Mouse Lethal Activity of a HEp-2 Vacuolation Factor, Cereulide, Produced by *Bacillus cereus* Isolated from Vomiting-Type Food Poisoning

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ABSTRACT. The HEp-2 vacuolation factor (or cereulide) produced by *Bacillus cereus* isolated from vomiting-type food poisoning, which is supposed to induce emesis, was found to give mouse and suncus lethality after intravenous and intraperitoneal administration. The emetic activity of the factor was also found to be resistant to heating at 121°C for 15 min, exposure to pH 2 and 11, and to digestion with proteolytic enzymes such as pepsin and trypsin. These findings suggest that the cereulide produced by *B. cereus* is stable in the digestive tracts, induce emesis, and show lethal activity leading to cellular damage. — KEY WORDS: *Bacillus cereus*, emesis, suncus.

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Bacillus cereus causes two different types of food poisonings such as diarrheal- and vomiting-types. Both food poisonings are associated with at least two different toxins produced by *B. cereus* [5]. The diarrheal-type food poisoning is well known to be caused by a diarrheagenic toxin (or an enterotoxin) [3, 4, 10], whereas the vomiting-type one is assumed to be associated with the heat-stable emetic toxin [5–7]. Szabo *et al.* [12] reported that the vacuole response to HEp-2 cells was observed with the heat-stable substance(s) in culture supernatants of *B. cereus* isolated from vomiting-type food poisoning. Agata *et al.* [1] have recently isolated a vacuolation factor to HEp-2 cells, which is a dodecadepsipeptide (a cereulide), produced by *B. cereus*. Agata *et al.* [2] and Shinagawa *et al.* [8] have reported that the cereulide induced emesis in monkeys and *Suncus murinus* (suncus). Although the substance may be concluded to be an emetic toxin itself produced by *B. cereus*, other *in vitro* biological activities of the substance are not yet fully studied. Therefore, attempts were made to study mouse lethality and *in vitro* stability of the cereulide to elucidate the mechanism of emetic action of the toxin in case of vomiting-type food poisoning.

B. cereus strain No. 55 was isolated from vomiting-type food poisoning. *B. cereus* No. 55 was cultured in 10% skim milk [9]. The vacuolation factor (cereulide) was isolated and purified from the culture supernatant by the following steps such as precipitation of 50% ammonium sulfate, extraction with ethanol, extraction with chloroform, and silica gel column chromatography as described previously [8].

As reported previously, vacuole response assay was determined using HEp-2 cells cultured in Eagle's Basal Medium (Flow Laboratories, New York, U.S.A.). The reciprocal of the highest dilution of the test sample producing vacuole in more than 25% of the HEp-2 cells at 10 vacuoles/well was defined as a vacuolation titer (one unit/m*l* corresponding to 5 *n*g/m*l*) [1, 9].

Stabilities of purified cereulide to pH 2 and 11 and by digestions with pepsin (Sigma Co., St. Louis, U.S.A. 2 mg/m*l*) and trypsin (Sigma Co., St. Louis, U.S.A. 2 mg/m*l*) were examined by the methods of Shinagawa *et al.* [11]. After treatment, either neutralization or boiling was carried out before suncus administration.

For determination of mouse lethality, the test sample was intravenously (0.2 m*l*) and intraperitoneally (0.5 m*l*) injected into ICR male mice weighing 20–30 g. The mice were observed for 24 hr. *Suncus murinus* (suncus) strain Jic-SUN weighing 50–70 g (for male) and 35–45 g (for female) were purchased from Charles River Japan, Inc., Yokohama, Japan. Oral and/or intraperitoneal administration (0.2 m*l*) into each suncus were performed. The interval from administration of cereulide to the first episode of vomiting (latency) and the number of vomiting episodes were recorded. A ED_{50} value was defined as the dose of cereulide which caused emesis in approximately 50% of the challenged animals.

To study animal lethality, mouse lethality was examined after intravenous and intraperitoneal administration with the purified cereulide. With intravenous administration of the cereulide containing 500 units of HEp-2 vacuolating activities, no mice died. Four out of 5 mice and 5 out of 5 mice died, respectively with the same cereulide containing 800 and 1,000 units (Table 1). On the other hand, none of 3 mice, 2 out of 3 mice, and 3 out of 3 mice were found to be dead after intraperitoneal administration with the same substance containing 500, 1,000, and 2,000 units, respectively (Table 1). From these findings, mouse LD_{50} of the cereulide was estimated to be 707.1 units for intravenous administration and 925.9 units for intraperitoneal administration, respectively.

Similar lethality was also found in suncus after intraperitoneal administration with the cereulide containing 1,200 and 2,000 units (Table 1). However, suncus lethality was not observed with oral administration of the cereulide containing 2,000–8,000 units (data not shown) although emesis was observed with the same amount of the cereulide (Table 2). From the results presented in Table 2, suncus ED_{50} of the cereulide was estimated to be 3,428 units for

oral administration. Depending on the findings, the cereulide may be less stable in the digestive tract than the abdominal cavity.

To study *in vitro* stability of purified cereulide, the emetic activity to suncus was examined after heating at 121°C for 15 min, exposure to pH 2 and 11, and digestions with pepsin and trypsin. As presented in Table 3, such treatment did not give significant effect to the emetic activity of the cereulide. Thus, the cereulide is suggested to be very stable in the digestive tracts.

From the present results, the cereulide produced by *B. cereus* is supposed to be a lethal toxin leading to cellular damage. More detailed studies will be needed to correlate the cereulide (or mouse lethal toxin) with the clinical symptom found in the vomiting-type food poisoning.

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Table 1. Lethal activity of purified vacuolation factor to mice and suncus

Injection of animal	Total units administered	Lethality
Mouse		
Intravenous injection	500	0/5[a]
	800	4/5
	1,000	5/5
Intraperitoneal injection	500	0/3
	1,000	2/3
	2,000	3/3
Suncus		
Intraperitoneal injection	800	0/2
	1,200	1/1
	2,000	2/2

a) Number of animal death/Number of animal tested.

Table 3. Emetic response of suncus administered orally with purified vacuolation factor (8,000U) after different physicochemical treatment

Treatment	Number of suncus with emesis	Period (min) to induce emesis
Positive control[a]	3/3[b]	26–85
Heating (121°C, 15 min)	6/6	23–90
pH 2	2/2	62 or 98
11	2/2	23 or 37
Enzyme		
Pepsin	2/2	63 or 84
Trypsin	2/2	28 or 34
Control buffer	0/8	

a) Purified vacuolation factor (8,000U) was administered.
b) Number of suncus with emesis/Number of suncus tested.

Table 2. Emetic response of suncus orally and intraperitoneally administered with purified vacuolation factor

	Method of administration	Total units administered	Number of suncus with emesis	Period (min) to induce emesis
Purified factor	Oral	2,000	0/2[a]	
		4,000	1/3	48
		8,000	3/3	10–68
	Intraperitoneal	800	0/2	
		1,200	1/1[b]	37
		2,000	2/2[c]	28 or 46
Control[d]	Oral	0	0/2	
	Intraperitoneal	0	0/2	

a) Number of suncus with emesis/Number of suncus tested.
b) Suncus was dead after 3 days.
c) Suncus was dead after 5 to 6 hr.
d) 10% ethanol (v/v) in physiological saline.

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