolysis was negative, but utilization of citrate, nitrate reduction to nitrite, Voges-Proskauer, and hydrolysis of gelatin were all positive. These results indicated that the strain of B. cereus that produced the heat stable toxin had the V serotype.

Discussion

There are two types of symptoms associated with food poisoning by B. cereus: one is vomiting and the other is enteropathogenic diarrhea. The former is caused by an emetic toxin [1], which is very difficult to detect because emesis has to be induced in the monkey by oral administration of the sample. Kramer and Gilbert [1] used monkeys to test vomiting and reported that emetic toxins are stable against heat, acidity, alkalinity, and proteinase. The emetic toxin was not antigenic in monkeys because, when orally administered for several months, there were no resistant properties against the toxin [6]. The emetic toxin passes through the membrane filter, indicating that its molecular mass is below 5000 [7]. However, it has not been purified or structurally analyzed. The antigenicity of the emetic toxin of B. cereus is very low, preventing detection by routine immunochemical means. Thus, a simple and convenient method for the detection of emetic toxin has not yet been developed. Hughes et al. [3]...
have reported that an emetic toxin of *B. cereus* produced vacuoles in HEp-2 cells.

Therefore, we investigated a new and simple method of detecting the emetic toxin of *B. cereus* using HEp-2 cells. We found that the color of the conditioned medium changed when metabolites of the HEp-2 cells were inhibited by emetic toxin. We thus examined the relationship between the growth inhibitory activity of the cytostatic toxin on HEp-2 cells and the characteristics of the emetic toxin. The *B. cereus* toxin produced in milk medium inhibited the growth and induced acid production in HEp-2 cells. The acid production changed the color of the medium, which was evaluated by measuring the absorption at 545 nm. The toxin was stable against heat, acidity, alkalinity and proteinase. It was possible that some strains of *B. cereus* produced heat stable hemolysin, which has cytostatic activity against HEp-2 cells. However, these strains of *B. cereus* had no hemolytic activities. These results suggest that the cytostatic and emetic toxins were identical. Using the HEp-2 assay, we analyzed the emetic toxin from 310 environmental strains of *B. cereus*. Only 16 strains produced an emetic toxin and all the strains were serotype H.1, which is frequently detected in emetic food poisoning.

On the other hand, the diarrheal type of food poisoning is due to heat unstable enterotoxins, and the main symptoms are stomach ache and diarrhea. A full-scale investigation of the diarrheal toxin was performed by Turnbull et al. [8], who reported that the toxin was a protein, with a molecular mass of 50 000 and pH 4.9. Thompson et al. [9] separated the enterotoxin by means of sequential column chromatography, and reported that the toxin was a complex of several proteins with a subunit structure. Shinagawa et al. [10] separated and purified a heat-labile protein from *B. cereus* and raised an antibody against it. According to these findings, a kit that can detect the enterotoxin has been commercially available. Here, we assayed the production of enterotoxin in 310 environmental strains of *B. cereus* using the commercial kit. It was notable that 228 environmental strains of *B. cereus* (73.5%) produced enterotoxin, since outbreaks of diarrheal food poisoning due to *B. cereus* do not frequently occur. Thus, the mechanism of transmission of the organism should be further examined.

### References