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Purification and Characterization of a *Shigella dysenteriae* 1-Like Toxin Produced by *Escherichia coli*

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A toxin from an enteropathogenic strain of *Escherichia coli* (*E. coli* H30) was purified to apparent homogeneity from cell lysates. The steps used to isolate the *E. coli* H30 toxin included French pressure-cell disruption of bacteria grown in iron-depleted media, Affi-Gel Blue chromatography, chromatofocusing, and anti-Shiga toxin affinity chromatography. The mobilities of the subunits of radioiodinated *E. coli* H30 toxin and Shiga toxin observed after the two toxins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis were identical. In the absence of 2-mercaptoethanol, a narrow band was seen at M_r 31,500 (±1,000), and a wide heavy band was observed between M_r 4,000 and 15,000. In the presence of 2-mercaptoethanol, bands were seen at M_r 31,500 (±1,000), 27,000, and 4,000 to 15,000. Other similarities between purified *E. coli* H30 and Shiga 60R toxins included identical isoelectric points (7.03 ± 0.02); comparable biological activities, i.e., cytotoxicity, lethality for mice, and enterotoxicity; and the same relative heat stabilities (up to 65°C for 30 min). Nevertheless, the two toxins had apparently different molecular weights as determined by sucrose gradient analysis, by gel filtration, and by cross-linking experiments with dimethyl suberimidate. The M_r of native *E. coli* H30 toxin estimated from cross-linking studies was 48,000, whereas the estimated M_r of Shiga 60R toxin was 58,000. These results suggest that like the cholera–*E. coli*–heat-labile toxin family, a family of Shiga-like toxins exists.

Escherichia coli is one of several agents responsible for acute bacterial gastroenteritis. Two pathogenic mechanisms have been described by which *E. coli* can mediate diarrhea (8). Some strains of *E. coli* colonize the mucosal surface of the small bowel and elaborate heat-labile or heat-stable enterotoxins or both that cause fluid secretion and, hence, diarrhea (30). In contrast to these noninvasive enterotoxigenic strains, other *E. coli* isolates are serologically related to the shigellae (10) and, like shigellae, they are able to penetrate and multiply within colonic epithelial cells (8). These enteroinvasive *E. coli* strains are recognized by their ability to evoke keratoconjunctivitis in guinea pigs (Séreny test, reference 27). Strains of a third group designated by serotype as enteropathogenic *E. coli* (EPEC) have been incriminated as agents of epidemic and endemic diarrheal disease in infants (1, 26), but they are not enteroinvasive, nor do they produce heat-labile or -stable enterotoxins (12). Recent studies from this laboratory (20) revealed that most EPEC and some other *E. coli* enteric isolates (heat-labile and -stable enterotoxin-positive organisms and an enteroinvasive strain) make a cell-associated cytotoxin that can be neutralized by antitoxin prepared against the purified toxin of *Shigella dysenteriae* 1 (Shiga). Moreover, like Shiga extracts, cell lysates of certain EPEC strains are also enterotoxic for rabbit ileal segments and lethal and paralytic for mice. That a cell-associated cytotoxin could be a virulence determinant in EPEC strains is suggested by the observation that the destruction of gut epithelial cell microvilli seen on small bowel biopsies of human infants with EPEC diarrhea is confined to regions of dense *E. coli* adherence (5, 25). In this investigation, the Shiga-like toxin from one EPEC strain, *E. coli* H30, was purified to apparent homogeneity, and its biological and biochemical characteristics were compared with those of Shiga toxin.

MATERIALS AND METHODS

Bacterial strains. *E. coli* H30, an 026 EPEC isolate (14), was used as the source of Shiga-like toxin (20). Shiga toxin was prepared from *Shigella dysenteriae* 1 strain 60R (7).

Antitoxin. Monospecific antitoxin against purified

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Shiga 60R toxin was raised in rabbits by injection of toxin-antitoxin complexes, as previously described (19).

Preparation of bacterial extracts. Iron-depleted syncase broth was prepared by a previously described method (18, 20). Organisms were incubated for 48 h with shaking (280 rpm) at 37°C. Culture volumes were typically 8 to 12 liters. Bacteria were harvested by centrifugation, washed, and disrupted in a French pressure cell, and extracts were clarified by ultracentrifugation as detailed elsewhere (19).

Toxin purification. French press extracts were dialyzed against 100 volumes of 10 mM sodium phosphate buffer (pH 7.4) and then were applied to a column (2.5 by 23 cm) that contained 50 ml of Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.), a chromatographic procedure originally used by Olsnes et al. (23). The column was then washed with 10 mM sodium phosphate buffer (pH 7.4) until the optical density at 280 nm (OD_{280}) was <0.050. Material which had bound to the column was eluted with 500 mM NaCl in 10 mM sodium phosphate (pH 7.4). A pool of this eluate was then dialyzed against 200 volumes of 25 mM Tris (pH 8.3). Next, the partially purified toxin was concentrated to a volume of 5 ml on an Amicon concentrator with a YM-10 membrane (Amicon Corp., Lexington, Mass.). The concentrated toxin was then applied to a column (0.9 by 30 cm) that contained 20 ml of polybuffer exchanger PBE 94 (Pharmacia, Uppsala, Sweden) which had been equilibrated with 25 mM Tris (pH 8.3). This ion-exchange column was eluted with a mixture of polybuffer 96 and polybuffer 74 as described by Pharmacia ("Chromatofocusing with Polybuffer and PBE," technical brochure) to generate a pH gradient between pH 8.0 and 5.0. Increments of 0.02 pH units were formed per 2-ml column fraction. By this chromatofocusing procedure, the HeLa cell cytotoxic peak fraction occurred between pH 6.9 and 7.1. The peak sample and the two fractions on either side of it were pooled and applied to a 40-ml column of rabbit anti-Shiga toxin linked to Sepharose 4B (Pharmacia) that had been prepared as previously described (19). The antitoxin column was washed with 140 mM NaCl in 10 mM phosphate (pH 7.4) until the OD_{280} was ≤0.005. Bound toxin was eluted with 3.5 M $MgCl_2$, and samples were pooled, dialyzed against 200 volumes of 140 mM NaCl in 10 mM phosphate (pH 7.4), and stored at −70°C.

Protein measurements. To conserve toxin, protein concentrations were monitored spectrophotometrically (15) at each stage of purification by the following formula: $1.55(OD_{280} - OD_{315}) - 0.76(OD_{260} - OD_{315})$, where OD_{280}, OD_{315}, and OD_{260} are the optical densities at 280, 315, and 260 nm, respectively. Subtraction of OD_{315} readings from OD_{280} and OD_{260} values, a modification of Layne's formula (15), was used to negate the effects of light scattering from any particulate matter present in the samples. The protein concentrations estimated by this method were no more than 2.5-fold different (greater or less) than values calculated for crude samples tested by the Lowry method (16).

Biological assays. Biological assays for toxin activity were performed as previously described for HeLa cell cytotoxicity (11), enterotoxicity in rabbit ileal loops (9), and lethality for mice (21). When required, Shiga toxin or *E. coli* Shiga-like toxin activities were neutralized by rabbit anti-Shiga toxin (21).

Radioiodination. Purified and partially purified preparations of toxin from Shiga 60R and *E. coli* H30 were labeled by a modification of the chloramine T method (13). Carrier-free sodium iodide (1 mCi; Amersham Corp., Arlington Heights, Ill.) was added to 10 to 100 μg of toxin in 500 μl of 140 mM NaCl in 10 mM phosphate (pH 7.4) buffer. The solution was then made 0.2 mM chloramine T by the addition of appropriate quantities of concentrated reagent. After a 5-min incubation on ice, the mixture was made 0.5 mM sodium metabisulfite and 10 mM NaI. One minute later, 100 μl of a 1 mg/ml solution of myoglobin was added to prevent nonspecific absorption of the radiolabeled toxin to the glassware. The mixture was then dialyzed extensively against 140 mM NaCl in 10 mM phosphate (pH 7.4).

Sucrose gradients. Radiolabeled toxin samples were layered onto 4-ml, 10 to 40% sucrose gradients in 140 mM NaCl–10 mM phosphate (pH 7.4) and centrifuged in a Beckman SW-60 rotor at 485,000 × *g* (60,000 rpm) for 20 h. Fractions of 200 μl were collected from the tops of the tubes with a Buchler Auto Densi Flow II C (Buchler Instruments, Fort Lee, N.J.), and the refractive index of each fraction was measured to quantitate the percent sucrose of the sample. To locate toxin, the radioactivity and cytotoxic activity of each fraction was determined. The OD_{280} reading and the refractive index of each fraction from a separate tube that contained bovine serum albumin and myoglobin were used to determine the position of the toxin relative to these protein standards.

Immunoprecipitation. Immunoprecipitations of ^{125}I-labeled toxin samples were carried out as previously described (19).

Polyacrylamide gel electrophoresis. Immunoprecipitates or samples of toxins were mixed with an equal volume of disruption buffer (100 mM Tris [pH 7.0], 4% [wt/vol], sodium dodecyl sulfate [SDS], 10% [wt/vol] sucrose, and 0.04% [wt/vol] bromophenol blue). Samples were incubated in this mixture with or without 0.7 M 2-mercaptoethanol for 20 min at 37°C followed by 2 min at 100°C and applied to slots of a 15% acrylamide slab or cylindrical gel cross-linked with *N,N'*-diallyltartardiamine. The buffer system used was that described by Morse et al. (17). The gels were run at 150 V constant voltage until the tracking dye reached the bottom of the gel. The slab gels were then fixed, dried, and exposed to Kodak XAR-5 film (5 by 7 in. [12.7 by 17.8 cm], Kodak, Rochester, N.Y.) with an intensifier screen (Cromex Xtra Life, Du Pont Co., Wilmington, Del.). Nondenaturing polyacrylamide gel electrophoresis was undertaken in cylindrical gels (0.5 by 10 cm) prepared as described above, except that 10% acrylamide was used and both SDS and 2-mercaptoethanol were omitted from the mixture. Cylindrical gels were fractionated into 2-mm slices, and the radioactivity in each sample was quantitated. For some experiments, toxin from gel slices was eluted into 250 μl of HeLa cell tissue culture media (11) by overnight incubation at 4°C, and the eluted material was subsequently analyzed for cytotoxic activity. Isoelectric focusing was done in 7% bis-acrylamide gels as described by Wrigley (32).

Enzyme treatment. Purified, radiolabeled toxin from

TABLE 1. Purification of a Shiga-like toxin from *E. coli* H30

Sample	Total protein (mg)[a]	Sp act (CD_{50}/mg of protein)[b]	Recovery of cytotoxic activity (%)	Immunoprecipitability of ^{125}I-labeled sample with anti-Shiga toxin (%)
French press lysate[c] clarified by ultracentrifugation	277	3×10^5	100	Not done
Affi-Gel Blue chromatography retentate	24	5×10^6	160	1
Chromatofocus fraction at pH 7.03	0.15	3×10^8	67	18
Antitoxin affinity chromatography	0.10	5×10^8	67	>90

[a] Protein concentrations were estimated spectrophotometrically by the formula given in the text.

[b] CD_{50} is the dose of toxin required to kill 50% of the approximately 16,000 cells in a microtiter well within 24 h.

[c] Cultures (8 liters) were grown for 48 h in iron-depleted (i.e., Chelex-treated) modified syncase broth.

Shiga 60R or *E. coli* H30 was added to an equal volume of trypsin (diphenyl carbamyl treated, Sigma Chemical Co., St. Louis, Mo.) or α-chymotrypsin (Sigma) in 10 mM phosphate with 140 mM NaCl (pH 7.4) to give a final enzyme concentration of 10 μg/ml. The mixtures were incubated for 4 h at 37°C. An excess (20 μg/ml) of egg white trypsin inhibitor (Sigma) was added to the trypsin-exposed toxin. All samples were frozen at −20°C.

Cross-linking. Radiolabeled, purified toxin from Shiga 60R or *E. coli* H30 was cross-linked with dimethyl suberimidate (Pierce Chemical Co., Rockford, Ill.) in 1 M triethanolamine–100 mM sodium carbonate (pH 8.5) as detailed by Davies and Stark (6). Dimethyl suberimidate was added at a final concentration of 1, 6, or 12 mg/ml and allowed to react with toxin (10^6 cpm/ml) for 19 h at room temperature. Disruption buffer was then added, and the samples were subjected to SDS-polyacrylamide slab gel electrophoresis.

RESULTS

Purification of Shiga-like toxin from *E. coli* H30. The method developed to obtain purified toxin from cell extracts of *E. coli* H30 incorporates several techniques which have been successfully used to purify Shiga toxin, i.e., Affi-Gel Blue column chromatography (23) and antitoxin affinity chromatography (19), plus a new step, chromatofocusing, that separates proteins by their isoelectric points (28, 29). During

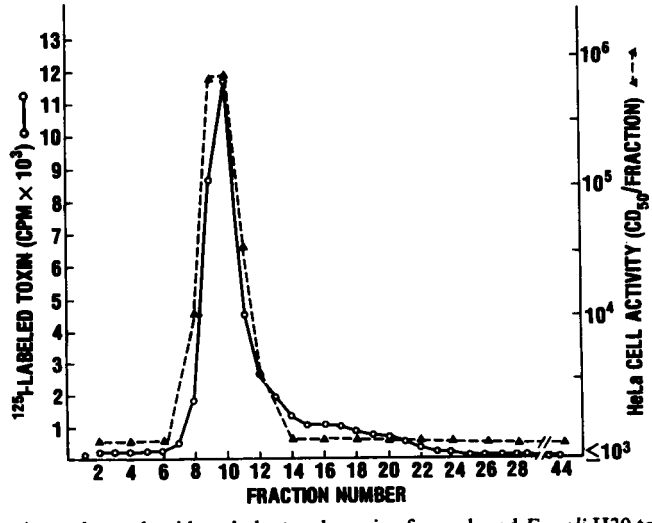

FIG. 1. Nondenaturing polyacrylamide gel electrophoresis of unreduced *E. coli* H30 toxin. *E. coli* H30 toxin was purified by the method outlined in Table 1, labeled with ^{125}I, and subjected to polyacrylamide gel electrophoresis. The gels were then fractionated, and the radioactivity and cytotoxicity associated with each 2-mm slice were quantitated.

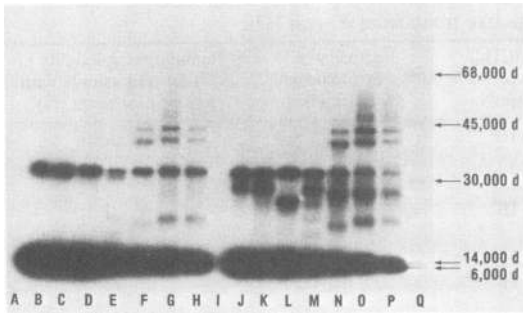

FIG. 2. SDS-polyacrylamide gel electrophoresis of unreduced *E. coli* H30 and Shiga 60R toxins. Purified toxins were labeled with ^{125}I and immunoprecipitated with immune or preimmune sera or treated with various enzymes or cross-linking reagents; the preparations were then subjected to SDS-polyacrylamide gel electrophoresis in 15% slab gels. The gels were dried and evaluated by autoradiography. Lanes: A, *E. coli* H30 toxin immunoprecipitated with preimmune rabbit serum (no bands); B, *E. coli* H30 toxin immunoprecipitated with rabbit anti-Shiga 60R toxin; C, *E. coli* H30 toxin, no treatment; D, *E. coli* H30 toxin pretreated with trypsin; E, *E. coli* H30 toxin pretreated with α-chymotrypsin; F, *E. coli* H30 toxin cross-linked with 1 mg of dimethyl suberimidate per ml; G, *E. coli* H30 toxin cross-linked with 6 mg of dimethyl suberimidate per ml; H, *E. coli* H30 toxin cross-linked with 12 mg of dimethyl suberimidate per ml; I, Shiga 60R toxin immunoprecipitated with preimmune rabbit serum (no bands); J, Shiga 60R toxin immunoprecipitated with rabbit anti-Shiga 60R toxin; K, Shiga 60R toxin, no treatment; L, Shiga 60R toxin, pretreated with trypsin; M, Shiga 60R toxin, pretreated with α-chymotrypsin; N, Shiga 60R toxin cross-linked with 1 mg of dimethyl suberimidate per ml; O, Shiga 60R toxin, cross-linked with 6 mg of dimethyl suberimidate per ml; P, Shiga 60R toxin, cross-linked with 12 mg of dimethyl suberimidate per ml; Q, ^{14}C-labeled molecular weight standards, bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), lysozyme (14,000), aprotinin (6,000).

the design of this protocol, the purity of toxin at each step was assessed by radiolabeling samples and by determining the percentage of radioactivity that was immunoprecipitable with monospecific antitoxin. As toxin is now routinely purified, only samples of the final product are radioiodinated and checked for purity by immunoprecipitability with antitoxin and by the mobility of radiolabeled toxin after SDS-polyacrylamide gel electrophoresis (see below). In addition, myoglobin is added at a final concentration of 0.1% (wt/vol) to all purified toxin samples to prevent the material from nonspecifically adhering to glassware.

The details of yield, purity, and specific activity of the *E. coli* H30 toxin for a typical preparation are given in Table 1. Two discrepancies in the data warrant comment. First, the apparent increase in toxin yield as determined by cytotoxic activity after Affi-Gel Blue chromatography was a reproducible phenomenon and could indicate that an inhibitor was removed from the crude extract by this step. Second, estimates of the purity of toxin after the chromatofocusing step or after the affinity chromatography step were probably evaluated more accurately by the percent immunoprecipitability of toxin with antitoxin than when determined by the specific activity of toxin because toxin was diluted in 10-fold increments for tests of cytotoxic activity, whereas undiluted radiolabeled toxin was tested for immunoprecipitability.

Evidence that *E. coli* H30 toxin was in fact purified to homogeneity by the protocol delineated in Table 1 was obtained from polyacrylamide gel electrophoresis. Under nondenaturing conditions, both cytotoxic activity of *E. coli* H30 toxin and its associated radioactivity comigrated (Fig. 1). Furthermore, when radiolabeled toxin was subjected to SDS-polyacrylamide slab gel electrophoresis (Fig. 2), two bands of M_r approximately 31,500 (±1,000) and 4,000 to 15,000 were seen. The broad band of M_r 4,000 to 15,000 could be narrowed to a range of M_r 4,000 to 11,000 by decreasing the time of X-ray exposure, but the 31,500 M_r band was much less distinct under those circumstances. These two bands were also evident when gels were stained with Coomassie blue, but the bands were too faint to be photographed well. In the presence of 2-mercaptoethanol, three bands of M_r 31,500 (±1,000), 27,000, and 4,000 to 15,000 were observed (Fig. 3). However, the 31,500 M_r band

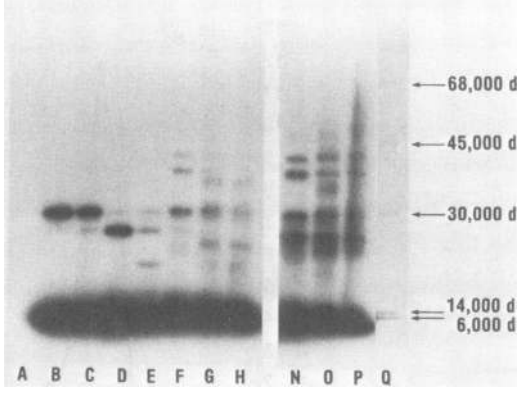

FIG. 3. SDS-polyacrylamide gel electrophoresis of reduced *E. coli* H30 and Shiga 60R toxins were labeled and treated as in Fig. 2, except that before electrophoresis all samples were reduced with 2-mercaptoethanol. Lanes A to H and N to Q contain the reduced samples corresponding to the unreduced samples in lanes A to H and N to Q in Fig. 2. Lanes I to M of this gel were not clearly resolved and are not shown.

TABLE 2. Biological activities of purified Shiga and *E. coli* Shiga-like toxins

Source of toxin	Cytotoxic dose 50% for HeLa cells[a] (pg)	Enterotoxic dose 50% for rabbit ileal segments[b] (μg)	Lethal dose 50% for mice[c] (μg)
S. dysenteriae 1 60R	1	1	0.10
E. coli H30	1	0.2	2

[a] The dose of purified toxin required to kill 50% of the approximately 16,000 HeLa cells (11) in a microtiter well within 24 h.

[b] The dose of toxin required to cause ≥10 ml of fluid secretion in a 10-cm ligated segment of rabbit ileum in 50% of the loops tested.

[c] The 50% lethal dose for a 20-g outbred CD-1 mouse given toxin intraperitoneally. All of the mice which succumbed to the effects of these toxins exhibited hind-leg paralysis before death.

was absent when toxin was both enzymatically nicked with trypsin and reduced with 2-mercaptoethanol (Fig. 3). The mobilities of these *E. coli* H30 toxin bands after the various treatment protocols and the observation that more radiolabel was associated with the 4,000 to 15,000 M_r band than with the 31,500 M_r component is consistent with the appearance of similarly treated Shiga 60R toxin (2, 19, 22, 23; Fig. 2 and 3).

Further evidence which substantiates the supposition that the *E. coli* toxin preparations were pure and which also verifies the antigenic relationship betwen Shiga toxin and *E. coli* H30 toxin was obtained from immunoprecipitation studies. The final *E. coli* toxin product was >90% immunoprecipitable with antitoxin prepared against purified Shiga 60R toxin but was not immunoprecipitable with monospecific antitoxin raised against purified cholera enterotoxin or purified *E. coli* heat-labile toxin (rabbit anti-cholera toxin and rabbit anti-heat-labile *E. coli* toxin were gifts of Randall Holmes, Uniformed Services University of the Health Sciences).

Comparison of the characteristics of *E. coli* H30 and Shiga 60R toxins. (i) Biological activities. The specific activity of purified *E. coli* H30 toxin varied from 5×10^8 CD_{50} (50% HeLa cell cytotoxic dose) to 1×10^9 CD_{50} per mg of toxin with separate lots of toxin. Thus, like Shiga toxin (18), approximately 1 pg of toxin was a cytotoxic dose for HeLa cells. Moreover, purified *E. coli* H30 toxin also resembled Shiga toxin (2, 19) in that it was enterotoxic for rabbit ileal segments and was paralytic and lethal for mice. The specific biological activities of the two toxins are compared in Table 2. The significance of differences between Shiga 60R and *E. coli* H30 in the dose of toxin required for enterotoxicity and lethality cannot be evaluated because of limitations in the amounts of purified toxins available to perform multiple assays. The molecular basis for these biological activities is believed to be inhibition of protein synthesis in eucaryotic cells (3, 4, 24, 31).

(ii) Estimates of molecular weights of native toxins. Olsnes and Eiklid (22) observed that the peaks of radioactivity and toxicity associated with Shiga toxin cosedimented on a sucrose gradient at a position which indicated a molecular weight for Shiga toxin greater than that of bovine serum albumin (68,000) but less than that of transferrin (85,000). The profile of purified Shiga 60R toxin on a sucrose gradient prepared in this laboratory (Fig. 4A) supports the finding of Olsnes and Eiklid of a molecular weight greater than 68,000. By contrast, radioactivity and cytotoxicity of *E. coli* H30 toxin cosedimented on a sucrose gradient at a location that suggests a molecular weight of less than 68,000 (bovine serum albumin) (Fig. 4B). Molecular weight estimates of Shiga 60R toxin and *E. coli* H30 toxin obtained by gel filtration also suggest differences in the sizes of the two toxins, although the values obtained for both toxins were aberrantly low for reasons that are not clear. Shiga 60R toxin appeared to have a molecular weight of 32,000 when chromatographed on a Sephacryl S-200 (Pharmacia) column (19), whereas the molecular weight estimate obtained for *E. coli* H30 toxin on the same column was consistently lower (25,000 to 28,000; data not shown). Shiga 60R toxin had an apparent molecular weight of 45,000 on a Bio-Gel P-100 column (Bio-Rad) run under the same buffer conditions and calibrated with the same standards as previously described for Sephacryl S-200 columns (19), a difference which may reflect an interaction of toxin with the carbohydrate-containing Sephacryl gel.

(iii) Arrangement of subunits. Cross-linking studies were performed to compare the molecular organization of the subunits of purified Shiga 60R and *E. coli* H30 toxins. Different concentrations of dimethyl suberimidate were used (1, 6, and 12 mg/ml), and the cross-linked toxin preparations were then subjected to SDS-polyacrylamide gel electrophoresis in the absence or presence of 2-mercaptoethanol (Fig. 2 and 3). A concentration of 6 mg/ml of the cross-linking reagent was judged to give the best results, i.e., the most well-resolved complexes. Therefore, the molecular weights of complexes formed under these conditions were estimated, and the possible subunit arrangements were determined (Table 3). The B subunit was given an estimated M_r of 4,000 from a plot of presumed numbers of B chains versus estimated molecular weight of the complex, a technique described by Olsnes et al. (23). From these findings, it again appeared

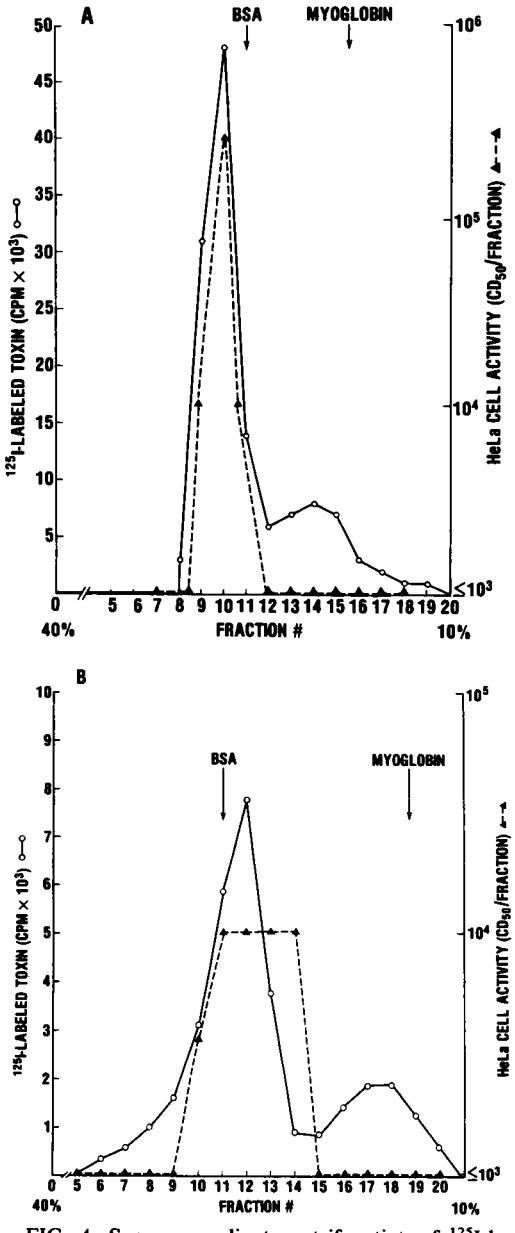

FIG. 4. Sucrose gradient centrifugation of ^{125}I-labeled Shiga 60R toxin (A) and *E. coli* H30 toxin (B). The 10 to 40% sucrose gradients were prepared and centrifuged as described in the text. Fractions were obtained and evaluated for refractive index, radioactivity, OD_{280}, or cytotoxic activity as detailed in the text.

that the molecular weight of native Shiga toxin (estimated as 58,000) was greater than that of *E. coli* toxin (estimated as 48,000). It should be emphasized that estimations of molecular weights from cross-linking data are accurate only if the capacity of the protomers to form cross-links reflects their spatial arrangement in the oligomer.

(iv) Isoelectric points. Biologically active Shiga 60R toxin had an isoelectric point of 7.03, as averaged from four separate analyses (Fig. 5A). The average isoelectric point of the biologically inactive B subunit was 5.67. Similarly, *E. coli* H30 cytotoxin had an isoelectric point of 7.01 (Fig. 5B, peak I), whereas the B subunit (peak II, as confirmed by appearance on a gel, not shown) had an isoelectric point of 5.66. Differences in the relative heights of peaks I and II for Shiga 60R and *E. coli* H30 toxin after isoelectric focusing (Fig. 5A and 5B, respectively) probably reflect different ratios of intact toxin to B subunit.

(v) Relative heat stability. Both Shiga 60R and *E. coli* H30 toxin retained full biological activity after exposure to temperatures between 37 and 65°C for 30 min in 10 mM phosphate (pH 7.4). Both toxins were completely inactivated by boiling for 2 min.

DISCUSSION

Toxin purified from *E. coli* H30 is biologically and structurally similar to Shiga 60R toxin. Toxins from both organisms are cytotoxic for HeLa cells at picogram doses, and both toxins are lethal for mice and enterotoxic for rabbits in microgram or submicrogram doses. Each toxin consists of an A subunit (M_r = 31,500 ± 1,000) and several copies of a B subunit (M_r about 4,000). The A subunit can be nicked by trypsin or by endogenous proteases to form an A_1 component (M_r = 27,000) and either an A_2 component ($M_r \simeq 4{,}500$), the existence of which has not been verified by resolution of a distinct band on polyacrylamide gel electrophoresis (23), or several peptide fragments. The function of each toxin in the normal physiology of the bacterial cell has not been investigated. Moreover, the genetic mechanisms responsible for toxin production have not been determined for either *E. coli* or shigellae.

Although the studies reported herein showed that *E. coli* H30 and Shiga 60R toxins were nearly indistinguishable, subtle differences between the toxins were observed. Despite the limitations described above for each method used to evaluate the molecular weights of the native toxins, i.e., sucrose gradient, gel filtration, and toxin cross-linking, the results from all three procedures indicated that *E. coli* H30 toxin had a lower molecular weight (estimated as 48,000 by cross-linking studies) than Shiga 60R toxin (estimated as 58,000 by cross-linking studies). The cross-linking results suggest that the variation in toxin size occurs because native *E. coli* H30 toxin has fewer copies of the B subunit

TABLE 3. Subunit complexes formed after cross-linking *E. coli* H30 toxin or Shiga 60R toxin

Source of toxin	Treated with 2-mercaptoethanol	Estimated M_r[b]	Possible subunit arrangement[c]
E. coli H30	No	48,000	A + 4B
		43,000	A + 3B
		40,000	A + 2B
		31,500*	A
		20,000	5B
		15,000–4,000*	Mixture of 1B, 2B, 3B, and 4B
E. coli H30	Yes	44,000	A + 3B
		40,000	A + 2B
		38,000	A_1 + 3B
		31,500	A
		27,000	A_1
		24,000–22,000	5B + A_2
		20,000	5B
		15,000–4,000*	Mixture of 1B, 2B, 3B, 4B, and A_2
Shiga 60R	No	58,000	A + 6B
		53,000	A + 5B
		49,000	A + 4B
		43,000*	A + 3B
		40,000*	A + 2B
		31,500*	A
		27,000*	A_1 (partially nicked sample?)
		20,000*	5B
		15,000–4,000	Mixture of 1B, 2B, 3B, and 4B
Shiga 60R	Yes	58,000	A + 6B
		44,000	A + 3B
		40,000	A + 2B
		35,000	A_1 + 2B
		31,500*	A
		27,000	A_1
		24,000–22,000*	5B + A_2
		15,000–4,000	Mixture of 1B, 2B, 3B, 4B, and A_2

[a] ^{125}I-labeled toxins were cross-linked by exposure to dimethyl suberimidate at a final concentration of 6 mg/ml as described by Davies and Stark (6). The reaction mixtures were then subjected to SDS-polyacrylamide gel electrophoresis in a 15% slab gel with or without pre-exposure to 0.7 M 2-mercaptoethanol.

[b] For the purpose of comparing these findings with previously reported cross-linking results on Shiga toxin, the arrangement of this table is patterned after the data presentation in Table 2 of reference 23. Very heavy bands are indicated with an asterisk. Very faint bands are underlined. Diffuse bands are indicated by a range of estimated molecular weights.

[c] B is given an estimated M_r of 4,000; A_2 is given an estimated M_r of 4,500 from the difference in M_r of A and A_1.

(estimated four) than does Shiga 60R toxin (estimated six). Alternatively, *E. coli* H30 native toxin may have the same M_r as Shiga 60R toxin but may dissociate more readily during purification because of differences in the strength of the hydrophobic interactions between the subunits. That Shiga 60R toxin may actually have more than six B subunits is suggested by the observation that toxin appears larger than bovine serum albumin on repeated analyses by sucrose gradient.

Another apparent difference between the *E. coli* H30 toxin and the Shiga 60R toxin is that little of the *E. coli* toxin in any preparation existed in the "nicked" (i.e., A_1) form (see Fig. 2). The *E. coli* H30 toxin had to be treated with trypsin and reduced with 2-mercaptoethanol to appear in the A_1 form. By contrast, most preparations of the Shiga 60R toxin were already partially nicked (presumably by endogenous proteases) and reduced, as could be seen on gels even in the absence of 2-mercaptoethanol (Fig.

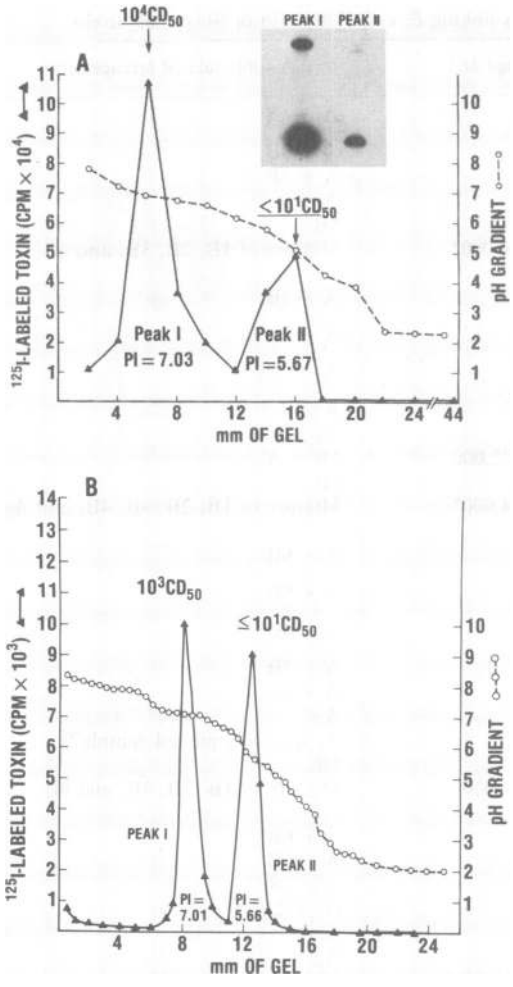

FIG. 5. Isoelectric focusing of ^{125}I-labeled Shiga 60R (A) and *E. coli* H30 (B). Samples were subjected to isoelectric focusing in 7% bis-acrylamide gels as described by Wrigley (32). Samples were eluted from gel fractions into 300 μl of 0.85% NaCl, and the pHs of the eluates were determined. For toxin-containing gels run in parallel, samples were eluted into 250 μl of tissue culture medium (19) and tested for radioactivity and cytotoxicity. The CD_{50} values shown reflect the activity associated with the most radioactive sample in each peak. The insert in panel #A is an autoradiograph of nonreduced samples from peaks I and II run on 15% SDS-polyacrylamide gels.

2). These observations could indicate that *E. coli* H30 toxin is more resistant to proteolytic cleavage than is Shiga 60R toxin. An equally tenable explanation is that bacteria of the Shiga 60R strain have higher levels of endogenous proteases than do *E. coli* H30 organisms.

In conclusion, the findings presented herein support the concept that a family of Shiga-like toxins exists. A more detailed comparative evaluation of the structure and function of Shiga 60R and *E. coli* H30 toxins at the molecular level, e.g., amino acid sequencing and mechanisms of inhibition of protein synthesis, is required to define more clearly the similarities and differences between the family members. Finally, the role of Shiga toxin (if any) in the pathogenesis of shigellosis or of the Shiga-like toxin in certain *E. coli* diarrheal diseases remains to be determined.

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