Quantitative Analysis of Cereulide, the Emetic Toxin of Bacillus cereus, Produced under Various Conditions

Max M Häggblom, Camelia Apetroaie, Maria A. Andersson, and Mirja S. Salkinoja-Salonen

Department of Applied Chemistry and Microbiology, University of Helsinki, FIN-00014 University of Helsinki, Finland, and Department of Biochemistry and Microbiology and Biotechnology Center for Agriculture and the Environment, Cook College, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901-8525

This paper describes a quantitative and sensitive chemical assay for cereulide, the heat-stable emetic toxin produced by Bacillus cereus. The methods previously available for measuring cereulide are bioassays that give a toxicity titer, but not an accurate concentration. The dose of cereulide causing illness in humans is therefore not known, and thus safety limits for cereulide cannot be indicated. We developed a quantitative and sensitive chemical assay for cereulide based on high-performance liquid chromatography (HPLC) connected to ion trap mass spectrometry. This chemical assay and a bioassay based on boar sperm motility inhibition were calibrated with purified cereulide and with valinomycin, a structurally similar cyclic depsipeptide. The boar spermatozoan motility assay and chemical assay gave uniform results over a wide range of cereulide concentrations, ranging from 0.02 to 230 μg ml⁻¹. The detection limit for cereulide and valinomycin by HPLC mass spectrometry was 10 pg per injection. The combined chemical and biological assays were used to define conditions and concentrations of cereulide formation by B. cereus strains F4810/72, NC7401, and F5881. Cereulide production commenced at the end of logarithmic growth, but was independent of sporulation. Production of cereulide was enhanced by incubation with shaking compared to static conditions. The three emetic B. cereus strains accumulated 80 to 166 μg of cereulide g⁻¹ (wet weight) when grown on solid medium. Strain NC7401 accumulated up to 25 μg of cereulide ml⁻¹ in liquid medium at room temperature (21 ± 1°C) in 1 to 3 days, during the stationary growth phase when cell density was 2 × 10⁸ to 6 × 10⁹ CFU ml⁻¹. Cereulide production at temperatures at and below 8°C or at 40°C was minimal.

Bacillus cereus is frequently diagnosed as a cause of gastrointestinal disorders (10, 18). The heat-stable toxin of B. cereus, cereulide, results in vomiting within 1 to 5 h of ingestion and has been linked to illness leading to fatalities or requiring hospitalization (27). Cereulide is a mitochondriotoxin (22, 28) and causes emesis in primates (30, 32). Cereulide present in foods and is resistant to inactivation by heat (2, 24). It is generally believed that any food exceeding 10⁴ to 10⁵ cells or spores per g may not be safe for consumption (8, 15, 24). This number is often exceeded in a wide range of foods that are actually consumed. Nonetheless, illness is relatively rare (23) considering the high levels (>10⁵ CFU) of B. cereus that are consumed (24). This probably reflects the wide variation of pathogenic potential and overall diversity among B. cereus strains (16).

Several studies indicated that only a minority of B. cereus isolates may produce cereulide (4, 21, 26). Cereulide is a small stable depsipeptide that is resistant to inactivation by heat (2, 4), proteases, acid, or alkali (21, 28). Bioassays that are currently used for measuring cereulide give an approximate toxicity titer, but not an accurate concentration (6, 13, 21). The dose of cereulide causing illness in humans is therefore not known. Safety limits for cereulide in foods thus cannot be indicated, such as have been set for the common fungally produced toxins aflatoxin B1, ochratoxin A, and trichothecenes (1).

In this paper, we describe a quantitative and sensitive chemical assay for cereulide [cyclo(1-O-Val–I-Val–D-0-Leu–D-Ala–)], and valinomycin [cyclo(1-Val–D-Hy1Va–D-Val–L-Lac–)], based on liquid chromatography connected to ion trap mass spectrometry (MS). In addition, we demonstrate the calibration between the chemical assay and a bioassay based on boar sperm motility inhibition (6). The boar spermatozoan motility assay and chemical assay gave uniform results over a wide range of cereulide concentrations, ranging from 0.02 to 230 μg ml⁻¹. We used the combined chemical and biological assays to define conditions and concentrations of cereulide production by B. cereus.

MATERIALS AND METHODS

Strains. B. cereus strain NC7401 was obtained from N. Agata (Nagoya City Public Health Institute, Nagoya, Japan). Strains F5881 and F528 were obtained from A. C. Scoging (Public Health Laboratory Service, London, United Kingdom). F4810/72 (SMR 178) was obtained from A. Christiansson (Swedish Dairy Association; strain originally from Public Health Laboratory Service) OH599 was obtained from M. Haapasalo (Institute of Dentistry, University of Helsinki, Helsinki, Finland). ATCC 14579 was obtained from the American Type Culture Collection, Manassas, Va.

Cultivation and preparation of cell extracts. The strains were grown on tryptic soy agar (Difco) plates or in Trypticase soy broth (BBL). Biomass from plates was collected, and cells were lysed by three repeated freeze-thaw cycles and extracted with 98% methanol (10 ml per g of biomass [wet weight]) overnight. The mixture was centrifuged at 3,800 rpm (2,500 × g) in a tabletop centrifuge, and the supernatant was collected. The sample was evaporated to dryness, and the supernatant was collected. The sample was evaporated to dryness.
RESULTS

Analysis of cereulide and valinomycin. An HPLC method for the separation and analysis of the two structurally similar depsipeptide ionophores cereulide and valinomycin was developed, with detection by ion trap MS. With a C3 column and a solvent made up of 95% acetonitrile, 4.9% H2O, and 0.1% trifluoroacetic acid, the retention times of valinomycin and cereulide were 5.32 and 5.78 min, respectively (Fig. 1a). The typical mass spectra of cereulide and valinomycin are shown in Fig. 1b and c. The NH4+ adducts of valinomycin and cereulide had molecular weights of 1,128.9 and 1,171.1, respectively.

Since cereulide is not commercially available, a calibration standard for routine use was made by using valinomycin. The responses of valinomycin and cereulide in HPLC-MS analysis were very similar (within 10% [data not shown]). Mass spectra were scanned from 500 to 1,300 m/z, and the total ion chromatogram was integrated after smoothing. The variability in peak areas of replicate injections of the same sample was below ±10% for valinomycin concentrations of 1 to 10 µg ml−1. As seen in Fig. 1d, a linear curve could be used for concentrations below 1 ng per 1-µl injection, while a second-order equation (y = −21.5 + 263.6x0.786) gave the best fit over a range of 0.1 to 20 ng per injection. Ten picograms per injection could readily be detected and quantified by using selected ions with molecular weights of 1,128.9 for valinomycin and 1,171.1 for cereulide (data not shown) without interference from other materials present in the pentane extracts (detected by A230 [data not shown]).

Comparison of HPLC-MS and toxicity bioassay. Methanol extracts from several independent preparations of the various emetic B. cereus strains were analyzed by HPLC-MS and the boar spermatozoon motility bioassay (Fig. 2). The cereulide concentrations as determined by HPLC-MS ranged from 0.02 to 230 µg ml−1. Based on twofold dilution steps, the toxicity bioassay gave a concentration range that corresponded well to the HPLC-MS assay results. The bioassay was independently calibrated with purified cereulide. The results from analysis of several different emetic strains demonstrated that the boar spermatozoon motility assay read after 1 day of incubation and the chemical assay gave uniform results over a wide range of cereulide concentrations, ranging from 0.02 to 230 µg ml−1. This also confirms that the compound responsible for the observed toxicity of the strains is cereulide.

Effect of growth and incubation conditions on cereulide production. The HPLC-MS and toxicity assays were used in combination to determine the effect of different growth and incubation conditions on cereulide production by the emetic B. cereus strains. The minimum growth temperature of the tested strains was 10 to 11°C, while the maximum growth temperature ranged from 42 to 47°C. The effect of temperature on cereulide production by B. cereus strains was tested after growth for 11 days at temperatures of 11, 21, 40, and 42°C (Fig. 3). The levels of cereulide production by the three emetic strains F5881, F4810/72, and NC7401 were very similar. At 21°C, the strains accumulated 80 to 166 µg of cereulide g−1 (wet weight). Assuming that dry weight is approximately 20% of wet weight, cereulide corresponded to approximately 0.1% of cell dry weight. At 11°C, cereulide accumulation ranged from 0.5 to 2.8 µg g−1 (wet weight), while cereulide production at 40 and 42°C...
was negligible (<0.2 µg g⁻¹ [wet weight], with the exception of strain NC7401, which accumulated 0.9 µg g⁻¹ at 40°C). The nonemetic strains F528, OH599, and ATCC 14579 did not produce cereulide (<0.02 µg ml⁻¹) at any temperature.

Cereulide production by strain NC7401 pregrown at 21°C was also tested at temperatures below the minimal growth temperature of 11°C (Table 1). Cell suspensions incubated at 21°C accumulated over 10 µg of cereulide ml⁻¹ within 7 days, while cereulide production at temperatures at and below 8°C was minimal.

Initial experiments suggested that agitation of broth cultures

FIG. 1. HPLC-MS analysis of cereulide and valinomycin. (a) HPLC chromatogram (500 to 1,300 m/z) showing separation of cereulide and valinomycin. (b) Mass spectrum of valinomycin. (c) Mass spectrum of cereulide. (d) Calibration curve for valinomycin determined from four injections of two separate dilution series.

FIG. 2. Comparison of chemical and biological assays for determining cereulide concentrations in methanol extracts from independent preparations of different B. cereus strains. The line indicates a 1:1 correlation. The inset shows the same data drawn to a linear scale.

FIG. 3. Production of cereulide by B. cereus strains NC7401, F5881, and F4810/72 compared to that by F528, ATCC 14579, and OH599 (mean) grown on tryptic soy agar plates for 11 days at different temperatures.
affected cereulide production. This was tested in detail with strains NC7401 and F4810/72. Broth cultures inoculated 1/1,000 with an overnight culture were incubated with or without shaking (Table 2). Although growth levels were nearly identical based on turbidity (data not shown), cereulide production in static incubations was minimal compared to that in cultures incubated on a rotary shaker. This suggests that aeration may be required for cereulide production.

Cereulide production at different phases of growth. Growth, spore formation, and production of cereulide by strain NC7401 grown at room temperature in Trypticase soy broth (on a rotary shaker at 150 rpm) are shown in Fig. 4. The culture reached stationary phase within 20 h, with a cell density of $2 \times 10^8$ to $6 \times 10^8$ CFU ml$^{-1}$ (corresponding to 430 ± 20 Klett units). Cereulide production did not commence until after the end of logarithmic growth, approximately 24 h into the stationary phase. Cereulide accumulated to 7 to 22 units). Cereulide production did not commence until after the 7 days of incubation.

TABLE 1. Effect of incubation temperature on cereulide production by B. cereus strain NC7401 pregrown at 21°C

| Temp (°C) | Cereulide production (µg ml of broth culture$^{-1}$)$^a$ |
|----------|----------------------------------------------------------|
| 4        | 0.016 ± 0.002                                           |
| 8        | 0.071 ± 0.003                                           |
| 21       | 0.03 ± 0.003                                            |
| Inoculum$^b$ | 0.03 ± 0.003                                            |

$^a$ Values represent the mean ± standard deviation of three replicate cultures after 7 days of incubation.

$^b$ Inoculum of pregrown cells.

FIG. 4. Growth of B. cereus strain NC7401 in Trypticase soy broth and production of cereulide.

TABLE 2. Comparison of levels of cereulide production by B. cereus strains NC7401 and F4810/72 incubated with or without shaking

| Strain   | Incubation time (h) | Cereulide production (µg ml of culture$^{-1}$)$^a$ |
|----------|---------------------|--------------------------------------------------|
|          |                     | Static                                           |
|          |                     | Shaken                                          |
| NC7401   | 24                  | 0.0 ± 0.0                                       |
|          |                     | 1.36 ± 0.12                                     |
| F4810/72 | 24                  | 0.0 ± 0.0                                       |
|          |                     | 1.07 ± 0.02                                     |
| NC7401   | 70                  | 0.16 ± 0.12                                     |
|          |                     | 21.98 ± 2.21                                    |

$^a$ The cereulide concentration was analyzed after 24 or 70 h of incubation at 21°C.

acceptability threshold) (12) are the limits. In Sweden for vanillina sauce powder and other dried products from nonfermented milk, $10^3$ CFU ml$^{-1}$ (m) and $10^4$ CFU ml$^{-1}$ (M) (19) are the limits. The Dutch authorities have set as a general rule that the level of B. cereus in all food products should be below $10^5$ CFU g$^{-1}$ (12).

Incidents of food poisoning, however, have been reported at B. cereus concentrations similar to or below these threshold values (15, 23). It thus appears that there is no clear margin of safety. B. cereus emetic syndrome is considered an intoxication caused by ingestion of the preformed vomiting-inducing toxin, cereulide (20). Identification of conditions favorable for cereulide production in foods has been difficult due to the lack of suitable assays for accurately measuring cereulide concentrations. Bioassays are error prone and when based on twofold dilution steps are able to detect only a difference in cereulide production of >50%. The work described in the present paper was aimed at developing accurate and rapid analytical methods for measuring cereulide, so that emetic toxin-producing strains can be eliminated and/or processing conditions that prevent toxin synthesis can be identified.

We developed a sensitive and rapid chemical assay for cereulide (and valinomycin), based on separation by HPLC followed by ion trap MS detection. The ion ($m/z$, 1,171.1; NH$_4^+$ adduct) was chosen as specific and selective for cereulide, based on its described chemical structure (2, 6, 17). The ion range of 500 to 1,300 $m/z$ was analyzed with minimal interference from other compounds present in the pentane or methanol extracts of biomass. One of the three emetic toxin strains chosen for the present study, F4810/72, is a proven emesis-provoking strain by the monkey feeding assay (3, 32). Strain NC7401 has been tested in Suncus murinus (house musk shrew) (3), and the third strain, F5881, has been shown to produce cereulide (26). The results in this paper show that the results obtained by the boar spermatozoan motility assay and the chemical assay matched well. The chemical assay is more accurate (standard deviation, ±10%) than the bioassay (50%, based on twofold dilution steps).

Motility of boar spermatozoa is exclusively dependent on oxidative phosphorylation in the mitochondria. Since the motility inhibition assay is sensitive also to other microbial mito-
and of emetic toxin production by Bacillus cereus in a defined medium with amino acids. Microbiol. Immunol. 43:15–18.
6. Andersson, M. A., R. Mikkola, J. Helin, M. C. Andersson, and M. Salkinoja-Salonen. 1998. A novel sensitive bioassay for detection of Bacillus cereus emetic toxin and related depsipeptide ionophores. Appl. Environ. Microbiol. 64:1338–1343.
7. Batt, C. A. 2000. Bacillus cereus, p. 119–124. In R. K. Robinson, C. A. Batt, and P. D. Patel (ed.), Encyclopedia of food microbiology, vol. 1. Academic Press, San Diego, Calif.
8. Beattie, S. H., and A. G. Williams. 2000. Detection of toxins, p. 141–158. In R. K. Robinson, C. A. Batt, and P. D. Patel (ed.), Encyclopedia of food microbiology, vol. 1. Academic Press, San Diego, Calif.
9. Christianson, A., A. S. Naidu, I. Nilsson, T. Wiström, and H.-E. Pettersson. 1989. Toxic production by Bacillus cereus dairy isolates in milk at low temperatures. Appl. Environ. Microbiol. 55:2595–2600.
10. Drobniowski, F. A. 1993. Bacillus cereus and related species. Clin. Microbiol. Rev. 6:324–358.
11. Elintarvikevirasto, 1998. Päätös lastenruokien mikrobio logistiasta laatuaav timuksista 1023/84. (Decision on the microbiological safety criteria of baby foods.) Elintarvikevirasto, Helsinki, Finland.
12. European Commission. 1997. Harmonization of safety criteria for minimally processed foods. Inventory report FAIR concerted actions. FAIR CT96-1026. European Commission, Brussels, Belgium.
13. Finlay, W. J., N. A. Logan, and A. D. Sutherland. 1999. Semiautomated metabolite staining assay for Bacillus cereus emetic toxin. Appl. Environ. Microbiol. 65:1811–1817.
14. Finlay, W. J., N. A. Logan, and A. D. Sutherland. 2000. Bacillus cereus produces most emetic toxin at lower temperatures. Lett. Appl. Microbiol. 31:385–389.
15. Gramann, P. E. 1997. Bacillus cereus. Detection of toxins, p. 327–336. In R. K. Robinson, C. A. Batt, and P. D. Patel (ed.), Encyclopedia of food microbiology, vol. 1. Academic Press, San Diego, Calif.
16. Helgason, E. D., D. A. Caugant, I. Olsen, and A.-B. Kolstø. 2000. Genetic structure of population of Bacillus cereus and B. thuringiensis isolates associated with periodontitis and other human infections. J. Clin. Microbiol. 38:1615–1622.
17. Isohe, M., T. Ishikawa, S. Suwan, N. Agata, and M. Ohta. 1995. Synthesis and activity of cereulide, a cyclic depsipeptide ionophore as emetic toxin from Bacillus cereus. Bioorg. Med. Chem. Lett. 5:285–288.
18. Kotiranta, A., K. Lounatmaa, and M. Haapaniemi. 2000. Epidemiology and pathogenesis of Bacillus cereus infection. Microb. Infect. 2:189–198.
19. Livsmedelsverket. 1998. Vägledning för mikrobio logisk bedömning av livs medel. Livsmedelsverket, Uppsala, Sweden.
20. McKillop, J. L. 2000. Prevalence and expression of enterotoxins in Bacillus cereus and other Bacillus spp.: a literature review. Antonie Leeuwenhoek 77:393–399.
21. Mikami, T., T. Horikawa, T. Murakami, T. Matsumoto, A. Yamakawa, S. Murooka, S. Katagiri, K. Shinagawa, and M. Suzuki. 1994. An improved method for detecting cytotoxic protein (emetic toxin) of Bacillus cereus and its application to food samples. FEMS Microbiol. Lett. 119:53–58.
22. Mikkola, R., N.-E. L. Saris, P. A. Grigoriev, M. A. Andersson, and M. Salkinoja-Salonen. 1999. Ionophoretic properties and mitochondrial effects of cereulide, the emetic toxin of Bacillus cereus. Eur. J. Biochem. 263:112–117.
23. Notermans, S., J. Dufrenne, P. Teunis, R. Beumer, M. te Giffel, and P. P. Weem. 1997. A risk assessment study of Bacillus cereus present in pasteurised liquid milk. Food Microbiol. 14:239–245.
24. Notermans, S., and C. A. Batt. 1998. A risk assessment approach for foodborne Bacillus cereus and its toxins. J. Appl. Microbiol. Suppl. 84:615–618.
25. Pirttiläjärvi, T. 2000. Contaminant aerobic spore-forming bacteria in the manufacturing processes of food packaging and food. Ph.D. thesis. University of Helsinki, Helsinki, Finland.
26. Pirttiläjärvi, T. S. M., M. A. Andersson, A. C. Scoping, and M. S. Salkinoja-Salonen. 1999. Evaluation of methods for recognizing strains of the Bacillus cereus group with food poisoning potential among industrial and environmental contaminants. Syst. Appl. Microbiol. 22:133–144.
27. Rowan, N. J., J. G. Anderson, and A. Anderton. 1997. The bacteriological quality of hospital-prepared infant feeds. J. Hosp. Infect. 36:259–267.
28. Sakurai, N., K. A. Koike, Y. Irie, and H. Hayashi. 1994. The rice culture filtrate of Bacillus cereus isolated from emetic-type food poisoning causes mitochondrial swelling in Hep-2 cell. Microbiol. Immunol. 38:337–343.
29. Setlow, P., and E. A. Johnson. 1997. Spores and their significance, p. 30–64. In M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.
30. Shinagawa, K., H. Konuma, H. Sekita, and S. Sugí. 1995. Emesis of rhesus monkeys is induced by oral administration with the Hep-2 vacuolation factor, cereulide, produced by Bacillus cereus. FEMS Microbiol. Lett. 130:87–90.
31. te Giffel, M. C., R. R. Beumer, S. Leijendekkers, and F. M. Rombouts. 1996. Incidence of Bacillus cereus and Bacillus subtilis in foods in The Netherlands. Food Microbiol. 13:531–535.
32. Turnbull, P. B. C., J. M. Kramer, K. Jorgensen, R. J. Gilbert, and J. Melling. 1979. Properties and production characteristics of vomiting, diarrheal and necrotizing toxins of Bacillus cereus. Am. J. Clin. Nutr. 32:219–228.