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Characterization of the Components of Hemolysin BL 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 08903-0231

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Previously we described the partial purification of a novel hemolysin from *Bacillus cereus* and showed that hemolytic activity required the combined action of at least two components, called B and L to signify their cell-binding and cell-lytic roles in this activity. On further purification, as described in the present article, a combination of anion-exchange chromatography and polyacrylamide gel electrophoresis separated three proteins, B, L_1, and L_2 (35, 36, and 45 kDa, respectively). Individually, these proteins were inactive in hemolytic and vascular permeability assays, and combinations of B and L_1 or B and L_2 were either inactive or slightly active. Combinations of all three moieties produced the unique ring-shaped zone of hemolysis, a previously described characteristic of hemolysin BL, as well as edema and bluing in the vascular permeability assay. Since the vascular permeability assay is known to correlate with enterotoxicity, these results suggest that hemolysin BL is enterotoxigenic. Furthermore, the molecular weights and isoelectric point values of the hemolysin BL components are consistent with those described by others for the multicomponent diarrheal enterotoxin of *B. cereus*. Immunofluorescent staining of B-treated erythrocytes confirmed that B binds to cells as an initial step required before the L components can act to cause cell lysis.

Bacillus cereus, which is best known as the cause of two distinct food poisoning syndromes, has also been implicated in a wide variety of illnesses, including systemic infections and panophthalmitis (19). The virulence factors of this bacterium remain ill defined, partly because it produces a large number of proteins that potentially possess toxigenic activity and partly because these metabolites are difficult to isolate. Possible virulence factors include three phospholipases, a diarrheal enterotoxin, an emetic enterotoxin, a factor lethal to mice, and several hemolysins (19).

Initially, *B. cereus* was described as the producer of two hemolysins, cereolysin (3, 14) and a poorly characterized "secondary hemolysin" (5, 15). Later, Ikezawa et al. (9) reported that the sphingomyelinase of *B. cereus* lyses ovine and bovine erythrocytes, and Gilmore et al. (6) found that this hemolytic activity is enhanced by the phosphatidylcholine-preferring phospholipase C of this organism. The sphingomyelinase-phospholipase C combination was designated cereolysin AB. More recently (1), we described hemolysin BL, which is also composed of more than one component, and showed that it is distinct from both cereolysin and cereolysin AB. This hemolysin exhibits a unique ring-shaped zone in the lytic clearing of erythrocytes in gel diffusion assays.

The components of hemolysin BL comigrated during preparative isoelectric focusing and were only slightly separable by gel filtration chromatography (Superose-12). However, by using anion-exchange chromatography, fractions that possessed no hemolytic activity individually but were hemolytic in combination were obtained. Sheep erythrocytes treated with one fraction and then washed did not lyse until addition of the other fraction. Hemolysis did not occur when the fractions were added in the reverse order, suggesting that a component (B) in the first fraction was required to bind to the erythrocytes before a component (L) in the second fraction was enabled to cause lysis. The complex was designated hemolysin BL.

The components of this hemolysin were only partially purified, and the B and L functions could not be assigned to particular proteins. In this article, we present a purification scheme, combining anion-exchange chromatography and preparative polyacrylamide gel electrophoresis (PAGE), that resulted in highly purified preparations and allowed identification of the proteins responsible for the B and L activities.

The B component has a molecular mass of 35 kDa, and its role as a binding protein was verified by immunofluorescent staining. Gel filtration chromatography suggested that the L function is performed by a combination of two proteins with molecular masses of 36 and 45 kDa designated L_1 and L_2, respectively. This suggestion was confirmed in additional experiments which showed that none of the components were active individually and that a combination of all three was necessary to produce the typical ring-shaped hemolysis pattern in a gel diffusion assay. The three components were also necessary to produce edema and bluing in a rabbit vascular permeability assay. As the latter test is considered a valid alternative to the rabbit ileal-loop assay for diarrheal activity of *B. cereus*, we conclude that the three components of hemolysin BL also compose the diarrheal enterotoxin of this organism.

MATERIALS AND METHODS

Production of hemolysin BL. Hemolysin BL was produced in culture supernatant of *B. cereus* F837/76 as described previously (1). The culture supernatant was concentrated by precipitation with ammonium sulfate (60% saturation at 0°C

* Corresponding author.

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‡ Present address: Food Research Institute, University of Wisconsin, Madison, WI 53706.

overnight). The concentrate was dialyzed (12,000- to 14,000-molecular-weight cutoff) against 50 mM bis-Tris-HCl (pH 6.0) for 12 h at 4°C and then filtered through a 0.22-μm-pore-size filter.

Anion-exchange chromatography. Ion-exchange chromatography was performed on Fast Flow Q Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.). Thirty milliliters (240 mg of protein) was applied to a column (1.6 by 25 cm) with a 30-ml bed volume. The column was equilibrated with 50 mM bis-Tris-HCl (pH 6.0) at a flow rate of 1.5 ml/min, and proteins which did not bind to the matrix were collected in three fractions of 15 ml each. Bound proteins were eluted first with 75 ml of the same buffer containing a linear gradient (0 to 0.25 M) of NaCl, then with 5 ml of 0.25 M NaCl in buffer, then with 10 ml of 1.0 M NaCl in buffer, and finally with 30 ml of buffer only. Proteins eluted from the column were collected in 2.5-ml fractions.

Preparative PAGE. Preparative PAGE was performed with a Hoefer SE 600 vertical slab unit (Hoefer Scientific Instruments, San Francisco, Calif.) by a modification of the method described by the manufacturer (8). Proteins were separated by either discontinuous sodium dodecyl sulfate (SDS)-PAGE or continuous nondenaturing PAGE in 1.5-mm-thick resolving gels (10% acrylamide) at 4°C. The buffers described by Laemmli (10) were used for SDS-PAGE, and 50 mM Tris–160 mM glycine buffer (pH 8.5) was used for nondenaturing PAGE.

The gels were poured without sample combs, and an even surface was obtained by overlay of water-saturated *n*-butanol. Samples from Fast Flow Q Sepharose columns were concentrated by ultrafiltration (Centricon 10 microconcentrators; Amicon, Danvers, Mass.) to 0.5 ml and added to 1.0 ml of Laemmli (10) sample buffer for SDS-PAGE or to a mixture of 0.4 ml of 6.25 mM Tris–20 mM glycine buffer (pH 8.5), 0.1 ml of glycerol, and 10 μl of 0.1% bromophenol blue for nondenaturing PAGE. The samples were applied to the surface of the gel under the reservoir buffer. Protein loads were up to 8 mg per gel.

For SDS-PAGE the samples were run through the stacking gel at 30 mA per gel (constant current) and through the resolving gel at 60 mA per gel. For nondenaturing PAGE, the samples were run at 20 mA per gel until they entered the gel and then at 50 mA per gel. The current was stopped either 15 min (SDS-PAGE) or 2 h (nondenaturing PAGE) after the tracking dye had run off the end of the gel.

After electrophoresis, 1.5-cm-wide strips were cut lengthwise (vertically) from each side of the gel, and the strips were stained with colloidal Coomassie brilliant blue G-250 (13). Since the staining process swells the gel, the strips were cut jaggedly to facilitate later realignment of stained bands with the proteins in the unstained central portion of the gel, which was stored at 4°C as the side strips were being stained.

By using the stained strips as vertical guides, horizontal gel strips each containing a desired protein band were excised from the unstained gel. A strip was covered with a support gel, and the protein was eluted from the strip into 50 mM Tris–160 mM glycine by using the Hoefer electrophoresis unit and procedures that were performed essentially as described by the manufacturer (8). During elution, the gel was maintained at 4°C for 2 h and the current was 50 mA. Solutions (ca. 1.5 ml) containing the eluted proteins were concentrated to 0.2 to 0.3 ml by ultrafiltration (Centricon 10; Amicon).

Analytical SDS-PAGE. Analytical SDS-PAGE (10% acrylamide) was performed in the Hoefer apparatus described above with the buffers described by Laemmli (10). Samples were prepared in sample buffer without 2-mercaptoethanol to avoid artifacts sometimes encountered with silver staining (17).

Assays for hemolytic activity. Hemolytic activity on sheep erythrocytes was assayed by the gel diffusion and spectrophotometric methods previously described (1), with the following modifications of the latter procedure. Samples were added to 25 μl of 10% (vol/vol) washed sheep erythrocytes, and the reaction volume was brought to 50 μl with Tris-buffered saline. Hemolytic activity was determined by spectrophotometric measurement of hemoglobin release. After an appropriate time at 37°C, 10 μl of the reaction mixture was removed and added to 100 μl of Tris-buffered saline, and the mixture was centrifuged at 13,000 × *g* for 30 s. One hundred microliters of the supernatant was then added to a well in a 96-well microtiter plate, and the A_{405} was measured in a plate reader. The A_{405} was also measured for a nonlysed control, and this value was subtracted from the sample values and also from a 100% lysed control value (i.e., erythrocytes lysed by the addition of saponin and H_2O [2]). The corrected sample value divided by the corrected value for the lysed control was used to calculate the percent hemolysis.

Immunoassays. Monoclonal antibody M2 was produced as described previously (1). This antibody binds to a 35-kDa protein in fractions of the culture supernatant exhibiting activity of the B component of hemolysin BL. To detect this antigen in purified preparations, 1-μl volumes were dotted onto nitrocellulose strips and probed with monoclonal antibody M2 as described previously (1).

An immunofluorescent staining procedure was used to detect attachment of the B component to sheep erythrocytes. One microliter of the purified B component was added to 50 μl of a 1:10 dilution of ammonium sulfate-concentrated monoclonal antibody M2 in Tris-buffered saline (pH 7.4), the solution was held at 37°C for 45 min, and 2 μl of sheep erythrocytes was added. After 2 h at 37°C, the erythrocytes were recovered by centrifugation, washed three times with 1 ml of Tris-buffered saline (pH 7.4), and resuspended in 50 μl of Tris-buffered saline containing goat anti-mouse antibody conjugated to fluorescein isothiocyanate (Sigma; 1:25). After 90 min at 37°C, the cells were washed two times with 1 ml of Tris-buffered saline and brought to a final volume of 50 μl. As a control, erythrocytes were treated as described above except that the B component was not present. Wet mounts were prepared, and photomicrographs were taken with an epifluorescence microscope (1,000× optics).

Other analytical procedures. Vascular permeability was assayed as described by Glatz et al. (7). In brief, hair was clipped (size 40 blades, Oster A5 clipper) from the back of a rabbit (New Zealand White, female, 4.5 kg) and 50-μl samples were injected intradermally. Evans blue dye (3%) was injected intravenously (1 ml/kg) to visualize the vascular permeability reactions. After 1 h, reactions were recorded as the diameters of the zones of edema and bluing.

Protein concentrations were estimated spectrophotometrically (A_{280} value = mg/ml) as described by Stoscheck (16) or with Coomassie protein assay reagent (Pierce, Rockford, Ill.) by the procedure described by the manufacturer.

Lecithinase activity was detected by a radial diffusion assay in egg yolk agar, and phosphatidylinositol phospholipase C activity was detected by a radial diffusion assay using crude phosphatidylinositol (Sigma) as the substrate (1).

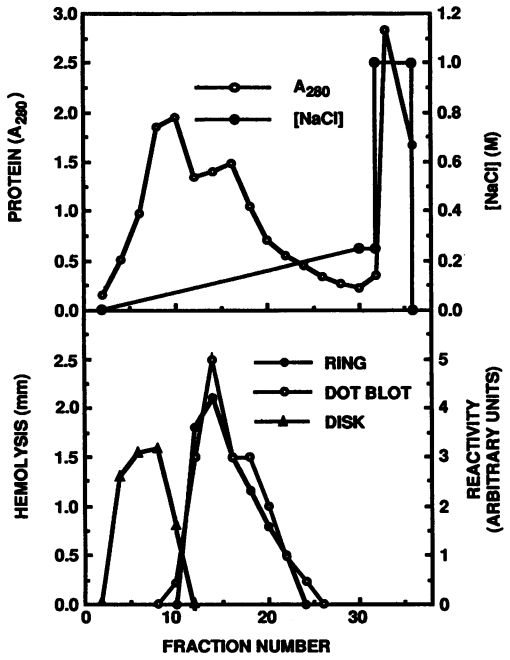

FIG. 1. Elution profile from anion-exchange chromatography. Proteins in a concentrated (60% saturated ammonium sulfate-precipitated) culture supernatant of *B. cereus* F837/76 were adsorbed to a Fast Flow Q Sepharose column at pH 6.0 and eluted with an NaCl gradient as indicated. (Top) Protein and NaCl gradient. (Bottom) Results of a dot blot assay of the B component, expressed in arbitrary units based on reactivity of monoclonal antibody to the B component. Hemolytic activity in the gel diffusion assay is expressed as the radius of the hemolytic zone minus the radius of the well (1.5 mm). Sample sizes were 6 μl. RING indicates hemolysin BL activity, and DISK indicates hemolytic activity due to hemolysins other than hemolysin BL.

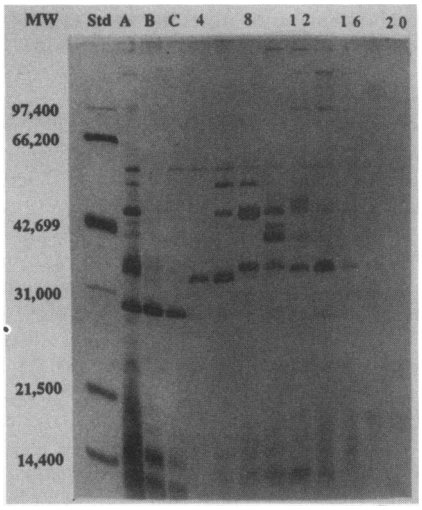

FIG. 2. SDS-PAGE of fractions from Fast Flow Q Sepharose column (Fig. 1). The column was run and proteins were eluted as described in Materials and Methods. Lane Std, Bio-Rad low-molecular-weight standards; lane A, proteins precipitated by ammonium sulfate; lanes B and C, fractions 1 and 2 (containing nonbound proteins). The other lanes contain even-numbered fractions from 4 to 20. Lane numbers correspond to fraction numbers (Fig. 1).

RESULTS

In our earlier article (1), we reported that the lytic activity of hemolysin BL required the combined action of two separate proteins. The component designated B had to bind to cells before component L could initiate cell lysis. Subsequently, in an attempt to further purify these components by gel filtration chromatography (Sephadex G-75), it was noted (data not shown) that L activity (i.e., lysis after addition of L to B-treated erythrocytes) occurred only on addition of fractions that contained two particular proteins of 36 and 45 kDa. As a number of other proteins were also present in these same fractions, it was necessary to explore other purification methods to purify the two proteins and determine their role in the action of hemolysin BL.

Anion-exchange chromatography. Previously, hemolysin components with B or L activity were separated by linear gradient elution (NaCl) from an ion-exchange column (1). However, as analytical-scale procedures were employed, protein yields were low. In the work described here, a preparative (30-ml) Fast Flow Q column was used to increase yields. Protein from 4 liters of *B. cereus* F837/76 culture supernatant was concentrated by ammonium sulfate precipitation, and after dialysis 30 ml (240 mg) was applied to the column. Figure 1 compares the elution profiles for total protein, protein reactive with monoclonal antibody M2, hemolysin BL, and another hemolysin(s) which forms a disk-shaped zone of clearing rather than the ring-shaped zone of hemolysin BL. Lecithinase did not bind to the column and was detected only in the initial three fractions containing nonadsorbed protein. Phosphatidylinositol phospholipase C activity was detected in fractions 4 to 8.

Some of the fractions collected from the column were subjected to SDS-PAGE, and the results are shown in Fig. 2. The proteins of interest with respect to L activity (36 and 45 kDa) both peaked in fraction 8, and the B component (35 kDa) peaked in fractions 14 and 15. Although L and B seemed well separated, residual L activity remained in the B preparation (Fig. 1) and further purification was necessary. It seemed unlikely that either anion-exchange or gel filtration chromatography would satisfactorily separate the two suspected L components. However, these two proteins were separated by SDS-PAGE, suggesting that preparative PAGE might provide a purification method.

Preparative PAGE. Initial experiments indicated that the 36- and 45-kDa proteins in the fractions possessing L activity (fraction 8 in Fig. 2) were not well separated by nondenaturing PAGE. Preparative SDS-PAGE would provide useful purification only if these proteins were stable under treatment with SDS. To test for stability, a preparation of hemolysin BL (fraction 15 in Fig. 1) was treated with 0.1% (wt/vol) SDS for 2 h at 4°C. Removal of SDS with ActiGel D (Pierce) resulted in nearly full recovery of hemolytic activity. Hemolysis was not due to residual SDS, as the ring-shaped pattern typical of hemolysin BL was obtained in a gel diffusion assay.

Two milliliters (~0.3 mg of protein as determined by A_{280}) of fraction 8 (Fig. 2) was used for the purification of the 36- and 45-kDa proteins by preparative SDS-PAGE. Because the B component and the 36-kDa suspected L protein are nearly the same size, they are difficult to separate by SDS-PAGE, but preliminary experiments indicated that they could be separated well by native PAGE. Fractions 14 and 15 (Fig. 1) were pooled, and 4 ml (~3.4 mg of protein as determined by A_{280}) was subjected to preparative nondenaturing PAGE for isolation of the B component. The proteins

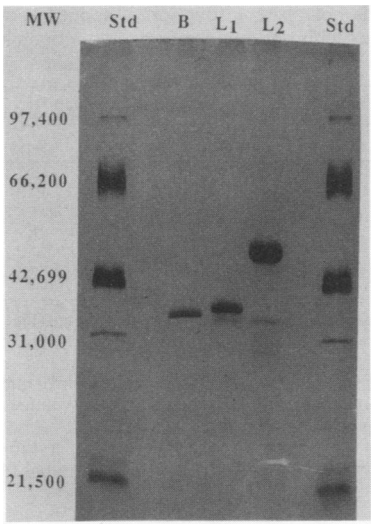

FIG. 3. SDS-PAGE of hemolysin BL proteins purified by preparative PAGE. Proteins in fractions from a Fast Flow Q Sepharose column (Fig. 2) were separated by SDS-PAGE or nondenaturing PAGE and recovered by electroelution as described in Materials and Methods. The fractions that were applied to the gel are described in the text. Lanes Std, Bio-Rad low-molecular-weight standards (0.3 μg per band). The other lanes are labeled with the designations assigned to the respective proteins in the text. Fifteen-microliter samples were applied to the gel.

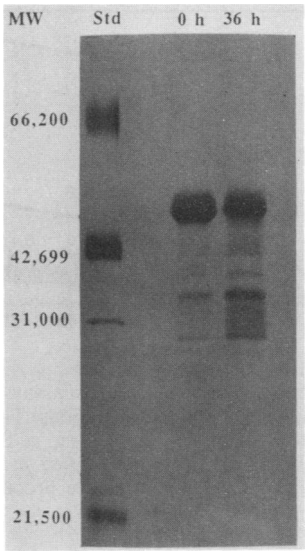

FIG. 4. SDS-PAGE illustrating degradation of L_2 with time. Lane Std, Bio-Rad low-molecular-weight standards; lane 0 h, L_2 that was not held at 37°C (t = 0 h); lane 36 h, L_2 that was held at 37°C for 36 h (t = 36 h). Protein loads were as described in the legend to Fig. 3.

were eluted from the gels and concentrated as described in Materials and Methods.

The concentration of protein in the purified preparation of the B component was 65 μg/ml, as measured by the Coomassie blue protein assay with bovine serum albumin as the standard. Since considerable experimentation was required in order to evaluate the role of the L components in the activity of the BL toxin, we decided not to waste purified material in protein determination. Instead, by comparing the bands in SDS-PAGE (Fig. 3), we roughly estimated the concentration of the purified L_1 preparation as 65 to 75 μg/ml and that of L_2 as 120 to 140 μg/ml.

SDS-PAGE of purified hemolysin BL components. The purity of the three proteins obtained by preparative PAGE was assessed by SDS-PAGE (Fig. 3). The B protein (lane B) was apparently homogeneous. The suspected L components, which had molecular masses of 36 and 45 kDa, were designated L_1 and L_2, respectively. The purified L_1 protein contained a minor, slightly smaller contaminant, and the L_2 protein was present with a large number of smaller proteins.

These smaller proteins in L_2 were unexpected, as this component had been purified by elution from a single band cut from an SDS-polyacrylamide gel. An experiment was performed to test the possibility that L_2 may have undergone proteolytic degradation resulting in the production of the smaller fragments. A sample of the L_2 preparation was held at 37°C for 36 h and analyzed by SDS-PAGE (Fig. 4). The intensity of the smaller bands increased and that of the 45-kDa band decreased in comparison with a sample that was not held at 37°C. This result confirms that the L_2 protein is the source of the smaller bands but does not indicate whether autolysis or a contaminating protease is the cause of the degradation.

Hemolytic activity of hemolysin BL components by gel diffusion assay. Combinations of the three preparations described above were tested in wells in blood agar gels to determine their contribution to the activity of hemolysin BL. The results differed depending on whether the components were added to the same well or allowed to diffuse toward one another from separate wells. In the blood agar gel shown at the bottom of Fig. 5 each preparation was put into a separate well, with B in the center well. None of the preparations were hemolytic alone. A zone of hemolysis formed between the wells containing B and L_1 but not between those containing B and L_2. The rounded zone is concave to B, which verifies it as the B component on the basis of earlier data (1). In the experiment illustrated at the top of Fig. 5, wells were filled with one of the following combinations: B plus L_1, B plus L_2, and B plus both L_1 and L_2. Rapid and complete hemolysis occurred only when all three proteins were added

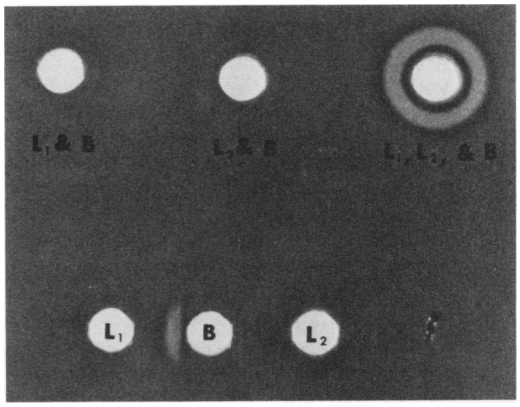

FIG. 5. Gel diffusion assay (hemolysis) of proteins purified by preparative PAGE (Fig. 3). The assay was performed as detailed in Materials and Methods. The samples (2 μl) were added to the wells as indicated and allowed to diffuse for 3 h at 37°C. (Top) Combinations of samples were added to individual wells as indicated. (Bottom) Individual samples were added to separate wells as indicated.

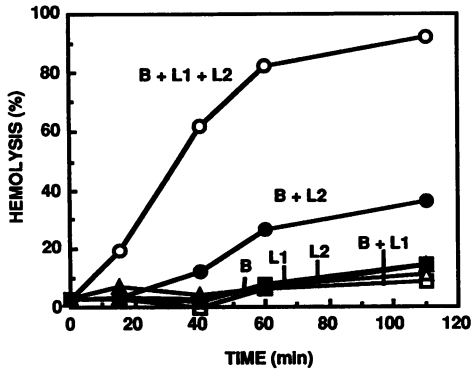

FIG. 6. Spectrophotometric hemolysis assay of hemolysin BL components individually and in combinations. The B and L preparations were diluted 1:10, and 10 μl (~65 ng) of B, 5 μl (~35 ng) of L_1, and 5 μl (~70 ng) of L_2 were assayed in the combinations indicated by using the spectrophotometric procedure described in the text.

to a well. There was a barely discernible ring of hemolysis for the B-plus-L_2 combination. Apparently, under these conditions, B must be complemented by both L_1 and L_2 for maximal hemolysis to occur.

That B and L_1 caused hemolysis when they diffused toward one another (Fig. 5) but not when they originated from the same well (Fig. 5) is puzzling. When L_2 was placed in one well and the nonhemolytic combination of B and L_1 was placed in an adjacent well, an arc of hemolysis formed between the wells (data not shown), which indicated that the inactivity of B plus L_1 was not due to inactivation or irreversible inhibition.

Hemolytic activity of hemolysin BL components by spectrophotometric assay. Kinetic plots of the hemolytic activities of hemolysin BL components assayed separately and in combinations are shown in Fig. 6. None of the components were hemolytic on their own, nor was the B-plus-L_1 combination. The combination of B and L_2 reached 37% hemolysis after 110 min at 37°C, and a combination of all three components was completely lytic.

The preceding results indicate that all three components play a role in the activity of hemolysin BL. An experiment was designed to determine whether a specific sequence of attack by components L_1 and L_2 occurs after B acts on cells. Erythrocytes were treated as shown in Table 1. Cells were treated first with one or two components, washed three times with 20 volumes of Tris-buffered saline, and then treated with the other component(s). When cells were treated first with B, then washed, and then subjected to a combination of L_1 and L_2, they lysed. Treatment with L_1 and L_2 first and then B did not result in lysis. These results are consistent with our earlier report (1), in which we hypothesized that L might be a single protein. Table 1 also shows that cells that were first treated with B plus L_1 or B plus L_2, then washed, and then treated with L_2 or L_1, respectively, also lysed. This result indicated that if B binds to cells, both L_1 and L_2 independently recognize and bind to, or act on, the B-erythrocyte complex.

Immunofluorescent staining of erythrocytes. We had always suspected that B acts as a binding moiety, and the results in Table 1 support but do not confirm this interpretation. Dot blots of the three purified preparations showed that monoclonal antibody M2 reacted only with the B component, suggesting that this antibody could be used to

TABLE 1. Hemolysis as a function of sequence of treatment of erythrocytes with various components and combinations of components of BL hemolysin[a]

Prewash treatment[b]			Postwash treatment[c]			Hemolysis (%)
B	L_1	L_2	B	L_1	L_2	
+	+	−	−	−	+	54
+	−	+	−	+	−	56
+	−	−	−	+	+	51
−	+	+	+	−	−	9
−	−	−	+	+	+	61

[a] Preparations containing hemolysin BL components (Fig. 3) were added to reaction mixtures as follows: B, 1 μl (~65 ng); L_1, 0.5 μl (~35 ng); and L_2, 0.5 μl (~70 ng).

[b] Erythrocytes (50 μl; 5%, vol/vol) were treated for 20 min at 37°C with the indicated components, washed three times with 20 volumes of Tris-buffered saline, and resuspended at their original volume.

[c] The indicated components were added to washed erythrocytes and held at 37°C for 30 min before hemoglobin release was measured (A_{405}).

determine whether this protein did indeed bind to the erythrocytes. The immunofluorescent staining procedure described in Materials and Methods was employed to test this possibility, and a photomicrograph of the results is shown in Fig. 7. The B component of hemolysin BL was detected as discrete spots on the surfaces of sheep erythrocytes and erythrocyte ghosts, and no fluorescence was detected on cells treated only with the monoclonal antibody and the anti-mouse immunoglobulin G-enzyme conjugate (data not shown). These observations confirm that B does bind to erythrocytes, as opposed to altering them without binding, for subsequent attack by L_1 and L_2.

The experiment described above was performed by reacting B first with the monoclonal antibody and then with the cells. In an experiment in which B was first reacted with erythrocytes, then washed, and then reacted with the antibody, a similar but less intense fluorescence pattern was observed (data not shown).

Vascular permeability assay of hemolysin BL components. Preparations of B, L_1, and L_2 were tested separately and in combination by the rabbit vascular permeability assay. All samples were tested in duplicate by using intradermal injections (50 μl) of phosphate-buffered saline containing either 4 μl (0.26 μg) of B, 3.5 μl (~0.25 μg) of L_1, 3.5 μl (~0.5 μg) of L_2, or various combinations of the three. There were no distinguishable reactions for B, L_1, L_2, B plus L_2, or L_1 plus L_2. The combination of B and L_1 produced minor edema (diameter, 10.5 ± 1.5 mm) with no bluing, and the combination of B, L_1, and L_2 produced edema (diameter, 18 ± 2 mm) with a central area of bluing (diameter, 6.5 ± 0.5 mm). A culture supernatant of *B. cereus* F837/76 (incubated for 8 h at 37°C in brain heart infusion broth [Difco] plus 1% glucose), used as a positive control, produced edema (diameter, 20 ± 2 mm), bluing (diameter, 15 ± 1 mm), and a central area of necrosis (diameter, 5 mm).

DISCUSSION

Earlier (1) we reported that the B and L components of hemolysin BL could be separated into two nonhemolytic fractions which were again hemolytic when recombined. The preparations used in those experiments were only partially purified, so a function in the lytic process could not be assigned to specific proteins. The results in the present work show that hemolysin BL is composed of three separate proteins and that maximal hemolytic and vascular perme-

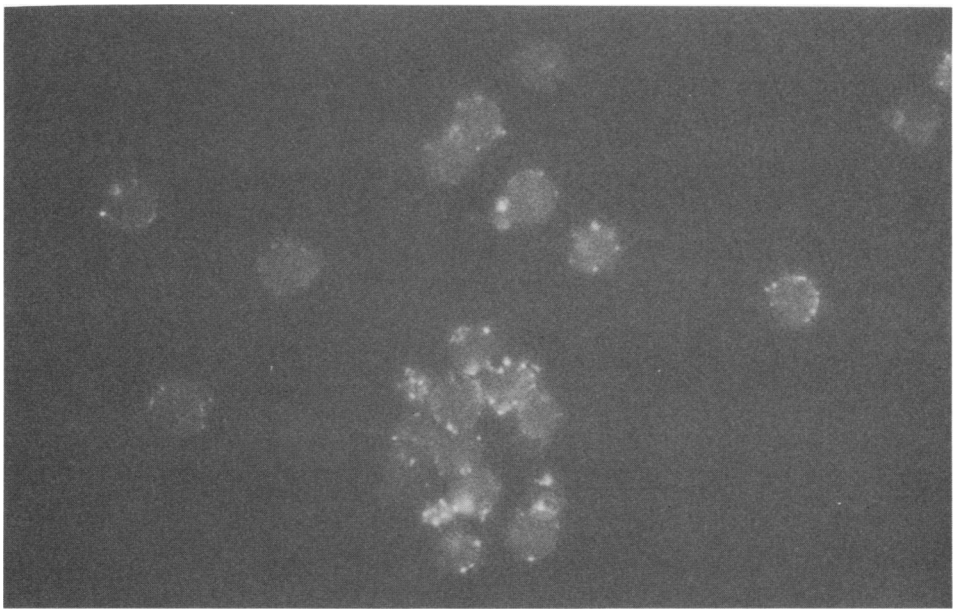

FIG. 7. Immunofluorescent staining of sheep erythrocytes. Sheep erythrocytes were treated with component B, monoclonal antibody M2, and fluorescein isothiocyanate-labeled conjugate. The staining procedure is described in the text.

ability activities require all three components. The B component is a 35-kDa protein, and the L component which complements the B component is split between two proteins of 36 and 45 kDa. We do not have direct evidence which proves that the two L components are distinct proteins. It is possible that the smaller one is a degradation product of the larger one. However, on the basis of their synergistic action in a variety of biological assay systems, we strongly suspect that they are separate components of the BL hemolysin.

In Fig. 1, the elution profile of hemolysin (ring-shaped hemolysis zone) from the Fast Flow Q column is nearly identical to that of the B component. This was unexpected, since one might assume, on the basis of the multicomponent nature of the hemolysin, that activity would peak in an area intermediate to the separated components. However, previous results (1) showed that the diameter of the hemolysis ring in gel diffusion assays is determined primarily by the concentration of the B component, and apparently only trace amounts of L are required to cause hemolysis with B. In anion-exchange chromatography, the tailing of trace amounts of the L components into fractions containing B made it difficult to obtain preparations of B that were devoid of hemolytic activity.

Earlier data (1) showed that the unique ring-shaped pattern of hemolysis exhibited by hemolysis BL was a function of the B component. We suggested that the area of nonlysed cells inside the ring might be due either to prohibitively high concentrations of B or to the presence of another protein that competed with B for binding sites on the cells. However, the data in Fig. 3 suggest that the latter possibility is not the case. The B component was purified to apparent homogeneity, and the ring pattern (Fig. 5) still occurred.

The combination of B and L_1 was hemolytic when the two components diffused toward one another, but cells did not lyse when they both diffused from the same well. Not enough is known about the toxin or its performance in this assay system to explain this observation satisfactorily. One possibility, however, is that a minor 34-kDa contaminant detected in the L_1 preparation (Fig. 3) is an inhibitor. This contaminant is the same size as the major degradation product of L_2 (Fig. 4) and could be a fragment of L_2 which forms a nonfunctional (i.e., nonlytic) assemblage with B and L_1. Since hemolysis on gels can occur only within a fixed area defined by the concentration of B (1), when diffusing from the same well as B and L_1, small amounts of inhibitor may be sufficient to prevent hemolysin within that limited area. The patterns of hemolysis that occur upon diffusion of various components toward each other are more difficult to explain on the basis of an inhibitor without experimentation in which the effect of concentration, diffusion rates, and binding efficiencies can be tested directly with purified hemolysin components and the suspected inhibitor.

The gel diffusion data shown in Fig. 5 were corroborated in a tube assay system (Fig. 6). The spectrophotometric assay for hemolysis indicated that none of the components are hemolytic on their own and that there was some activity for a combination of B and L_2 and maximal activity for a combination of B, L_1, and L_2. The combination of B and L_1 had no more activity than any of the individual components, which is consistent with the inhibition model discussed above. Isolation of L_1 without the putative inhibitor might result in an active B-plus-L_1 combination in the spectrophotometric assay.

In the immunofluorescence experiments (Fig. 7), the occurrence of discrete fluorescent spots may reflect the binding of B to specific, discretely spaced receptors or the aggregation of B molecules which initially bind over the entire cell surface but move through the fluid membrane and associate with each other. The data from Table 1 indicate that once B binds to the cell, either L component can perform its particular function independently of the other. Apparently the L components recognize either B or the B-erythrocyte complex, and several possible events may occur after that. For example, the L components may enter the cell and alter metabolic functions, or they may interact with the B molecules and form membrane lesions.

The high correlation between the rabbit ileal-loop assay and the rabbit vascular permeability assay prompted Turnbull (19) to adopt the latter for assay of the *B. cereus* enterotoxin. The activity of hemolysin BL in the vascular permeability assay suggests that it possesses enterotoxic activity. However, interpretation of the rabbit vascular permeability data is difficult. Necrosis is often seen at the center of zones of bluing and is interpreted as a potent manifestation of the bluing response (18, 20). In our experiments we did not observe necrosis, probably because the concentrations of the components were too low. Despite the current trend towards reliance on vascular permeability for assay of the *B. cereus* enterotoxin, we feel that it is necessary to show that hemolysin BL causes fluid accumulation in the rabbit ileal-loop assay to prove conclusively that it is the enterotoxin. However, to test this will require much larger amounts of purified components than are currently available.

Despite these problems, it seems likely that hemolysin BL is the same as the enterotoxin described by Turnbull et al. (20) and that the hemolytic activity in their preparations was due to the toxin itself. These authors reported that the enterotoxin had a pI value of 4.9; the pI of the hemolytic activity of hemolysin BL ranged between 4.8 and 5.1 (1). Thompson et al. (18) determined that the enterotoxin is composed of at least two components. Their published data showed that preparations also possessed hemolytic activity; however, these results were not discussed. We reported (1) that the monoclonal antibodies which bind to L_2 and B also bind to the major proteins in enterotoxin preparations which had been partially purified by Thompson and coworkers.

The work of Bitsaev and Ezepchuk (4) on a three-component toxin they called the DL-toxin confirmed that the *B. cereus* enterotoxin consists of at least two components. They concluded that the DL-toxin possesses a binding (B) moiety of 43.5 kDa that acts with another component (A) of 37 kDa to cause edema (in a mouse paw assay) and fluid accumulation but that these components are not lethal to mice. The B component and a C component of 35.3 kDa are lethal to mice but do not cause edema or fluid accumulation. Additionally, A is inhibitory to the lethal activity of C against mice, and C is inhibitory to the activity of A. This scenario is suggestive of the protective antigen, edema factor, and lethal factor of anthrax toxin (11).

In contrast, for the two activities of hemolysin BL that were tested (vascular permeability/edema and hemolysis), a combination of all three components had much greater activity than any combination of two components. That hemolysin BL has maximal activity when all three components are present suggests that the proteins involved are not identical to those of the DL-toxin, which acts more like two distinct toxins that share a ligand-binding moiety. It is improbable, though, that a single organism produces two different three-component toxins with similar biological activities and nearly identical molecular weights. Thus, DL-toxin and hemolysin BL may be the same, despite differing observations which could be due to laboratory anomalies or to minor differences in one or another of the protein components.

It is not uncommon for separate proteins of bacterial origin to act synergistically to cause hemolysis (12). Usually, only two components are involved, and they generally arise from separate species. In addition to its unusual ring-shaped hemolytic zone in gel diffusion assays, hemolysin BL appears unique in that it is composed of three components all arising from the same organism. The role which this hemolysin might play in the virulence of *B. cereus* is not known.

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