Purification and Some Properties of a *Bacillus cereus* Mouse Lethal Toxin

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ABSTRACT. A mouse lethal toxin (MLT) produced by *Bacillus cereus* isolated in vomiting-type food poisoning was purified by chromatography on DEAE-Sephadex A-25 followed by gel filtration on Sephadex G-75. Purified MLT possessed a molecular weight of 33,000–34,000. It showed mouse lethality and hemolytic (HL) activity on sheep and rabbit erythrocytes; the latter erythrocytes were more weakly hemolyzed than the former ones. However, fluid accumulation in mouse ligated intestinal loops was not induced by purified MLT at the highest concentration used. Both MLT and HL activities were stable at pH 6–9, during storage at −20°C for 8 weeks, and resistant to papain, cholesterol, lecithin, and dithiothreitol treatments. Most activity was lost during storage at 4°C or 25°C for 2 weeks or upon treatment with trypsin, trypanblue, or ethanol. The activities were resistant to heating at 37°C for 5 min, less resistant at 98°C for 5 min, and sensitive at 60°C for 5 min. It can be concluded from the results that MLT is different from the diarrheagenic toxin produced by *B. cereus* isolated in diarrheal-type food poisoning, but is similar to, if not identical, hemolysin II.—KEY WORDS: *Bacillus cereus*, hemolysin II, mouse lethality, toxin.

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Some strains of *Bacillus cereus* cause food poisoning characterized by either diarrhea or vomiting [5, 6, 16]. Hemolytic (HL) activity, mouse lethal toxin (MLT), lecithinase (LC) and vascular permeability (VP) activities, and a factor responsible for fluid accumulation in ligated intestinal loops of rabbits and mice have been found in culture filtrates of *B. cereus* isolated from food poisoning cases [5–8, 12, 15, 16]. While the MLT activity has been reported in culture filtrates of *B. cereus* strains isolated in outbreaks of both diarrheal- and vomiting-type food poisonings, VP activity has been observed in culture filtrates of those isolated only in diarrheal-type food poisoning [12, 17]. It has been suggested that the VP activity of *B. cereus* culture filtrates may be an indicator correlated with fluid accumulation in rabbit ligated intestinal loops. Purified diarrheal toxin is associated with MLT [13, 14] like cereolysin (hemolysin I) [1–3]. To distinguish between the MLT activity of cereolysin and that of the diarrheal toxin, purification of the MLT produced by a *B. cereus* strain isolated in an outbreak of vomiting-type food poisoning was attempted.

MATERIALS AND METHODS

Bacterial strain and production of MLT: *B. cereus* strain FS-1, originally isolated in vomiting-type food poisoning, was used to purify MLT. For production of MLT, a fresh slant culture of brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich, U.S.A.), incubated for 12 hr at 32°C, was used to inoculate 50 m*l* of BHI broth in a 500-m*l* Sakaguchi flask. After shaking at 100 cycles/min in a water-bath for 14–16 hr at 32°C, 10 m*l* of the seed culture was transferred to 1,000 m*l* of BHI broth containing 10 g glucose (BHIG) in a 3-liter Sakaguchi flask. The culture was incubated with shaking (90 cycles/min) for 5 hr at 32°C. Bacterial cells were removed by centrifugation at 8,000 rpm for 20 min at 4°C; the supernatant was used as the starting material for purification of MLT.

Purification of MLT: Ammonium sulfate was added to the supernatant to 70% saturation. An hour later, the precipitate was collected by centrifugation at 8,000 rpm for 20 min at 4°C. It was dissolved in 30 m*l* of distilled water and the suspension was dialyzed against 50 mM Tris-HCl, pH 8.6. The insoluble substance formed during dialysis was removed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was stirred for 20 min with DEAE-Sephadex A-25 equilibrated with the Tris-HCl buffer. The gel was allowed to settle and the supernatant was decanted after washing thoroughly with the same buffer. The adsorbed proteins were eluted from the gel with 0.1 M NaCl in the same buffer. The eluate was collected and concentrated by precipitation with 70% saturated ammonium sulfate. After dialysis, the concentrate (partially purified MLT) was applied to a

Sephadex G-75 column (1.5 × 80 cm) equilibrated with 5 mM Tris-HCl, pH 8.6, containing 50 mM NaCl. The fractions possessing MLT and HL activities were collected and concentrated by precipitation with 70% saturated ammonium sulfate. The concentrate was subjected again to gel filtration on the Sephadex G-75 column. The fractions possessing both MLT and HL activities were pooled and was used as purified MLT.

The diarrheagenic toxin was purified by the method described previously [13].

Biological assays: MLT was determined by intravenous injection of a 0.2-m*l* dose of a sample into an ICR mouse (20–22 g) according to Johonson and Bonventre [8]. Death within an hour was considered as a positive response. One MLT unit (MLTU) was expressed as the reciprocal of the highest dilution giving lethal toxicity in mice. HL activity was determined also by the method of Johonson and Bonventre [8]. A 0.9-m*l* portion of each of serially twofold dilutions of a sample was mixed with 0.1 m*l* of 5% sheep erythrocytes and the mixture was incubated for 30 min at 37°C to determine hemolysis. One HL unit (HLU) was expressed as the reciprocal of the highest dilution inducing 50% hemolysis. Vascular permeability and fluid accumulation in mouse intestinal loops were assayed by the methods described previously [7, 11, 14, 19].

Preparation of antiserum and immunological methods: Purified MLT was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich, U.S.A.). The mixture was injected intraperitoneally into rabbits. Injections of 3, 6, 12, 25, 50, and 100 μg of purified MLT were made on days 0, 3, 6, 9, 12, and 15, respectively. After three weeks, the rabbits were boosted with 100 μg of purified MLT without adjuvant. The antigenicity of MLT (10 μg) was determined by microslide double gel diffusion. Antigenic titers (immunodiffusion unit, IDU) were expressed as the reciprocal of the highest dilution forming a precipitin line(s). For neutralization tests, 0.5 m*l* of partially purified MLT (4 MLTU, 320 HLU) was mixed with 0.5 m*l* of antiserum and the mixture was incubated for an hour at 37°C. After incubation, the mixture was tested for MLT, HL, and antigenic activities to determine if any loss in these activities had occurred.

In vitro stability: The buffers used for stability tests at different pH values were: 0.1 M citrate-phosphate buffer at pH 3, 4, 5, and 6; 0.1 M phosphate buffer at pH 7 and 8; and 0.1 M glycine-NaOH for pH 9, 10, 11, and 12. A 2-m*l* portion of partially purified MLT was dialyzed for 24 hr at 4°C against each buffer. After dialysis, each sample was tested for MLT, HL, and antigenic activities.

Three 500-μ*l* samples of partially purified MLT were incubated for 5 min at 37, 60, or 98°C. After incubation, each sample was immediately cooled in ice-water and tested for MLT, HL and antigenic activities to determine if any loss had occurred.

For stability tests during storage at different temperatures, 0.1-m*l* samples of partially purified MLT were stored at 25, 4, or −20°C for 1, 2, 4 or 8 weeks.

To study the effects of different reagents, 0.5-m*l* samples of partially purified MLT were incubated with 0.5 m*l* of trypsin (Difco Laboratories) at 100 μg/m*l*, papain (Katayama Chemical Co., Osaka) at 100 μg/m*l*, cholesterol (Daiichi Chemical Co., Tokyo) at 100 μg/m*l*, lecithin at 100 μg/m*l*, 0.05 M dithiothreitol (Wako Pure Chemical Co., Osaka), or 0.1% trypanblue. The mixtures were incubated for 30 min at 25°C or 37°C. After incubation, each sample was tested for HL activity.

Other methods: Electrophoresis and determination of protein contents were performed by the methods described previously [4, 9, 10, 18].

RESULTS

Purification of MLT: The culture supernatant contained 1 MLTU/m*l* and 160 HLU/m*l*, but no VP activity. MLT and HL activities in the ammonium sulfate concentrate were adsorbed on DEAE-Sephadex A-25 gel. They were eluted with 0.1 M NaCl. The eluate was gel filtered on Sephadex G-75 (Fig. 1). The fractions exhibiting MLT and HL activities were pooled and concentrated by precipitation with ammonium sulfate. The concentrate was further gel filtered on Sephadex G-75 (Fig. 2). The single protein peak eluted contained both MLT and HL activities. Approximately 200–300 μg of MLT were obtained from 1,000 m*l* of culture supernatant fluid.

Some properties of purified MLT: In polyacrylamide gel electrophoresis at pH 8.3 and 4.3, purified MLT was resolved into one major and two or three very faint protein bands. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, on the other hand, a single protein band was observed under

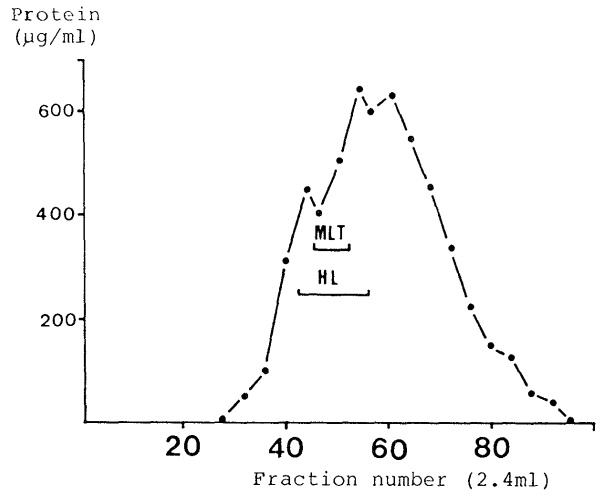

Fig. 1. Gel filtration on Sephadex G-75 (superfine). Column size: 1.5×85 cm, Eluant: 50 mM NaCl in 5 mM Tris-HCl buffer, pH 8.6, Flow rate: 6.2 ml/hr.
MLT: Mouse lethal toxin, HL: Hemolytic activity.

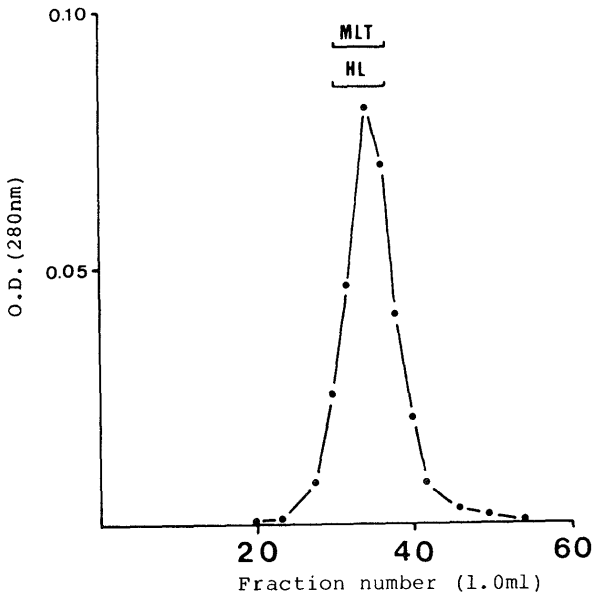

Fig. 2. Gel filtration on Sephadex G-75 (superfine). Column size: 1.5×85 cm, Eluant: 50 mM NaCl in 5 mM Tris-HCl buffer, pH 8.6, Flow rate: 6.2 ml/hr.
MLT: Mouse lethal toxin, HL: Hemolytic activity.

reducing condition (Fig. 3), suggesting that MLT is composed of uniform subunits. In double immunogel diffusion, purified MLT formed a major and a faint precipitin line with rabbit antiserum against purified MLT (Fig. 4). Purified MLT was antigenically different from diarrheagenic toxin purified from culture filtrate of *B. cereus* from diarrheal-type food poisoning. Its molecular weight was estimated to be 33,000–34,000 by gel filtration. The biological activities of purified MLT are summarized in Table 1. Intravenous injection of 8 μg of purified MLT into mice resulted in death within 2 min. Neither VP activity in rabbit skin nor fluid accumulation in mouse ligated intestinal loops was found with purified MLT at the highest concentration tested. However, purified MLT showed HL activities on sheep and rabbit erthrocytes (Table 1).

Neutralization tests: After mixing anti-MLT (rabbit IgG) with partially purified MLT, 87–94% of MLT, HL, and antigenic activities disappeared, whereas most of these activities remained after mixing with normal rabbit IgG.

In vitro stability: MLT, HL, and antigenic activities of partially purified MLT were stable at pH between 6 and 9, whereas they were unstable at pH values lower than 3 and higher than 12 (Table 2). Although MLT and HL activities of partially purified MLT were unaffected by exposure to 37°C for 5 min, 90–94% of the MLT, HL, and antigenic activities disappeared after exposing partially purified MLT to 60°C for 5 min. On the other hand, 20% of the MLT and 50% of the HL activities were still remained after treating at 98°C for 5 min (Table 2). The antigenicity was not affected by such treatment.

Upon storage at 4°C or 25°C, both MLT and HL activities of partially purified MLT gradually decreased; 90–95% of the activities were lost within 4 weeks. Biological activities of partially purified MLT were unaffected by storage at −20°C.

HL activity of partially purified MLT was affected by treatment with trypsin, trypanblue, or ethanol, whereas it was unaffected by treatment with papain,

Fig. 3. Disc electrophoresis of purified MLT (20 μg) in polyacrylamide gel containing SDS.
BPB: Brome phenol blue.

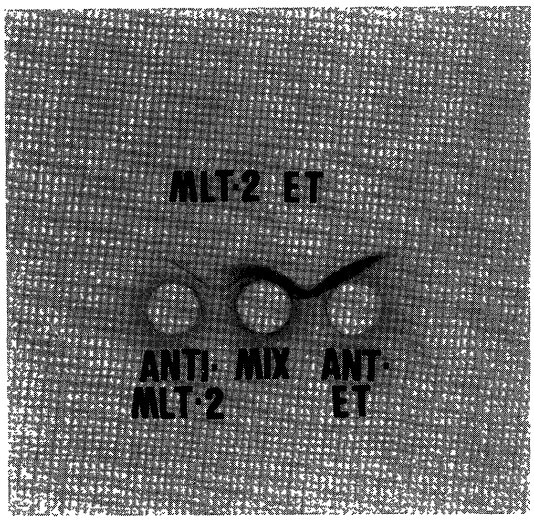

Fig. 4. Agar gel diffusion with anti-MLT, anti-ET, purified MLT, and crude ET.
MLT: Mouse lethal toxin, ET: *B. cereus* enterotoxin
Anti-MLT-2: 3-fold diluted rabbit anti-MLT with PBS.
Anti-ET: 3-fold diluted rabbit anti-ET with PBS.
MIX: Mixture of twofold diluted anti-MLT with twofold diluted anti-ET.
MLT-2: purified MLT (30 μg/ml), ET: crude ET (100 μg/ml).

Table 1. Biological activities of purified MLT

Assay	Minimum dose to give biological activity
Mouse lethal activity	: 8 μg/mouse
Hemolytic activity	
Sheep erythrocyte	: 0.1–0.2 μg
Rabbit erythrocyte	: 2.6-2.8 μg
Vascular permeability activity	: 1 μg/rabbit
Fluid accumulation activity	: 50 μg/mouse
Neutralized with anit-MLT serum-immunogloblin	: Yes
Neutralized with normal serum-immunogloblin	: No

cholesterol, lecithin, or dithiothreitol (Table 2).

DISCUSSION

B. cereus is known to cause two types of food poisoning: diarrheal and vomiting-types [5, 6, 16]. In the former type of food poisoning clinical symptoms are caused mainly by a *B. cereus*-produced diarrheagenic enterotoxin; those in the latter type are due to a toxic factor(s) responsible for vomiting [5–7, 15–17]. A highly purified diarrheage-

Table 2. Some properties of purified MLT

Treatment		Remaining activity
Stability		
pH	; 6–9	: MLT; 80–100%, HL; 80–90%
	; 3 and 12	: MLT; 16–20%, HL 10–16%
Heating	; 37°C, 5 min	: 100%
	; 60°C, 5 min	: MLT; 10%, HL; 6.3%
	; 98°C, 5 min	: MLT; 20%, HL; 50%
Storage	; 25°C, 2 weeks	: MLT; 0%, HL; 20%
	; 4°C, 2 weeks	: MLT; 10%, HL; 20%
	; −20°C, 8 weeks	: MLT; 90%, HL; 100%
Properties[a)]		
Trypsin (100 μg/ml)		: 13%
Papain (100 μg/ml)		: 100%
Cholesterol (100 μg/ml)		: 100%
Lecithin (100 μg/ml)		: 100%
Dithiothreitol (DTT)		: 100%
Trypanblue (0.1%)		: 3%
Ethanol (10%)		: 5%

a) The activity of MLT was determined in HL only in some cases.

nic toxin has recently been isolated [13, 14]. It has been shown to exhibit vascular permeability and mouse lethal activities and to cause fluid accumulation in rabbit and mouse ligated intestinal loops. Although culture filtrate of *B. cereus* isolated in an outbreak of vomiting-type food poisoning did not exhibit vascular permeability activity nor caused fluid accumulation in ligated intestinal loops, although it showed mouse lethal activity [17]. These findings indicate that *B. cereus* produces an MLT which is different from the diarrheagenic toxin. This was confirmed by the results obtained in this study; (1) it did not induce fluid accumulation in ileal loops of mice or rabbits, (2) it did not have vascular permeability activity, (3) it had a different molecular weight (33,000–34,000 vs 45,000), and (4) it differed antigenically from diarrheal toxin (ET) (Fig. 4).

Purified MLT had an estimated molecular weight of 33,000–34,000 as determined by gel filtration; this is close to the one (29,000–31,000) reported for hemolysin II [2]. Besides the mouse lethal activity, it showed hemolytic activity on sheep and rabbit erythrocytes, the activity on the sheep erythrocytes being 10 to 20-times greater than on the rabbit erythrocytes. It did not show any vascular permeability activity nor did it cause fluid accumulation in ligated intestinal loops. It differed antigenically from the diarrheagenic toxin (Fig. 4). Since MLT was sensitive to trypsin and to storage at 4°C for longer than 2 weeks, the biologically active site(s) of MLT may be located on the protein moiety. These findings are similar to those with *B. cereus* diarrheagenic toxin [13, 14].

With respect to hemolytic activities in culture filtrates, *B. cereus* produces two different kinds of hemolysins; hemolysin-I (cereolysin) and hemolysin-II [1–3, 8]. Although cereolysin has been shown to exhibit mouse lethal toxicity [1], its biological activity is activated by dithiothreitol, but inactivated by cholesterol and by exposure to 60°C or a higher temperature [2, 3]. In contrast, the biological activities of MLT obtained in this study were not activated by dithiothreitol, nor were they inactivated by cholesterol. Moreover, the biological activities of MLT were found to be more stable on exposure to 98°C than to 60°C. The behavior is different from that of cereolysin. In addition, cereolysin is a larger protein (52,000 daltons) than MLT (33,000–34,000 daltons). Thus, it can be concluded that MLT is not cereolysin. Although minimal information is available about the characters of hemolysin II, the properties of MLT purified in this study are similar to those reported for hemolysin II [2]. The molecular weights are similar, 33,000–34,000 vs 29,000–31,000, neither is activated by dithiothreitol nor affected by cholesterol, both retain some activity after heating at 98°C, and both are hemolytic. Hemolysin II has not been tested for mouse lethality, hence this characteristic can be added to its properties. It can be concluded from the results that MLT is hemolysin II.

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