Characterization of a Parasporal Inclusion Body from Sporulating, Enterotoxin-Positive Clostridium perfringens Type A

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Inclusion bodies (IB) synthesized during sporulation and enterotoxin formation by Clostridium perfringens NCTC 8239 and 8798 were isolated and characterized. IB were isolated by disruption of sporangia by sonication in the presence of tetrasodium EDTA and phenylmethylsulfonfluoride. Fractionation was carried out in a linear gradient of sodium bromide, sucrose, or diatrizoate sodium. Denaturing and reducing agents were necessary to solubilize the IB. An alkylating agent was required to prevent reaggregation of the subunits. Molecular weight, compositional, and serological analyses and peptide mapping revealed strong similarities between the IB subunits and the enterotoxin synthesized during sporulation by C. perfringens. IB appear to represent the structural component where enterotoxin accumulates intracellularly. Enterotoxin-like subunits in the IB appeared to be held together by noncovalent and disulfide bonds, which were generally resistant to the action of intracellular proteases of C. perfringens, trypsin, or trypsin plus bile salts.

Clostridium perfringens type A is a common etiological agent in human food poisoning. An enterotoxin synthesized in large amounts only during sporulation is known to be responsible for the pathological effect in humans. Two other events have been reported to appear concomitantly with enterotoxin formation. These are the synthesis of intracellular proteases and, under certain conditions, the production of intracellular inclusion bodies (IB) (4, 18). Since these IB appeared only in enterotoxin-positive organisms, it has been suggested that they could represent a structural component where enterotoxin accumulates intracellularly (4, 19). The role of these IB in sporing cells of C. perfringens has never been determined, nor have they ever been characterized. We recently described a reproducible technique for the isolation and purification of the IB produced by this organism (19). In this work we describe the biochemical composition of the IB synthesized by C. perfringens type A and their relationship to enterotoxin.

MATERIALS AND METHODS

Bacterial strains and culture conditions. C. perfringens NCTC 8239 (Hobbs’ serotype 3), NCTC 8679 (Hobbs’ serotype 6), and FD-1 were used in this study.

Active cultures were obtained by overnight (16 to 18 h) growth in 10 ml of fluid thiglycolate medium (FTG; BBL Microbiology Systems, Cockeysville, Md.). Active cultures of NCTC 8239 and 8679 were inoculated (1%) directly into Duncan-Strong (DS) sporulation medium (5). For NCTC 8679, caffeine (Sigma Chemical Co., St. Louis, Mo.) was added (1 mM) to the DS medium. Caffeine has been shown to stimulate the sporulation of NCTC 8679 (16). After 7 to 8 h of growth at 37°C, the cells were mature sporangia with well-developed IB. The cultures were then washed twice with 0.05 M Tris hydrochloride buffer (pH 7.4) containing 100 mM NaCl to eliminate any adhering extracellular protein, washed twice with buffer without NaCl, and frozen until used.

Isolation and purification of IB. IB of C. perfringens type A were isolated as described elsewhere (19). Briefly, sporing cells were disrupted by sonication. IB were separated in a linear gradient of sucrose, sodium bromide, or diatrizoate sodium (1.12 to 1.44 gm/cm³) and then in step gradients of diatrizoate sodium (1.03, 1.10, 1.30, and 1.40 gm/cm³). Those fractions containing the inclusions were washed twice with double-distilled water and once with 100 mM NaCl to remove any adhering soluble enterotoxin. Inclusions were then treated with 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) to remove any attached membrane.

Protein determination. Protein was determined by the Warburg-Christian method of absorption at 260 and 280 nm (24).

Amino acid analysis. Samples were analyzed as described elsewhere (2), except that a Beckman 121 automatic amino acid analyzer with a one-column system was used.

Carbohydrate analysis. The presence of glycopeptides in the IB was determined by the method of Hirs (13) with glucose as a standard at concentrations of 1, 2, and 3 μM. We measured the A₄₉₉ for hexoses and the A₄₈₉ for pentoses and uronic acid.

Poly-β-hydroxybutyric acid analysis. Poly-β-hydroxybutyric acid was determined by the procedure described by Law and Slepecky (17) based on the ability of poly-β-hydroxybutyric acid to produce crotonic acid when treated with hot sulfuric acid.

Nucleic acid analysis. The presence of nucleic acids was determined by a modification of the method of Keleti and Lederer (14) which consisted of incubating the isolated inclusions with 0.6 N HClO₄ and then centrifuging them at 3,060 × g for 15 min. The precipitate was washed with 0.2 N HClO₄ and allowed to air dry. The dried pellet was then incubated with 0.3 N KOH at 37°C for 1 h in a shaking water bath. After the pellet was chilled for 15 min in an ice bath, 0.6 N HClO₄ was added, and the mixture was centrifuged at 3,060 × g for 15 min. The presence of RNA in the supernatant fluid was determined by measuring its A₂₆₀. The pellet was analyzed for the presence of DNA. The precipitate was dissolved in 0.3 N KOH and incubated overnight at 37°C in a shaking water bath. An equal volume of a 0.06% indole solution prepared in 2.5 N HCl was added, and the mixture...
was then cooled and removed. The 4% aqueous layer was read with KOH as a blank.

**Lipid analysis.** Inclusions were extracted three times with petroleum ether and centrifuged at 2,500 \( \times g \) for 15 min at 4°C. The supernatant solution was dried, and lipids were assayed by thin-layer chromatography (TLC) in a one-dimensional solvent system (chloroform-methanol-water, 65:25:4, vol/vol/vol). Lipids were detected on the TLC plates by charcoaling lipids being sprayed with 70% sulfuric acid. Octanoic acid was used as a standard.

**Solubilization of the IB.** Several methods were used to solubilize the IB. The effectiveness of each procedure was determined by electrophoresis in 7% polyacrylamide gels and serological reactivity with enterotoxin antiserum. Isolated inclusions (30 \( \mu l \); 1.5 to 2.0 mg/ml) were added to 30 \( \mu l \) of each solubilizing solution. When specified, 20 \( \mu l \) of the alkylating agent iodoacetic acid (0.1 M, pH 9.0) was added. The solubilizing methods tried were as follows: (i) 1% sodium dodecyl sulfate (SDS; Fisher Scientific Co., Pittsburgh, Pa.), 1 h, 38°C; (ii) heating at 70°C, 15 min; (iii) adjustment to pH 12 with NaOH, 1 h, 38°C; (iv) 4 M KSCN (Fisher), 1 h, 38°C; (v) 1 M urea (Sigma), 1 h, 38°C; (vi) 10 mM diithiothreitol (DTT), 30 min, 38°C, followed by iodoacetic acid, 1 h, 38°C; (vii) 2% mercaptoethanol (ME; Sigma), 1 h, 38°C; (viii) 1% SDS–2% ME–38.4 mM glycine–Tris hydrochloride (pH 8.4), 15 min, 70°C, followed by iodoacetic acid, 1 h, 38°C; (ix) 20% SDS–10 mM DTT (pH 7.4), 15 min, 25°C, followed by iodoacetic acid, 1 h, 38°C; (x) 1 M KSCN–0.1 M morpholinepropanesulfonic acid (MOPS)–0.05 M DTT (pH 7.5), 30 min, 38°C, followed by iodoacetic acid, 1 h, 38°C; and (xi) 1 M urea–0.1 M MOPS–0.05 M DTT (pH 7.5), 30 min, 38°C, followed by iodoacetic acid, 1 h, 38°C.

**PAGE.** Polyacrylamide gel electrophoresis (PAGE) was carried out in 7% polyacrylamide gels. The compositions of the stacking and separating gels were as described by Maurer (20).

**Determination of molecular weight.** Molecular weight determination was done by gel filtration chromatography. Sephadex G-150 in a 20- by 50.0-cm column (Pharmacia Fine Chemicals, Piscataway, N.J.) was equilibrated in 0.02 M NaH₂PO₄ (pH 6.8). The elution pattern of solubilized inclusions was compared with that of proteins of known molecular weight: lysozyme (14,400), chymotrypsinogen A (25,000), ovalbumin (OVA; 43,000), and bovine serum albumin (BSA; 68,000). Inclusions were solubilized by method ix, x, or xi as described above, except that phosphate buffer was used instead of water to prepare the solubilizing mixtures. Solubilized material (500 \( \mu l \)) was layered on top of the column. Purified enterotoxin and enterotoxin treated with solubilizing mixtures x and xi were run under the same conditions as the IB.

**Purification of enterotoxin.** The enterotoxin in the dialyzed material from *C. perfringens* NCTC 8239 was purified by a modification of the method of Granum and Whitaker (10). The cell extract of sporulating *C. perfringens* was precipitated with equal volumes of 30% (NH₄)₂SO₄ and passed through Sephadex G-150. Sterile phosphate buffer (0.2 M, pH 6.8) without NaN₃ was used to elute the enterotoxin. The purity of the enterotoxin was assayed by PAGE in 7% polyacrylamide gels. An extinction coefficient (ɛ₂₈₀) of 1.33 \( \text{mg}^{-1} \text{cm}^{2} \) was used to determine the concentration of the purified enterotoxin (10).

**Production of immune sera.** Antienterotoxin was produced by using purified enterotoxin from strain NCTC 8239 in a manner similar to the method of Stark and Duncan (23). A series of seven injections (one per week) was administered to two female New Zealand rabbits weighing approximately 2 kg. The enterotoxin, emulsified in an equal volume of Freund complete adjuvant, was injected intramuscularly for the first 6 weeks. At days 52, 58, and 65, the rabbits were bled from the ear with a bleeding apparatus (Bellco Glass, Inc., Vineland, N.J.). Titers were determined by counter-current immunoelectrophoresis as described elsewhere (18).

**Determination of proteases adhering to the IB.** Inclusions were isolated as described above, except that they were not washed with either NaCl or CHAPS, which could inactivate adhering proteases. Sucrose or diatrizoate sodium gradients were used. NaBr was avoided, since it is known to inactivate proteases (1). The presence of proteases was determined by the Azocoll method as described by Loffler and Labbe (18). A 1-ml amount of isolated IB was used for the analysis (protein concentration, 2.1 mg/ml).

**Endogenous radioactive labeling of IB and enterotoxin.** *C. perfringens* NCTC 8679 (100 ml) was grown in a defined medium. A mixture of \(^{14}C\)-labeled amino acids (ICN Chemicals, Cleveland, Ohio) was added at a final concentration of 1 \( \mu C \)/ml. Labeled IB were thus isolated as described above. Enterotoxin was partially purified from the \(^{14}C\)-labeled cell extract of *C. perfringens* NCTC 8679 by a two-step precipitation procedure. The cell extract was first treated with 0.01% polyethyleneimine (Sigma) to remove nucleic acids, and the precipitate was discarded. The enterotoxin was then precipitated from the cell extract by the addition of an equal volume of 30% (NH₄)₂SO₄. The purity of the enterotoxin was assessed as described above.

**Solubilization of \(^{14}C\)-labeled inclusions and enterotoxin.** Isolated \(^{14}C\)-labeled inclusions (30 \( \mu l \); 0.2 to 0.8 mg/ml) were treated with the following: (i) 30 \( \mu l \) of an unlabeled sonicated cell extract of *C. perfringens* NCTC 8679 containing 237.5 \( \mu \)U of intracellular proteases per ml, incubation at 37°C in a shaking water bath for 1 h; (ii) 30 \( \mu l \) of a 10-\( \mu \)g/ml solution of trypsin (Sigma), incubation at 37°C in a shaking water bath for 1 h; (iii) 30 \( \mu l \) of 0.1% bile salts (Sigma) plus 10 \( \mu g \) of trypsin per ml, incubation at 37°C in a shaking water bath for 1 h; (iv) 30 \( \mu l \) of a 10-\( \mu \)g/ml solution of *Staphylococcus aureus* V8 protease (Sigma), incubation at 37°C in a shaking water bath for 1 h; (v) similar to method 10 above, i.e., 30 \( \mu l \) of 1 M KSCN–0.1 M MOPS–0.05 M DTT (pH 7.5), incubation for 1 h at 38°C, followed by the addition of 20 \( \mu l \) of 0.1 M iodoacetic acid (pH 9.8), incubation for 1 h at 38°C; (vi) 30 \( \mu l \) of distilled water, incubation at 37°C in a shaking water bath for 1 h.

**Solubilization of \(^{14}C\)-labeled IB was assessed by PAGE.** Two gels were run for each solubilizing treatment. After the electrophoretic run, one of the gels was stained with Coomassie brilliant blue G-250. After being destained, the gel was scanned with a soft-laser densitometer. The second gel was sliced laterally at 1.0-mm intervals. Each slice was solubilized in a scintillation vial in the presence of TS-1 Solubilizer (Research Products International Corp., Mount Prospect, Ill.) and held for 2 h at 55°C. 2,5-Diphenyloxazole (POO; 0.5%, 5 ml; Fisher) in toluene was then added to each vial. Radioactivity was counted by using a liquid scintillation system (Delta 300; Searle Analytic, Inc., Chicago, Ill.).

Procedures iii, iv, and vi were also used for the digestion of \(^{14}C\)-labeled enterotoxin.

**Avidin-biotin-peroxidase immunochemistry.** *C. perfringens* NCTC 8679 and the nonenterotoxigenic strain FD-1
were fixed with 0.2% glutaraldehyde and 5% formaldehyde in isotonic phosphate-buffered saline (PBS) buffer (pH 7.2) for 1 h. Fixed cells were enrobed in purified agar in PBS and dehydrated in a graded ethanol series from 30 to 100%. Samples were infiltrated in LR White (London Resin Co. Ltd., Basingstoke, Hampshire, England) in accordance with the specifications of the manufacturer. Polymerization was carried out at 60°C for 24 h. Ultrathin sections were prepared with an LKB Ultratome 111 ultramicrotome and placed on nickel grids. Before being stained, the grids were floated on drops of 0.3% H2O2 in methanol for 30 min at 37°C and then washed for 20 min with PBS. The grids were then floated on drops of 1:20-diluted normal goat serum ( Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.) in PBS for 20 min at 37°C.

Excess serum was blotted, and the grids were incubated for 30 min with rabbit antienterotoxin serum diluted to 1:32 in buffer. The grids were then washed with PBS as described above and floated for 30 min at 37°C on diluted biotinylated antirabbit serum (1:20; Vectastain). After being washed for 10 min with PBS, the grids were floated for 1 h at 37°C on drops of avidin-biotin-peroxidase complex prepared in accordance with the specifications of the manufacturer (Vector Laboratories). The grids were washed and floated for 5 min on a solution of 0.02% H2O2 in distilled water plus 0.1% dianinobenzidine tetrahydrochloride (DAB; Polyscience, Warrington, Pa.) in Tris hydrochloride buffer (pH 7.2). After being washed with distilled water, the grids were dried and examined in a Zeiss EM 9S-2 electron microscope with an accelerating voltage of 60 kV. Controls consisted of the following: (i) Negative control: C. perfringens FD-1 (which produces no enterotoxin or insignificant amounts of enterotoxin) treated similar to strain NCTC 8679. (ii) Substrate control: The primary antiserum was substituted with normal serum, and (iii) positive control in which the thin sections were pre-adsorbed with purified enterotoxin.

**Biological activity.** The biological activity of the IB was determined by the method of McClane and McDonel (21) with Vero cells.

**RESULTS**

**Compositional analyses.** An example of IB formation in C. perfringens NCTC 8679 is shown in Fig. 1. Isolated inclusions were found to contain no carbohydrate, poly-β-hydroxybutyrate, or nucleic acids. However, they reacted with Coomassie blue and absorbed strongly at 280 nm, suggesting a protein composition. Lipids were not detected in those inclusions treated with CHAPS, a detergent with non-denaturing characteristics. On the other hand, lipids were detected in those inclusions not treated with this detergent, as determined by TLC. It is possible that the cell membrane remained attached to the isolated inclusions and was removed after treatment with the detergent. This possibility was supported by electron-microscopic data reported earlier (19). The amino acid composition of the solubilized IB closely resembled that of the purified enterotoxin (Table 1). Both had a high content of aspartic acid, glutamic acid, serine, and leucine. The results of the enterotoxin amino acid composition analysis were in agreement with those of Granum et al. (11).

**Solubilization.** A critical aspect in the characterization of the IB produced by C. perfringens was the method used for their purification and solubilization to render stable and functional subunits. Solubilization of the native inclusions could be determined by an increase in the A280 when the inclusions were incubated with solubilizing agents (Fig. 2). A plateau in the absorbance occurred when solubilization was complete. The effectiveness of the solubilization method was

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>IB</th>
<th>Purified enterotoxin</th>
</tr>
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<tbody>
<tr>
<td>Aspartic acid</td>
<td>14.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Serine</td>
<td>10.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.8</td>
<td>8.4</td>
</tr>
<tr>
<td>Proline</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.2</td>
<td>6.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Valine</td>
<td>6.6</td>
<td>6.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.6</td>
<td>6.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.9</td>
<td>5.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>
determined by PAGE. Intact inclusions were unable to enter the 3% stacking gel, producing a broad smear on top of the stacking gel and no bands in the separating gel. After solubilization, a single band was evident, suggesting that IB are composed of a single polypeptide. Therefore, it was assumed that successful solubilization was indicated by the presence of a single band after PAGE. The results of the solubilization studies are shown in Table 2. Both a reducing agent (DTT) and a denaturing agent (KSCN or urea) were required for the successful solubilization of the IB. The requirement for reducing agents for the solubilization of the IB suggests the importance of disulfide bonds in maintaining the structure of the IB. However, reducing agents alone (e.g., method vi in Table 2) were unable to solubilize the IB. This suggests that in intact inclusions, the disulfide bonds are not accessible without disruption of noncovalent intermolecular forces, which is achieved when denaturing agents are present. On the other hand, method vi has been successfully used for the solubilization of spore coat proteins (15), showing that the IB and the spore coat are structurally different. Solubilizing methods x and xi (Table 2) resulted in one band after PAGE. The serological relationship of the solubilized material to enterotoxin was maintained with all treatments except NaOH. Inconsistent results were observed when method viii was used (Table 2). Various numbers of bands appeared in the separating gel, possibly because of the presence of SDS. It is known that purified enterotoxin precipitates in the presence of SDS (22). Thus, since it is likely that solubilized IB are composed of enterotoxin or enterotoxinlike subunits, anomalous behavior in the presence of SDS is not surprising. A recurrent problem was the reaggregation of the solubilized components when they were stored for a few hours. This was made apparent by the inability of the stored material to enter the stacking gel during PAGE and by observations with a scanning electron microscope. Reaggregation was prevented by treatment with iodoacetic acid, again suggesting the importance of disulfide bonds. The \( R_f \) of solubilized inclusions was 0.70 to 0.74. Purified enterotoxin from \( C. \ perfringens \) NCTC 8239 had an \( R_f \) of 0.63. When this enterotoxin was treated with solubilizing method x or xi (Table 2) and subjected to PAGE, it produced a fast-moving band with an \( R_f \) higher than 0.75. This could be the result of an increase in the net charge of the molecule.

Molecular weight. The molecular weight of the inclusions produced by \( C. \ perfringens \) was determined by gel filtration chromatography. SDS-PAGE was not performed because of the known property of the IB to aggregate in the presence of SDS, preventing their entrance into the 3% stacking gel. \( C. \ perfringens \) NCTC 8679 IB solubilized by method x or xi (Table 2) yielded a single peak after gel filtration chromatography with Sephadex G-150. This result suggested the presence of one subunit with an apparent molecular weight of 38,000, which is very similar to the molecular weight of 35,500 obtained for purified enterotoxin from the same strain under the same solubilizing conditions. Erratic results were observed when IB were solubilized by method viii. A large peak eluted at the void volume. Another peak corresponded to a molecular weight of 38,000 and occasionally a molecular weight of 75,000 (data not shown). The protein eluting at the void volume could indicate a nonsolubilized or reaggregated inclusion. Inconsistent results were also obtained when purified enterotoxin was subjected to the solubilizing conditions of method viii. Many peaks were observed. The major peak eluted at a molecular weight of 35,500, with the remainder eluting at the void volume, indicating aggregation of the enterotoxin, presumably because of the presence of SDS.

In situ localization of inclusions by immunocytochemistry. Additional evidence of the relationship between enterotoxin and IB was determined with the avidin-biotin-peroxidase complex. Since nonspecific absorption reactions on the plastic are concentration dependent, it was essential to determine the optimum antiserum dilution. The best dilution for whole serum was found to be 1:32, since lower backgrounds with higher specific staining were observed.

Immunocytochemical staining in the avidin-biotin-peroxidase system indicated that enterotoxin was localized in the sporulating cells of \( C. \ perfringens \) around the cell wall, in the IB, and on the spores (Fig. 3). No staining was observed in the negative and substrate controls.

Potential biological role of IB. Both intact and solubilized IB possessed biological activity when assayed with Vero cells (21). The activity was neutralized by preincubation with antienterotoxin serum.

Intracellular proteases in \( C. \ perfringens \) were first described by Löffler and Labbé (18). Their effect on the IB was studied here by incubating \(^{14}C\)-labeled IB with unlabeled protease-containing cell extracts of the same strain. The ratio of proteolytic enzymes to inclusions was kept much higher than presumably exists in vivo. Solubilization of the

![Graph showing change in A280 during solubilization of IB of \( C. \ perfringens \) NCTC 8679. Isolated IB were incubated at 37°C in water or solubilized by method x.](https://example.com/graph.png)

**TABLE 2.** Solubilization of \( C. \ perfringens \) IB by physical and chemical methods

<table>
<thead>
<tr>
<th>Method</th>
<th>PAGE result</th>
<th>Serological relationship with enterotoxin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>No bands*</td>
<td>+</td>
</tr>
<tr>
<td>ii</td>
<td>No bands</td>
<td>+</td>
</tr>
<tr>
<td>iii</td>
<td>No bands</td>
<td>-</td>
</tr>
<tr>
<td>iv</td>
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<td>+</td>
</tr>
<tr>
<td>v</td>
<td>No bands</td>
<td>+</td>
</tr>
<tr>
<td>vi</td>
<td>No bands</td>
<td>+</td>
</tr>
<tr>
<td>vii</td>
<td>No bands</td>
<td>+</td>
</tr>
<tr>
<td>viii</td>
<td>No bands to many bands</td>
<td>+</td>
</tr>
<tr>
<td>ix</td>
<td>No bands</td>
<td>+</td>
</tr>
<tr>
<td>x</td>
<td>One band ( (R_f, 0.70 to 0.74) )</td>
<td>+</td>
</tr>
<tr>
<td>xi</td>
<td>One band ( (R_f, 0.70 to 0.74) )</td>
<td>+</td>
</tr>
</tbody>
</table>

* As determined by Ouchterlony gel diffusion. +, Homology with enterotoxin; –, failed to show homology with enterotoxin.

* Protein failed to enter the gel.
inclusions was determined by PAGE. As mentioned previously, intact inclusions are unable to penetrate the stacking gel. Therefore, any radioactive band in the gel would indicate some solubilization of the IB by the proteases in the cell extract. A scan of a Coomassie blue-stained gel is shown with the radioactive counts of each slice of a duplicate, unstained gel in Fig. 4. The stained gel had an array of bands corresponding to various proteins of the cell extract (Fig. 4B). One small band, however, coincided with a radioactive peak, and both had an Rf similar to that of enterotoxin. This indicates that some solubilization of the IB occurred. However, most of the radioactivity remained on top of the gel, suggesting that most of the IB material remained unaltered.

No proteolytic activity was detected by the Azocoll method in isolated IB synthesized by C. perfringens. This indicates that the IB were not degraded by attached proteases.

The effect of trypsin on the IB was also studied. Trypsin had some effect on the 14C-labeled IB (Fig. 4C). A number of radioactive bands were observed in the gel. One had a mobility similar to that obtained with solubilized IB and coincided with a stained band. Most of the radioactivity, however, remained near the top portion of the gel, indicating that most of the IB remained unaltered. A fast-moving radioactive peak was also detected at the dye front. This band moved with the tracking dye and coincided with a stained band, suggesting that trypsin could produce small protein fragments from the IB. In fact, it has been reported by Granum (8) that when trypsin acts on the 35,000-molecular-weight enterotoxin, three bands are formed: a large band with a molecular weight of 33,000 and two small ones with molecular weights of 16,000 and 12,500. The fast-moving radioactive band may correspond to one of these low-molecular-weight proteins. It is possible that trypsin acted on the IB and then on the enterotoxin subunits released from the 14C-labeled IB.
A different pattern, however, was observed when bile salts were added to trypsin (Fig. 4D). Radioactivity was detected only on top of the gel, and no peaks were observed in the separating gel. Similar results were obtained when 14C-labeled enterotoxin was treated with bile salts and trypsin (data not shown). These results suggested that bile salts, due to their detergentlike properties, promoted the aggregation of enterotoxin and thus the aggregation of IB subunits.

For comparison, 14C-labeled IB were solubilized by method x (Table 2). In this case, the stained band corresponded completely with the radioactive counts (Fig. 4E). No radioactive or stained peaks were observed on top of the gel, again suggesting that the procedure was a very efficient solubilization method.

IB and enterotoxin were also subjected to proteolytic digestion with S. aureus protease V8 (Fig. 5A and B). The radioactive pattern was similar in both, except that some IB material remained on top of the gel. The similar pattern on the separating gel suggested a similar primary structure in enterotoxin and IB.

**DISCUSSION**

*Clostridium perfringens* is one of the major causes of food poisoning in humans. An enterotoxin is known to be responsible for the pathological effect. Although the characteristics of this toxin are well described in the literature, the mechanism for its production is still unknown. The present work suggests that the enterotoxin accumulates intracellularly as an IB. This idea is supported by biochemical, serological, and immunocytochemical studies.

Our results indicated that the isolated IB contained only protein. No lipids, carbohydrates, or nucleic acids were detected. Denaturing and reducing agents were necessary to solubilize the IB into a single subunit component. An alkylating agent was required to prevent reaggregation of the subunits, suggesting the importance of disulfide bonds in maintaining the structure of the inclusions. Subunits resulting from solubilization of the IB had several characteristics in common with enterotoxin: (i) a molecular weight of 38,000, which was similar to the molecular weight of 35,500 obtained for purified enterotoxin subjected to the same conditions, was obtained (ii) the amino acid compositions of solubilized IB and purified enterotoxin were found to be very similar, (iii) they aggregated in the presence of SDS (this same characteristic has been previously observed in purified enterotoxin [22, 23]), (iv) they exhibited a reaction of complete identity with purified enterotoxin, as detected by gel diffusion (19), suggesting the presence of a common protein, and (v) upon digestion with *S. aureus* protease V8, solubilized IB and enterotoxin displayed a similar peptide pattern, indicating similarities in their primary structure.

Additional evidence supporting the hypothesis that enterotoxin accumulates intracellularly in distinctive IB included the in situ localization of enterotoxin by immunocytochemistry. The reactivity in the IB strongly supported the hypothesis that the IB represent the structural component where enterotoxin accumulates. Staining of the spores was expected, since spore coat proteins share one or more common proteins (6, 7). The presence of staining around the cell wall suggested that some synthesis of enterotoxin or enterotoxinlike proteins may occur in the cell membrane of sporulating cells of *C. perfringens*.

The question still remains as to whether the IB are solubilized to release free enterotoxin in vivo. The IB produced by *C. perfringens* seem to represent compartmentalized enterotoxin. If this is true, there may be a mechanism by which the IB are degraded into the 35,000-molecular-weight native unit. Proteolytic activity could be responsible for this mechanism. Proteases could have three origins. (i) The intracellular proteases of *C. perfringens* could act on their IB either before or after sporangial lysis. (ii) Proteolytic enzymes could be attached to the IB; after lysis of the sporangia in the human intestine, the IB could be degraded by their own proteases. (iii) IB could be degraded into enterotoxin subunits by proteases present in the human intestine. No proteolytic activity was associated with the IB. Thus, an autodigestion process is not likely, as was proposed for the IB of *Bacillus thuringiensis* (3). The role of intracellular proteases of *C. perfringens* seems minimal. Furthermore, after sporangial lysis in the human intestine, the concentrations of the intracellular proteases and IB would be so low that their interaction would seem very unlikely. No definite conclusions could be drawn on the potential effect of intestinal proteases on the IB, since only trypsin was tested. Trypsin appeared to have some effect on the inclusions, but it did not seem to be dramatic. The effect of trypsin on the IB was reversed in vitro by the addition of bile salts. This could be attributed to a reaggregation of the enterotoxin subunits by the detergentlike characteristics of the bile salts. IB may be solubilized in vivo by a combination of factors, including pH and various bacterial and intestinal proteases. However, the IB may very well remain intact after their release from the sporangia. It should be noted that the substantial amount of free-molecular-weight soluble enterotoxin is present in the sporulating cells of most food-poisoning strains. We have not attempted to quantitate the
distribution of the toxin between the “aggregated” state, i.e., IB, and the soluble form.

The question remains as to why the enterotoxin is synthesized in the first place. Some authors suggest that enterotoxin is a part of the spore coat which remains free, owing to loosely regulated synthetic processes (6). This would imply that enterotoxin synthesis is intimately related to the synthesis of spore proteins. However, Granum et al. (9) detected the presence of small amounts of enterotoxin in vegetative cells. It is possible that enterotoxin from only a few early sporulating cells contributed to these results. Enterotoxin, like spore coat proteins (15), may be an early (stage II) event in the sporulation process. Extensive electron-microscopic studies would be necessary to resolve this issue.

On the other hand, the enterotoxin could represent an intermediary or end product of metabolism related to the protein turnover known to occur after exponential growth ceases (12). In any event, genetic control must be responsible for the large amounts of enterotoxin produced during sporulation of food-poisoning strains.

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LITERATURE CITED