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A Novel Bicomponent Hemolysin from *Bacillus cereus*†

DOUGLAS J. BEECHER AND JAMES D. MACMILLAN*

Department of Biochemistry and Microbiology, New Jersey Agricultural Experiment Station, Cook College, Rutgers University, New Brunswick, New Jersey 08903

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A procedure combining isoelectric focusing (Sephadex IEF) and fast protein liquid chromatography (Superose 12; Mono-Q) removed hemolytic activity (presumably a contaminant) from partially purified preparations of the multicomponent diarrheal enterotoxin produced by *Bacillus cereus*. However, when the separated fractions were recombined, hemolytic activity was restored, suggesting that hemolysis is a property of the enterotoxin components. Combined fractions exhibited a unique ring pattern in gel diffusion assays in blood agar. During diffusion of the hemolysin from an agar well, the erythrocytes closest to the well were not lysed initially. After diffusion, hemolysis was observed as a sharp ring beginning several millimeters away from the edge of the well. With time the cells closer to the well were also lysed. This novel hemolysin consists of a protein (component B) which binds to or alters cells, allowing subsequent lysis by a second protein (component L). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isoelectric focusing, and Western blot analysis showed that hemolysin BL has properties similar to those described previously for the enterotoxin and that both components are distinct from cereolysin and cereolysin AB.

Bacillus cereus is a widely distributed bacterium that is commonly found in soil and has been isolated from a variety of foods, including rice, spices, meat, eggs, and dairy products (42), and from drugs, including both topical and oral pharmaceutical products (1). This organism is well established as the cause of two distinct types of food poisoning, known as the diarrheal and emetic syndromes, and a variety of nonenteric illnesses ranging from wound infections to panopthalmitis (38). Evidence suggests that the diarrheal syndrome is caused by an enterotoxin (31, 37, 39, 41) consisting of two, or possibly three, components (34). These components have to be combined to exhibit positive ileal loop, vascular permeability, and cytotoxic activities.

B. cereus reportedly produces two distinct hemolysins (8, 11, 29), but only the thiol-activated cereolysin has been purified and well characterized (3). Gilmore et al. (14) demonstrated that the phosphatidylcholine-preferring phospholipase C and the sphingomyelinase of *B. cereus* act synergistically to lyse erythrocytes. These authors designated the duplex cereolysin AB and suggested that it is responsible for the secondary hemolytic activity mentioned above.

The question of whether the diarrheal toxin is hemolytic remains open. No one has yet demonstrated enterotoxic activity devoid of hemolytic activity, but Turnbull et al. (41) detected separate peaks of hemolytic activity (distinct from cereolysin) and enterotoxic activity (loop-fluid-inducing factor). Since then, hemolytic activity associated with enterotoxin has been considered a residual contaminant (35), although Turnbull and co-workers (38, 40) pointed out that it is puzzling that such a toxin could be highly necrotic without also exhibiting hemolytic activity.

The original aim of our research was to completely separate the components of the enterotoxin, eliminate presumably contaminating hemolytic activity, and develop monoclonal antibodies for use in enterotoxin detection. Early in these studies, monoclonal antibodies were prepared by using partially purified (hemolytic) components of enterotoxin as immunogens, and we selected two antibodies that reacted with the major proteins in these preparations. Hemolytic activity was lost during purification of these major components from culture broth. However, when these two fractions were recombined, hemolytic activity was restored, suggesting that hemolysis was caused by enterotoxin components. This hemolytic activity exhibited some unique properties when it was assayed by gel diffusion in blood agar and is the focus of this report. When the fractions were combined in a well and allowed to diffuse out into the blood agar, cells close to the well were not lysed initially. After continued diffusion, hemolysis was observed as a sharp, clear, ring-shaped zone which began several millimeters away from the edge of the well. The biochemical mechanism involved in ring formation is not known. However, further study showed that the hemolysin is distinct from cereolysin AB and may consist of a binding protein (component B) and a lytic protein (component L).

MATERIALS AND METHODS

Toxins and antibodies. Partially purified preparations containing enterotoxin components previously designated 575, 577, and 580 (35) and rabbit polyclonal antisera which reacted to proteins in the toxin preparations were obtained from R. Bennett (Food and Drug Administration, Washington, D.C.). Electrophoretic and Western blot analyses (silver staining and rabbit antisera) showed that the toxin preparations contained two major proteins having molecular masses of 34 to 36 and 43 to 45 kilodaltons (kDa) along with a number of minor contaminants.

Production of monoclonal antibodies. Hybridomas capable of producing monoclonal antibodies were made by using toxin preparations 575, 577, and 580 (see above) as immunogens. Mice were injected intraperitoneally with 100 μg of protein in complete Freund adjuvant and boosted after 4 weeks with the same amount of protein in incomplete Freund adjuvant. The final boosting schedule was as described by Stähli et al. (32). For hybridoma production, cells were fused by using the method of Siriganian et al. (28), except that the spleen cell suspension was prepared as

* Corresponding author.

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described by Galfré and Milstein (12) and erythrocytes were lysed with 0.17 M NH_4Cl to facilitate counting of lymphocytes (19). Hybridomas capable of producing monoclonal antibodies were detected by using enzyme-linked immunosorbent assays, and antibody specificity was determined by using Western blots. Hybridomas were cloned twice by using the limiting dilution technique (7).

Antibodies from two hybridoma strains were used for antigen detection. Strain M1 was prepared by fusing myeloma cells (strain P3-X63-Ag8.653; Human Genetic Mutant Cell Repository, Camden, N.J.) with lymphocytes from an immunized BALB/c mouse (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Strain M2 was the product of a fusion between myeloma cells (strain FOX-NY; licensed from Hyclone Laboratories, Logan, Utah) and spleen cells from mouse strain RBF/Dn (Jackson Laboratory, Bar Harbor, Maine) (34).

Production of hemolysin. *B. cereus* F837/76, an isolate from a surgical wound infection, was obtained from J. M. Kramer, Public Health Laboratory Services, London, England. This strain is a high producer of enterotoxin (41), and it produces relatively large amounts of antigens that are reactive with antibodies M1 and M2.

For production of hemolysin, cells were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% glucose. One-liter cultures in 4-liter flasks were inoculated (0.5%, vol/vol) with an 18-h (stationary-phase) broth culture, and the preparations were incubated at 37°C on a rotary shaker at 200 rpm for 8 h. Cells were removed by centrifugation at 10,000 × *g* for 10 min, and proteins were concentrated by precipitation with 60% saturated ammonium sulfate at 4°C. The resulting concentrates were dialyzed overnight at 4°C against 20 mM Tris hydrochloride (pH 7.4) and were the source of toxin for additional purification studies.

Methods used for protein purification. The procedures of Radola (27) were used for preparative flat-bed isoelectric focusing (IEF) with 1-mm-thick gels of Sephadex IEF (Pharmacia Fine Chemicals, Piscataway, N.J.). The pH gradients were determined with a surface electrode (Ingold Electrodes Inc., Andover, Mass.). After IEF, the entire gel was cut into 0.5-cm strips that were cut perpendicularly to the direction of migration. Proteins were eluted from the strips with 1.5 volumes of distilled water; the Sephadex gel was removed by filtration through glass wool.

Gel filtration fast protein liquid chromatography (FPLC) was performed with a Superose-12 column (type HR10/30; Pharmacia) as recommended by the manufacturer. Samples (0.5 ml) were applied to the column, and proteins were eluted with 50 mM Tris hydrochloride (pH 7.4) at a flow rate of 1.0 ml/min.

The methods used for anion-exchange FPLC (Mono-Q column, type HR5/5; Pharmacia) were the methods described by the manufacturer. The proteins were applied to the column in 50 mM Tris hydrochloride (pH 7.4) and were eluted with a linear salt gradient (0 to 1 M, 30 ml).

Assays for hemolytic activity. Defibrinated sheep blood (BBL Microbiology Systems, Cockeysville, Md.), calf blood (BBL), horse blood (BBL), and rabbit blood (BBL) were purchased from Fisher Scientific Co., Springfield, N.J. Mouse blood was obtained by retroocular bleeding of C57BL mice (Jackson Laboratory), and human blood was obtained by finger puncture. Blood drawn directly was defibrinated by shaking the sample with glass beads (30).

A radial diffusion assay was used to monitor hemolytic activity. Blood agar consisting of washed, defibrinated sheep erythrocytes (5%, vol/vol), purified agar (1%, wt/vol), and Tris-buffered saline (pH 7.4) (50 mM Tris in 150 mM NaCl) or Alsever solution (pH 6.8) was prepared and poured onto glass slides or Gel-Bond film (FMC BioProducts, Rockland, Maine) to a depth of 1 mm (0.1 ml/cm^2). Wells (diameter, 3 mm) were punched into the gel, and samples (3 to 6 μl) were added. The blood agar gels were maintained at 37°C in a humidified chamber, and hemolysis was evident after 1 to 3 h. In some experiments the amounts of hemolytic activity were compared on the basis of the radius of the hemolytic zone (in millimeters) minus the radius of the well (1.5 mm).

Spectrophotometric assays for hemolysis were performed as described by Bernheimer (2), with the following modifications. Samples (2 to 3 μl) were added to 20-μl portions of 5% (vol/vol) erythrocyte suspensions in Tris-buffered saline (pH 7.4) in 1.5-ml Microfuge tubes. The suspensions were kept at 37°C for 30 or 60 min and then centrifuged at 13,000 × *g* for 15 to 20 s. Twenty microliters of each supernatant was removed and added to 100 μl of Tris-buffered saline in a 96-well microdilution plate. The percentage of hemolysis was calculated by dividing the A_{490} of the sample by the A_{490} of a control suspension of erythrocytes which had been lysed completely by treatment with saponin (2).

Analytical methods. Lecithinase activity was detected on agar gels containing 2% (vol/vol) egg yolk, 50 mM Tris hydrochloride, and 150 mM NaCl or on gels containing 1% (wt/vol) crude phosphatidylcholine (Sigma Chemical Co., St. Louis, Mo.), 50 mM phosphate buffer, and 150 mM NaCl. Test samples were added to wells in the agar as described above for the hemolysin assay. Slides were kept at 37°C in a humidified chamber, and after 12 to 18 h lecithinase activity was recorded as the radius (in millimeters) of the opaque zone minus the radius of the well (1.5 mm).

Phosphatidylinositol-specific phospholipase C activity was determined by a gel diffusion assay (21), using a gel containing 1% crude phosphatidylinositiol (Sigma) in phosphate-buffered saline (pH 7.4).

Sphingomyelinase activity was measured by using a modification of the colorometric method of Gatt et al. (13). Each reaction mixture consisted of 25 μl of a solution containing 30 mM trinitrophenylaminolauryl (TNPAL)-sphingomyelin (Sigma) and 5 mM $MgCl_2$ and a 5-μl sample brought to a volume of 50 μl with Tris-buffered saline (pH 7.4). After 6 h at 37°C, the reaction was stopped by adding 0.188 ml of 2-propanol–heptane–5 M H_2SO_4 (40:10:1).

The colored reaction product was extracted by adding 0.1 ml of water and 0.113 ml of *n*-heptane. The A_{340} of the heptane fraction (100 μl) was measured with a microplate reader. Absorbance values for samples were compared with values for culture supernatant and buffer controls.

Dot blot assays were performed as described by Tijssen (36). One-microliter samples were dotted onto nitrocellulose paper (Millipore Corp., Bedford, Mass.) and probed with monoclonal antibodies M1 and M2. The secondary antibody was goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). The peroxidase substrates were 4-chloro-1-napthol and hydrogen peroxide. Reactivity was recorded on an arbitrary scale of 1 to 5, with the darkest reactions recorded as 5.

Analytical agarose IEF was performed in gels consisting of 0.8% (wt/vol) agarose IEF (Pharmacia), 12% (wt/vol) sorbitol, and 2% (vol/vol) Pharmalytes (Pharmacia) in the pH range from 4 to 9 (1:1 mixture of Pharmalytes pH 4-6.5 and 6.5-9). The gels were 1 to 1.5 mm thick and backed with Gel-Bond film (FMC). The anolyte was 2.6% (vol/vol) Phar-

malyte pH 4-6.5 in cellulose electrode wicks (FMC), and the catholyte was 2.6% (vol/vol) Pharmalyte pH 6.5-9.

Zymograms were used for locating hemolytic and lecithinase activities on IEF gels. Zymograms for hemolytic activity were composed of blood agar prepared as described above for the radial diffusion assay. Lecithinase activity was detected by the development of turbidity on egg yolk gels prepared as described above. After agarose IEF gels were run, the pH gradient was determined, and the gel and Gel-Bond backing were cut lengthwise into strips. The strips were placed onto the surface of an appropriate zymogram gel and kept in a humid chamber at 37°C until reactions were visible.

For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) the buffer system described by Laemmli (20) was used in a slab gel apparatus. Proteins were visualized by using the silver staining method of Heukeshoven and Dernick (16). Proteins on SDS-PAGE gels were electroblotted onto membranes of polyvinylidine difluoride (Immobilon; Millipore). Specific antigens were located on these Western blots by using either rabbit polyclonal immunoglobulin G or murine monoclonal immunoglobulin G as the primary antibody. The secondary antibodies and substrate were the same as those described above for the dot blot assays except that goat anti-rabbit immunoglobulin G conjugated to horse radish peroxidase (Kirkegaard and Perry) was used as the second antibody when blots were probed with rabbit antiserum.

RESULTS

Numerous hybridomas were produced by using enterotoxin preparations obtained from the Food and Drug Administration as immunogens, and monoclonal antibodies were screened by using enzyme-linked immunosorbent assays and Western blots for reactivity with the major proteins present in these preparations (data not shown). Two monoclonal antibodies were selected for use in the studies described below. SDS-PAGE and Western blot analysis showed that antibody M1 bound strongly to a protein having a molecular mass of ca. 45 kDa (designated Ag-1) and cross-reacted with a protein having a molecular mass of ca. 36 kDa. Antibody M2 bound to a protein having a molecular mass of ca. 36 kDa (designated Ag-2) and cross-reacted slightly with a protein having a molecular mass of ca. 45 kDa.

We found that rabbit antisera made to these immunogens reacted with more than 20 proteins present in *B. cereus* F837/76 culture supernatants. Thus, the preparations which were used as immunogens for monoclonal antibody production were quite heterogeneous.

In an attempt to purify antigens Ag-1 and Ag-2 to determine whether these antigens had biological activity, we discovered a hemolysin that exhibited some unique properties. Dot blot assays showed that the concentrations of antigens Ag-1 and Ag-2 both peaked in preparative IEF fractions collected in the pH range from 4.8 to 5.0. These fractions also contained lecithinase activity and hemolytic activity. IEF fractions tested in wells in blood agar plates exhibited two markedly different patterns of hemolysis surrounding the wells (Fig. 1). Fractions at pH values above 5.5 produced a typical disk-shaped pattern which began immediately at the edge of the well and proceeded outward. However, fractions containing the two antigens formed an unusual ring-shaped zone of hemolysis (i.e., hemolysis started initially some distance from the well, resulting in a sharp circular band of clearing). Intermediate fractions exhibited a combination of the two patterns (Fig. 1). IEF fractions containing the two antigens were pooled and subjected to gel filtration FPLC (Fig. 2). The two antigens eluted close to one another, and the hemolytic activity (ring pattern) eluted concurrently with antigen Ag-2 (Fig. 2). Fractions containing the antigens and hemolytic activity but not lecithinase activity were pooled and subjected to anion-exchange FPLC (Fig. 3). This resulted in good separation of the antigens, but again the hemolytic activity eluted with antigen Ag-2. However, the total amount of hemolytic activity was much less than expected, based on the amount that had been applied to the column.

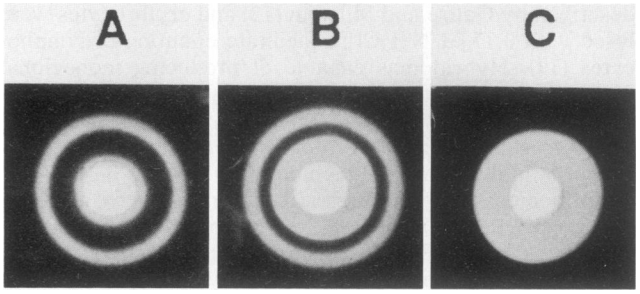

FIG. 1. Patterns of hemolysis produced by preparative IEF fractions analyzed by using the blood agar diffusion assay. The pI values of the fractions tested were 5.0 (A), 5.5 (B), and 6.0 (C).

Since the components of the diarrheal toxin are inactive separately (35) and the possibility that the toxin is hemolytic remains open (38), we recombined FPLC fractions to determine whether hemolytic activity could be restored. Figure 4 shows that fractions 50 and 54 from the Mono-Q column (Fig. 3), containing antigens Ag-1 and Ag-2, respectively,

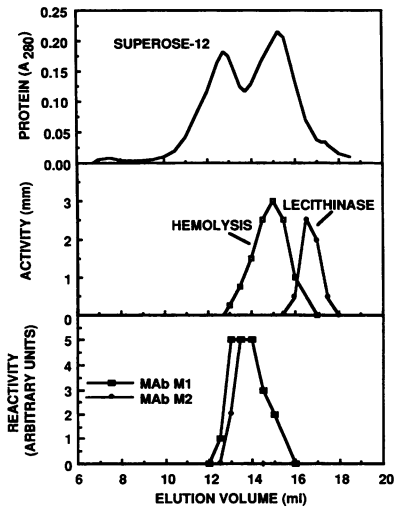

FIG. 2. Gel filtration FPLC elution profile. Proteins from preparative IEF fractions, collected in the pH range from 4.8 to 5.1, were pooled, applied to a Superose-12 column, and eluted as described in the text. Samples (6 μl) from eluted fractions were assayed for hemolytic and lecithinase activities by using the gel diffusion procedures. Activities were recorded as the radius (in millimeters) of the clear zone (hemolysis) or the opaque zone (lecithinase) minus 1.5 mm (the radius of the well in the diffusion gel). Dot blots of fractions were probed with monoclonal antibodies M1 and M2, and relative reactivity was estimated on a scale of 1 to 5, with 5 being the darkest reaction observed. MAb, Monoclonal antibody.

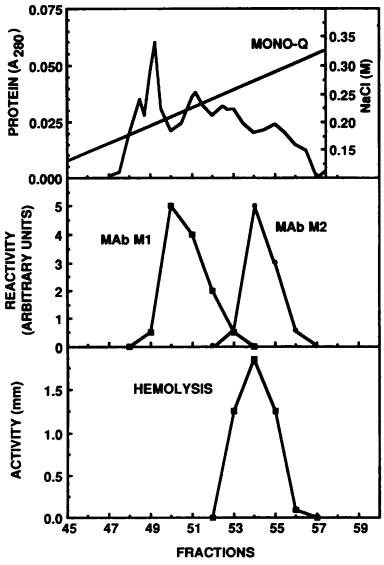

FIG. 3. Ion-exchange FPLC elution profile. Fractions from gel filtration FPLC (Fig. 2) containing antigen Ag-1, antigen Ag-2, and hemolytic activity (but not lecithinase activity) were pooled, applied to a Mono-Q column, and eluted with a salt gradient as described in the text. Hemolytic activity and reactivity with monoclonal antibodies were tested as described in the legend to Fig. 2. MAb, Monoclonal antibody.

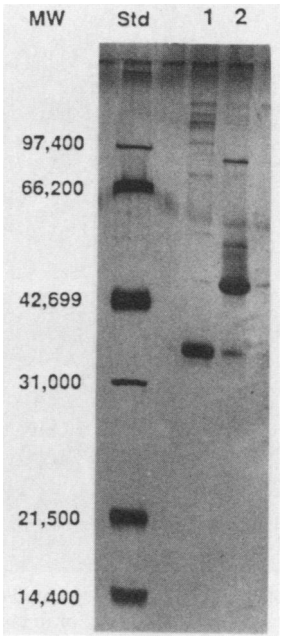

FIG. 5. SDS-PAGE of Mono-Q fractions Ag-1 (lane 2) and Ag-2 (lane 1). Bands were visualized with silver stain and compared with low-molecular-weight standards (Std) (Bio-Rad Laboratories, Richmond, Calif.). MW, Molecular weight.

had little or no hemolytic activity on blood agar alone, but when they were recombined, activity was greatly enhanced. Fractions 48 to 52 were pooled and applied to a Mono-Q column, and the proteins were eluted with a shallower gradient (50 to 300 mM, 25 ml). A pool representing fractions 53 to 56 was treated similarly. This improved the separation, and fractions containing only one or the other of the antigens were now completely devoid of hemolytic activity, except after recombination.

The results described above indicated that at least two components are required for activity of this hemolysin. Two Mono-Q fractions, each containing only one or the other of these components and either antigen Ag-1 or antigen Ag-2, were examined in subsequent experiments to further explore this possibility. Figure 5 shows that these two fractions (designated fractions Ag-1 and Ag-2) exhibited considerably different patterns when they were subjected to SDS-PAGE. Major bands were detected at 36 kDa (fraction Ag-2) and 45 kDa (fraction Ag-1); these values are similar to the molecular mass values (38 and 43 kDa) described previously for components of the enterotoxin (35).

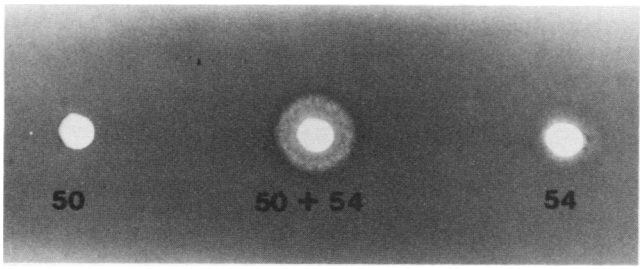

FIG. 4. Restoration of hemolytic activity by recombining Mono-Q fractions 50 and 54 (Fig. 3). The center well contained 3 μl of each fraction, and the end wells contained 3-μl portions of the fractions indicated and 3 μl of Tris-buffered saline.

Multicomponent toxins often have moieties which recognize cell surface receptors and either allow other components to bind to cells or mediate their entry into cells (33). The other components cannot act without prior attachment of the binding moiety to the cells. The experiment described below was performed to determine whether either fraction Ag-1 or fraction Ag-2 contained a component that might act as a binding moiety essential for the subsequent hemolytic activity of the other fraction. Two microliters of fraction Ag-1 was added to 10 μl of sheep erythrocytes (5%, vol/vol), and after 15 min at room temperature the volume was brought to 200 μl with Tris-buffered saline (pH 7.4). After another 15 min, the cells were washed three times with 1 ml of the buffer and suspended to a volume of 200 μl. Concurrently, a second erythrocyte suspension containing fraction Ag-2 was treated as described above. Each of the two suspensions then received 2 μl of the other Mono-Q fraction (i.e., fraction Ag-2 was added to cells previously sensitized with fraction Ag-1 and vice versa). After 30 min the extent of hemolysis was determined by spectrophotometrically measuring hemoglobin release. Erythrocytes treated first with fraction Ag-2 were lysed after fraction Ag-1 was added (A_{405}, 0.22) even though they had been washed thoroughly between additions. Cells treated first with fraction Ag-1 did not lyse when fraction Ag-2 was added (A_{405}, 0.04). For fractions Ag-1 and Ag-2 assayed individually, the A_{405} values were 0.03 and 0.04, respectively. This showed that fraction Ag-2 may have contained a binding moiety and fraction Ag-1 may have contained a component that was necessary for subsequent cell lysis. On this assumption we designated the active components in fractions Ag-2 and Ag-1 component B (binding) and component L (lysis), respectively.

Fraction Ag-2 (component B) and fraction Ag-1 (component L) were added separately to adjacent wells in a blood agar plate and together in a third well. The zones of

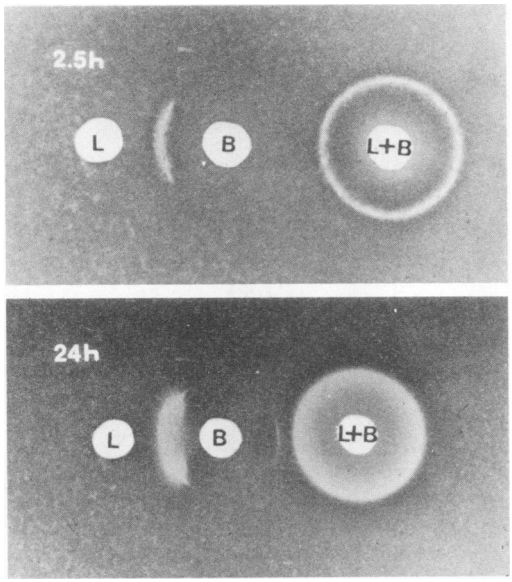

FIG. 6. Patterns of hemolysis when separated components of the hemolysin were tested either individually or in combination in wells in blood agar plates. Wells L contained 3 μl of fraction Ag-1 plus 3 μl of Tris-buffered saline. Wells B contained 3 μl of fraction Ag-2 plus 3 μl of Tris-buffered saline. Wells L+B contained 3 μl of each fraction.

hemolysis resulting after 2.5 and 24 h are shown in Fig. 6. Several things are apparent from Fig. 6. Components B and L were nonhemolytic individually, and hemolysis occurred only after the two components diffused into each other. The area of hemolysis between the wells containing components B and L was crescent shaped, with the concave side toward component B. When components B and L diffused from the same well, the pattern of hemolysis was a ring of lysed cells surrounding an area of nonlysed cells. Lysis began at a point several millimeters from the edge of the well, after the hemolysin components had diffused for about 2 h. After this time, lysis proceeded from the ring inward toward the well. By 24 h, hemolysis was complete to the edge of the well; however, from 2 to 24 h there was only a slight increase in the overall diameter of the outside edge of the ring.

We designed an experiment to evaluate the role of each fraction in determining the shape and extent of the zone of hemolysis. Dilutions of fraction Ag-2 (component B) and fraction Ag-1 (component L) were added to wells and troughs cut in blood agar (Fig. 7). The gel was kept at 37°C for 3.5 h before the following observations were made: (i) the zones of hemolysis between the component L wells and the component B trough were straight, while the zones of hemolysis between the component B wells and the component L trough were crescent shaped, suggesting that the shape of the zone of hemolysis was controlled by diffusion of component B; (ii) the location of the hemolysis zone between the wells and the troughs (i.e., the distance of the zone from a well) varied with the concentration of component B, but not with the concentration of component L, and the zone between the troughs was parallel to the component B trough; (iii) the hemolysis zone at the ends where the two troughs converged circumscribed only the component B trough end, where component L, because of distance, was at a relatively low concentration, suggesting again that the concentration of component B determined the location of the zone and the diameter of the ring as shown in Fig. 6; and (iv) hemolysis

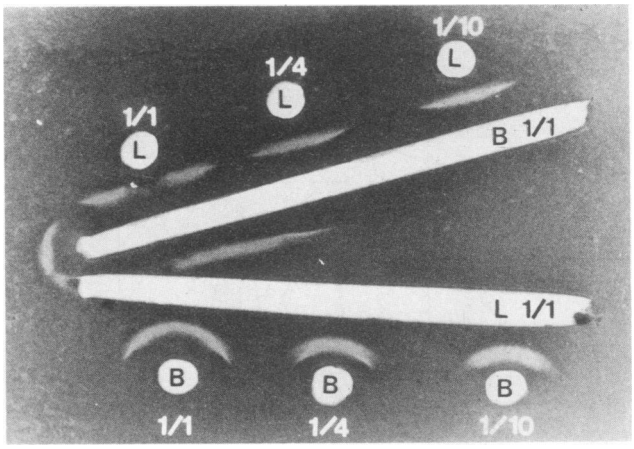

FIG. 7. Patterns of hemolysis when separated components of the hemolysin were tested at different concentrations in wells and troughs in a blood agar plate. The dilutions used were 1/1, 1/4, and 1/10 for fraction Ag-1 (component L) and fraction Ag-2 (component B). Six microliters of each dilution was added to wells, and 24 μl was added to troughs.

occurred up to the edge of the component L trough but only at a fixed distance from the component B trough, indicating that the central area of nonlysed cells within the ring (Fig. 6) was a function of component B or some other component also present in fraction Ag-2.

Next, erythrocytes from a number of different mammalian species were subjected to the combined action of components B and L for 30 min, and the results (Fig. 8) showed that there were considerable differences in the susceptibility of these erythrocytes to hemolysis. Sheep and calf erythrocytes were the most sensitive, while human and horse erythrocytes were the least sensitive. The suspensions of human and horse erythrocytes were incubated for an additional 24 h without further lysis, and we concluded that the hemoglobin release observed at 30 min was due to background and that these two types of erythrocytes were not susceptible to hemolysis by components B and L at the concentrations tested.

Similar results were found in another experiment in which the gel diffusion assay was used to test erythrocyte lysis.

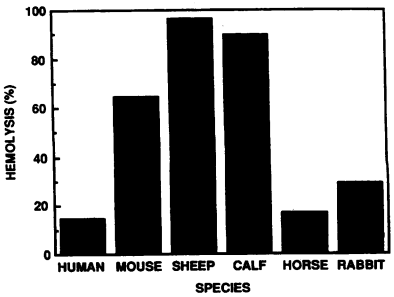

FIG. 8. Species differences in sensitivity of erythrocytes to hemolysin BL. Erythrocytes were washed with Tris-buffered saline until they were free of hemoglobin, and suspensions were adjusted to an A_{490} that was equal to the A_{490} of a 5% sheep erythrocyte suspension. Fractions Ag-1 and Ag-2 (2 μl of each per 20 μl of cell suspension) were added. After 30 min at 37°C cells were removed by centrifugation, and hemoglobin release was determined spectrophotometrically. Percentages of hemolysis were based on 100% lysis of controls consisting of the same types of erythrocytes.

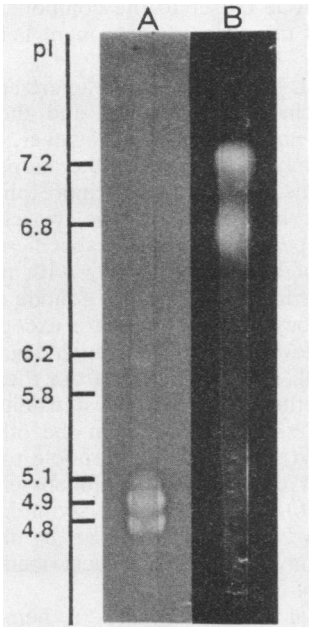

FIG. 9. Separation of hemolysins and lecithinase by IEF of a culture supernatant from *B. cereus*. After the proteins were electrofocused, the gel was cut into strips, and the strips were applied to gels containing sheep erythrocytes (lane A) and egg yolk (lane B) for identification of hemolytic and lecithinase activities. See Materials and Methods for details concerning IEF and the activity assays.

Hemolysin BL was inactive against human, horse, and rabbit erythrocytes and was active against sheep, calf, and mouse erythrocytes. The ring pattern was evident for sheep and calf blood but not for mouse blood, which exhibited a typical disk-shaped pattern. However, hemolysis of the mouse cells was not complete as the zone remained partially turbid, indicating that some of the cells had not been affected.

Figure 8 shows that erythrocytes from mice (64% hemolysis after 30 min) were less sensitive to hemolysin BL than sheep or bovine erythrocytes (96 and 90% hemolysis, respectively, after 30 min). However, we found that hemolysis of mouse erythrocytes initially seemed more rapid than hemolysis of sheep erythrocytes. In kinetic studies in which hemoglobin release was measured we found that the sheep erythrocytes were completely lysed in 20 min. However, hemolysis of the mouse erythrocytes began more rapidly and reached a plateau at 64% hemolysis. Ponder (25) indicated that hemolysin is an all-or-none phenomenon and that once hemoglobin begins to leave cells, it continues to leave rapidly until essentially all of it has been released. This suggests that in the experiment described above 36% of the mouse erythrocytes were not lysed.

The hypothesis that hemolysin BL differs from cereolysin AB is supported by the data in Fig. 9, which shows zymograms of Agarose IEF gels prepared with culture supernatant from *B. cereus* F837/76. Three bands of hemolytic activity were focused at pI values of 4.8, 4.9, and 5.2. These bands corresponded to hemolysin BL; components of this hemolysin have pI values in this range (see above). In addition, monoclonal antibodies M1 and M2 reacted with proteins which focused in the pI range from 4.8 to 5.0.

Lecithinase activity was well separated from the three bands representing hemolysin BL and was focused at pI values ranging from 6.0 to 7.7, with two major bands at pI 6.8 and 7.25. Sufficient amounts of the original fractions Ag-1 and Ag-2 were not available for direct testing of lecithinase activity. However, similarly prepared fractions containing component B or L were assayed directly, and no lecithinase activity was detected when samples were added to wells in a gel containing crude phosphatidylcholine as the substrate.

These same fractions were also assayed for sphingomyelinase activity. Some activity was detected in both component B and component L fractions after 6 h at 37°C. Heptane extracts of the two reaction mixtures containing component B or component L both had A_{340} values of about 0.17, compared with A_{340} values of 0.28 for a positive control (*B. cereus* culture supernatant) and 0.09 for a negative control (buffer). Phosphatidylinositol-specific phospholipase C was detected in concentrated (ammonium sulfate) crude culture broth from *B. cereus* F837/76, but no phospholipase C was detected in the fractions containing components B and L.

DISCUSSION

We isolated a hemolysin composed of at least two components (components B and L) which exhibit unique patterns of cell lysis in a gel diffusion assay. Hemolysin BL cannot be the same as the cereolysin AB described by Gilmore et al. (14), as components B and L separated on a Mono-Q column did not exhibit lecithinase activity. It seems likely that the lecithinase activity detected in fractions of components B and L from the preparative IEF gel was a residual activity from the culture broth. Figure 9 shows that the lecithinase activity produced by the *B. cereus* strain which we used was well separated from the activity of hemolysin BL by IEF.

Although there has been some dispute concerning the pI value of the phosphatidylcholine-preferring phospholipase C (38), our data agree with those of Ikezawa et al. (17), who determined that there are two isoelectric species of this enzyme (pI 6.8 and 7.5), and Bjorklid and Little (6), who reported a pI value of 6.8 for the native form. Furthermore, at the concentrations tested, hemolysin BL is not active on human erythrocytes whereas cereolysin AB is (14).

Only limited amounts of purified components B and L were available for the assay for sphingomyelinase activity, requiring the use of a modified assay procedure which was only qualitative at best. On the basis of A_{340} values obtained with a microplate reader, apparently identical amounts of sphingomyelinase activity were present in fractions containing either component B or component L. This is inconclusive evidence that these two components are both sphingomyelinases, and we do not believe that they are. Sphingomyelinase from *B. cereus* has a pI value of 5.6 (17), and thus if this enzyme is a part of hemolysin BL, lysis should have occurred at a pI value higher than 5.0, where it was detected (Fig. 9). Also, sphingomyelinase itself is hemolytic for sheep erythrocytes (18), and no hemolytic activity was detected in separate fractions of components B and L. However, this does not rule out the possibility that components B and L are sphingomyelinases which are not hemolytic individually.

The three hemolytic bands between pI 4.8 and 5.2 (Fig. 9) were probably not due to other known phospholipases of *B. cereus*, as the lowest reported pI value is 5.4 for the phosphatidylinositol-hydrolyzing phospholipase C (17). However, a pI of 5.15 has been reported (21) for the phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis*, and this enzyme has a high level of nucleotide sequence homology with the enzyme produced by *B. cereus*.

This enzyme was not detected in fractions containing separated components B and L.

The three bands of hemolysis at about pI 5.0 in Fig. 9 cannot represent cereolysin, at least not as described by Cowell (9), who found that this hemolysin from *B. cereus* has a pI of 6.8. Also, cereolysin is a single protein which is inhibited by cholesterol (3); hemolysin BL is at least two proteins and is not inhibited by cholesterol (data not shown). Hemolysin BL may be the same as the secondary hemolysin described by Slein and Logan (29), which was highly active against sheep erythrocytes but only weakly active against rabbit erythrocytes.

Pendleton et al. (23) purified and characterized the following two hemolysins from *B. thuringiensis*: thuringiolysin (analogous to cereolysin) and a secondary hemolysin. These authors found that the secondary hemolysin was most active against sheep erythrocytes, had intermediate activity against rabbit erythrocytes, and was nearly inactive against horse erythrocytes. These results agree with those found for hemolysin BL. The production of similar or identical hemolysins by *B. cereus* and *B. thuringiensis* is not unexpected since these bacteria are closely related and it has been proposed that they are actually members of the same species (15, 26).

The patterns of hemolysis produced by hemolysin BL are unique, and their shape and extent appear to be controlled primarily by component B. We cannot yet propose a precise mechanism for the action of hemolysin BL, although we are considering the two models described below which might explain the early formation of the ring-shaped hemolytic zone and the subsequent lysis of the cells in the area between the ring and the well. (i) The concentration of component B is critical. At high concentrations near a well, component B may saturate the membrane and prevent the appropriate association of component L, which can lead to cell lysis (i.e., high concentrations of component B inhibit component L from causing a lytic reaction). Farther from the well, where the concentration of component B is lower, component L can act to form the ring. With time, continued diffusion causes the concentration of component B to decrease to levels that allow lysis to occur near the well. (ii) There is another molecule, as yet undetected in our preparations, that inhibits or competes with component B for binding sites. When the competitor is bound to receptors on the erythrocyte surface, the component B-component L assemblage is prevented from forming, and lysis is inhibited. The inhibitor may have a higher affinity for binding to cells than component B and may effectively block component B binding to cells near the well. At some distance from the well, the concentration of the inhibitor is lower, allowing component B to bind and component L to lyse the cells, forming a ring-shaped zone of hemolysis. With time, component B may slowly displace the competitor, allowing an accumulation of the component B-component L assemblage and subsequent lysis of cells in the area between the ring and the well.

Another possibility is that a specific ratio of component B to component L is required for hemolysis. This does not seem likely, as we would then expect the zone of hemolysis formed between adjacent component L- and component B-charged wells to be shaped symmetrically about a line perpendicular to the line between the centers of the two wells (i.e., a pattern similar to precipitin lines in immunodiffusion assays). We observe instead a crescent or arc shape centered about the component B well. Furthermore, increasing the concentration of component L does not cause formation of the zone closer to the component B well as we would expect if the ratio of component L to component B were involved.

Hemolysin BL has not been characterized well enough to propose a mechanism of action, and the nature of the components is not yet known. However, as reviewed by Linder (22), hemolysis caused by the cooperative action of bacterial proteins, usually one or more phospholipases, is not uncommon. In most cases, the two components arise from different species and act in a specific sequence.

Correlation of hemolytic activity with membrane phospholipid content may point toward a mode of action. Sheep and calf erythrocyte membranes are exceptionally high in sphingomyelin levels (10), and the high degree of sensitivity to hemolysin BL of erythrocytes from these species compared with the other erythrocytes tested (Fig. 8) suggests the involvement of sphingomyelin. On the other hand, while rabbit erythrocytes are more susceptible to lysis by hemolysin BL, they contain less sphingomyelin than human erythrocytes (10). A sequential enzymatic attack of cell membranes may be involved, possibly by phospholipases of *B. cereus* that have not yet been described or by a nonenzymatic mode of action.

Staphylococcal gamma-toxin is a hemolysin with no known enzymatic activity or other mechanism of action (24). This hemolysin has long been considered a two-component toxin, although that view has been challenged recently (4). Other examples of multicomponent toxins (subunit toxins) are fairly well known (33) but are generally nonhemolytic. These toxins usually involve two distinct, nonidentical proteins. One protein moiety binds to susceptible cells and facilitates the entry of the second moiety into the cytoplasm, where it exerts its lethal effect. On the assumption that hemolysin BL will prove to be a subunit toxin involved with diarrheal illness, we designated the components as binding (component B) and lytic (component L).

We are currently attempting to resolve the question of whether hemolysin BL is indeed the multicomponent enterotoxin (5, 35). Turnbull (38) was puzzled that the enterotoxin could be necrotic but not hemolytic (41). Data reported by Thompson et al. (35) demonstrated enhanced hemolytic activity after recombination of enterotoxin components, but this point was not addressed in their discussion.

Our data suggest that the enterotoxin and the hemolysin are the same. Monoclonal antibodies to hemolysin BL were produced by using partially purified preparations of the diarrheal enterotoxin of *B. cereus*. Both the enterotoxin and the hemolysin require two, or possibly more, components for biological activity. Furthermore, SDS-PAGE, IEF, and Western blot analysis showed that components of the hemolysin are similar in isoelectric point and molecular weight to components of the enterotoxin (5, 35, 41).

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