Chromatofocusing: A New Method for Purification of Staphylococcal Enterotoxins B and C_1

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A new chromatographic procedure was developed which obtained highly purified preparations of staphylococcal enterotoxins B and C_1 in yields of 60% from cultures of *Staphylococcus aureus* and which is faster than any of the separation methods used previously. The procedure involves chromatography on carboxymethylcellulose, removal of alpha-toxin by adsorption to rabbit erythrocyte membranes, and, finally, chromatofocusing as the fundamental new step. Enterotoxins were obtained in highly purified form and behaved in a homogeneous manner as determined by ultracentrifugation and electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate, with molecular weights of 34,000 for staphylococcal enterotoxin B and 30,000 for staphylococcal enterotoxin C_1. Using chromatofocusing as the final purification step, we isolated three B and six C_1 distinct but immunologically identical enterotoxin fractions, which were found to be devoid of any impurities and to possess a marked degree of toxicity in monkeys.

The staphylococcal enterotoxins (SEs), the causative agents of food poisoning, are a closely related group of water-soluble exoproteins produced by some strains of *Staphylococcus aureus* under certain conditions in food and culture media.

So far, six antigenically distinct enterotoxins have been identified (A, B, C_1, C_2, D, and E [4, 8]) based on their reactions with specific antibodies. Intoxications caused by SEs appeared to be restricted to humans and certain monkeys. The effects on other animals are very contradictory (2).

Since opportunities to study this type of intoxication in humans and rhesus monkeys are rather limited, little is known about the internal mechanisms of biological activities of SEs. For oral application resulting in diarrhea and vomiting, several investigators (15, 42) have postulated that the primary effect of SE is located in the digestive tract.

To simplify finding the site of action, SE has been tested for cytotoxicity in a variety of mammalian cell cultures. The available data from these investigations are in disagreement as to whether SE has cytotoxic properties (26, 29–31).

Since the criteria of purity of SE have not been defined unambiguously in most biological studies, it is quite possible that the cytotoxic effects described so far might have been caused by contaminants possessing broader substrate specificity than SE.

For this reason, it was the aim of the present study to purify SE more rigorously, i.e., free from any contaminants with potential cytotoxicity for mammalian cells.

As a first step, this communication deals with chromatofocusing as a novel procedure for purifying enterotoxins B (SEB) and C_1 (SEC$_1$), in a simple, gentle, and speedy way, leading to a highly purified product.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. aureus* 10-275, selected for large-scale production of SEB (50), and strain 137, a producer of SEC$_1$ (19095 ATCC), were used throughout this study.

Unless otherwise stated, all biochemicals were obtained from Serva (Heidelberg, Federal Republic of Germany); α-lactalbumin and phosphorylase were purchased from Sigma Chemie GmbH, Munich, Federal Republic of Germany. Seed cultures of strain 10-275 were prepared by inoculating flasks containing 3% peptone from soya S/ELB (Serva) and 3% peptone from casein C/ESX (Serva), pH 6.8, supplemented with 0.5% yeast extract with a porcelain bead culture (19) and incubating at 37°C on a rotatory shaker (270 rpm) for 10 h. An inoculum of 0.5% (vol/vol) was added to 800-ml batches of fresh media and incubated for 37 h under the same conditions for SE production.

Strain 137 was cultivated in medium containing 1% yeast extract and 0.5 mg of thiamin (Sigma) per ml and 10 mg of niacin (Sigma) per liter, pH 7.6, as described

previously (3); instead of 3% N-Z-Amine NAK and 3% protein hydrolysate powder (3), 4% peptone from casein C/ELP was used.

Preparation of crude toxin. Cells were removed by centrifugation at 24,000 × g at 4°C for 20 min, and the resulting supernatants were sterilized by filtration through a 0.2-μm filter (Schleicher & Schüll, Dassel, Federal Republic of Germany). Subsequently, the supernatant was adsorbed on Amberlite CG-50 (Serva) and eluted by column chromatography under the conditions described by Schantz et al. (32).

For purification of SEC_1, the method of Borja and Bergdoll (6) was used with minor modifications, omitting the dialysis, concentration, and lyophilization of the culture filtrate. SEC_1 was absorbed on carboxymethylcellulose CM 52 (Whatman Inc., Maidstone, United Kingdom) equilibrated with 0.01 M phosphate buffer, pH 5.5, by a batch procedure, and the elution was subsequently performed in a stepwise manner.

For desalting, ion exchange fractions containing 100 to 200 mg of protein were applied to a column (3.5 by 100 cm) of Sephadex G-50 fine (Pharmacia, Uppsala, Sweden) and eluted with distilled water at a flow rate of 142 ml/h. The profile was monitored by conductivity measurements with an LBR meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Federal Republic of Germany).

Preparation of erythrocyte membranes and further purification steps. For membrane preparation, rabbit erythrocytes collected in Alsever solution (7) were washed three times with 0.15 M phosphate buffer, pH 7.4. Subsequently, the number of erythrocytes per milliliter was determined, and hemoglobin-free membranes were prepared with hypotonic 0.005 M phosphate buffer, pH 7.4, by the method of Dodge et al. (14). Membranes were washed with the same buffer until the supernatant was colorless.

Chromatofocusing. Chromatofocusing (35, 36) was performed according to the instructions of the manufacturer (Pharmacia). Ethanolamine (0.025 M) was degassed and adjusted with 1 N HCl to pH 9.5. Pharmalyte 6.5-9 (Pharmacia) was diluted 1:45 with distilled water, degassed, and adjusted with 1 N HCl to pH 7.7. Polybuffer exchanger type 94 was dispersed in 0.025 M ethanolamine, pH 9.5, and degassed, filled into a column (1 by 40 cm), and packed at a flow rate of 144 ml/h.

Sephadex G-25 preswollen in 0.025 M ethanolamine and degassed was layered on the packed gel. The column was equilibrated with 10 to 15 bed volumes of 0.025 M ethanolamine until the pH and conductivity of the effluent approached that of the starting buffer. Pharmalyte (5 ml, pH 7.7) was applied followed by addition of the sample, adjusted to pH 7.7.

Elution with Pharmalyte, pH 7.7, was performed at a flow rate of 15 ml/h; protein was monitored at 280 nm, and the pH of each fraction was measured immediately. The pH gradient was stopped at pH 8.0 by washing the column with degassed 1 M NaCl until all proteins were eluted.

Removal of Pharmalyte was accomplished by gel filtration on Sephadex G-50 (2.5 by 100 cm) at 4°C with 0.15 M NaCl, pH 6.5, at a flow rate of 18 ml/h. If completely desalted samples are needed, rechromatography on Sephadex G-25 fine with distilled water is recommended.

Concentration of enterotoxin-containing fractions

was performed by cold trap rotary evaporation (Büchi, Eislingen, Federal Republic of Germany) at room temperature.

Analytical methods. Protein was determined by the procedure of Lowry et al. (22), using bovine serum albumin (>99% pure; Serva) as standard. The actual concentration of the stock solution was determined by the electrobalance method (21). SEB and SEC_1 were measured by UV absorbance (277 nm), using absorptivity values ($A_{1cm}^{1\%}$) of 14.4 and 12.1, respectively (2).

The presence of enterotoxin at each purification step was analyzed by Ouchterlony double diffusion analysis (27) by the method of Sommerfeld and Terplan (37).

Antisera against both SEB and SEC_1 as well as standard toxins were purchased from Serva. The titer of precipitating antibodies was found to be 1:40 for anti-SEB and 1:30 for anti-SEC_1 as determined by double diffusion analysis on microslides with a limiting sensitivity of 1 to 2 μg of SE per ml.

Sodium dodecyl sulfate-polyacrylamide disk gel electrophoresis was performed as described by Davies and Stark (13), using 30 μg of protein per gel. Calibration proteins were purchased from Pharmacia. Isoelectric focusing was performed with thin-layer polyacrylamide gels (46) and pH 7 to 10 Ampholines (LKB, Bromma, Sweden). Solutions containing 0.3 to 1 mg of protein per ml were applied to the gel via rectangular pieces of filter paper. The pH was determined at 4°C with an Ingold LKB electrode (type 10 433 131), and the measured linear pH gradient (pH 7.5 to 9.5) was used for pI determination of distinct bands.

Amino acid analysis was performed on a Durrum D-500 analyzer (Durrum Instrument Corp., Palo Alto, Calif.) after hydrolysis in 6 N HCl (Ultrapur; E. Merck AG, Darmstadt, Federal Republic of Germany) for 24 h at 110°C. Before analysis, the dried samples were dissolved in citrate buffer, pH 2.0. For the determination of cysteine, the material was oxidized by dimethyl sulfoxide before hydrolysis (38). Amide nitrogen was determined by the method of Stegemann (41).

Sedimentation velocity measurements were made on a Spinco model E analytical ultracentrifuge with a Schlieren optical system at a speed of 52,000 rpm (4°C, 12 mm). A 74°C single-sector analytical cell was used.

Assay for contaminating materials. In samples containing 1 mg of protein per ml, DNase activity was tested by a minor modification of the procedure of Heins et al. (17). Briefly, DNA substrate (Boehringer Mannheim, Mannheim, Federal Republic of Germany) was incubated at 37°C in a water bath before addition of 0.1% bovine serum albumin (Serva) and 0.1 ml of the SE-containing solution. The reaction was stopped after incubating for 5 min at 37°C by addition of 7% perchloric acid and cooling in an ice bath for 10 min. After centrifugation at 2,100 × g for 10 min, the supernatant was decanted, and absorbance was determined at 260 nm in a spectrophotometer (Zeiss, Oberkochen, Federal Republic of Germany). Purified DNase from *S. aureus* (Boehringer Mannheim) was used as a standard with a detection limit of 0.1 μg/ml.

The amounts of alpha-, beta-, and gamma- and delta-toxin were determined on rabbit, sheep, and human erythrocytes, respectively, by hemolytic titration at a single dose of 1 mg of SEB per ml. After washing the erythrocytes three times with 0.15 M phosphate buffer, pH 7.4, a 1% (vol/vol) suspension was mixed with

the sample (1 mg/ml) to be tested and incubated at 37°C for 1 h. (For beta-toxin, the test tubes were subsequently kept at 4°C overnight.) After centrifugation at 1,000 × g for 15 min, the amount of toxin in the supernatant was measured at 412 nm (1 hemolytic unit [HU] = 50% hemolysis). Purified α-hemolysin (kindly supplied by Behringwerke, Marburg/Lahn, Federal Republic of Germany) was used as a standard with a limiting sensitivity of 0.1 µg/ml).

Protein A was determined (with a detection limit of 30 µg/ml) by double diffusion technique by the method of Ouchterlony (27), using partially purified human immunoglobulin G (20) as developing agent. Purified protein A (Pharmacia) was used as standard. The procedure of Charney and Tomarelli (11) was used for determination of protease, and 1 µg/ml was detectable.

The presence of staphylokinase was tested for with euglobulins (28) isolated from human serum as described by Weir (51). Euglobulins were suspended in water at a protein concentration of 1 mg/ml and added to an equal volume of enterotoxin (1 mg/ml). The mixture was incubated at 37°C for 15 min. After recalcification with 0.025 M calcium chloride, samples were incubated for up to 90 min at room temperature and checked every 15 min for clot lysis. Streptokinase (Sigma) was used as a positive standard.

Toxicity. The biological activity of the purified toxin was assayed by feeding rhesus monkeys (*Macaca fascicularis*; weight, ca. 3 kg) a piece of stale bread containing 5 µg of SEB per kg of body weight. Food and water were provided ad libitum, and the animals were observed continuously for 6 h after administration of the toxin. Untreated monkeys served as controls by eating stale bread with no toxin.

RESULTS

Desalting of enterotoxin by gel filtration through Sephadex G-50.

SEB was removed from the culture medium of *S. aureus* 10-275 by binding to the carboxylic acid exchange resin Amberlite CG-50 (32) and elution with 0.4 M phosphate buffer, pH 6.8–0.25 M NaCl. Fractions containing SEB as detected by immunodiffusion were pooled. Portions (200 ml) with protein concentrations of 2.0 mg/ml were desalted by gel filtration through Sephadex G-50 (3.5 by 100 cm). Elution was performed with degassed distilled water at a flow rate of 142 ml/h, and a representative elution pattern is shown in Fig. 1.

The first fraction contained the majority of SEs and low salt concentrations as reflected by a conductivity of about $3 \times 10^{-4} \, \Omega^{-1} \, cm^{-1}$. Some low-molecular-weight proteins as well as the bulk of salt (conductivity, $2.9 \times 10^{-2} \, \Omega^{-1} \, cm^{-1}$) were found in the second fraction. About 10% of the SEB eluting in the second fraction was discarded.

SEC_1-containing supernatant of strain 137 was treated with carboxymethyl-cellulose type 52 (6) and desalted as described above.

Since SEB and SEC_1 both have molecular weights close to the exclusion limit of Sephadex
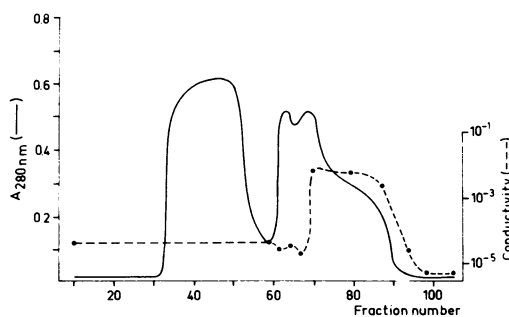

FIG. 1. Desalting of SEB by gel filtration through Sephadex G-50 (3.5 by 100 cm) and elution with distilled water. Fractions (14.4 ml) were collected at a flow rate of 144 ml/h. A_{280nm}, Absorbance at 280 nm. Conductivity is given in ohms^{-1} centimeters^{-1}.

G-50, columns could be eluted at high flow rates without losing resolution capacity.

Individual SE-containing fractions were pooled and tested for contaminating exoproteins. Using standard commercial reagents, we found SEB fractions to be devoid of lysostaphin, lysozyme, coagulase, and protein A. These fractions did, however, contain detectable quantities of DNase, alpha-toxin, beta-toxin, and staphylokinase. SEC_1 fractions contained alpha-, beta-, gamma-, and delta-toxin and staphylokinase activity but were found to be free of protein A, DNase, and protease.

Removal of an alpha-toxin by rabbit erythrocyte membranes.

In pilot studies it was established that 1 HU of purified alpha-toxin bound to an average of 10^7 erythrocytes without causing lysis. Therefore, erythrocyte membranes derived from 6 ml of whole blood were found to be sufficient to completely absorb alpha-toxin (8 HU/mg) from 100 mg of protein from the SE-containing fraction. Membranes suspended in 0.005 M phosphate buffer, pH 7.4, were centrifuged at 20,000 × g for 40 min at 4°C. The colorless supernatant was decanted, and the membrane pellet was suspended in the desalted SE-containing solution. After incubation overnight at room temperature, membranes were removed by centrifugation (20,000 × g, 4°C, 40 min) and washed again with distilled water. The resulting supernatants were combined and cleared by passing through 0.2-µm filters. The recovery of total protein was 90 to 95%.

Chromatofocusing.

The alpha-toxin-free and desalted sample was concentrated to 12 to 15 ml by cold trap rotary evaporation until a conductivity not exceeding $10^{-3} \, \Omega^{-1} \, cm^{-1}$ was reached. The pH was adjusted to 7.7, and the sample was applied to the chromatofocusing column (1 by 40 cm). Elution was performed with degassed Pharmalyte type 6.5–9, pH 7.7, at a flow rate of 15 ml/h.

The pH of each fraction was measured immediately at room temperature, and the linear part of the pH gradient for the elution of SEB-containing fractions was established in the range of pH 8.7 to 8.0 (Fig. 2). The fractions eluting above pH 8.7 represented a mixture of exoproteins with pI > 8.7. The three following fractions, P1, P2, and P3 with pI values of 8.6, 8.45, and 8.25, respectively, could be eluted in the linear part of the gradient (Fig. 2). P3 appeared to be inhomogeneous, consisting of two components with very close isoelectric points. Since no proteins were eluted between pH 8.0 and 7.7, the experiment was in general terminated at pH 8.0 by changing the eluent from Pharmalyte to 1 M NaCl.

The mixture of components eluted from the column under these conditions had a pI < 8.0. As shown by immunodiffusion analysis, fractions P1, P2, and P3 contained SEB, and only trace amounts of SEB were eluted with 1 M NaCl. The recovery compared to the desalted crude toxin was 70%.

Individual SE-containing fractions were combined and chromatographed over Sephadex G-50 (2.5 by 100 cm) and equilibrated with 0.15 M NaCl, pH 6.5, which was also used for elution. A typical elution pattern is depicted in Fig. 3. Only the first fraction contained SE specificity. Individual fractions were pooled and frozen at −25°C. The yield of SEB after all purification steps was about 60% of the desalted crude toxin.

The purification of SEC_1 by chromatofocusing was performed under conditions similar to those for SEB and provided comparable results (not shown). By using a pH gradient of 9.5 to 7.5, six

FIG. 3. Gel filtration on Sephadex G-50 (2.5 by 100 cm) to remove Pharmalyte from pooled SEB-containing fractions from chromatofocusing. Elution was performed at a flow rate of 18 ml/h in 7.2-ml samples. Symbols: – – –, absorbance at 280 nm; ———, absorbance at 206 nm.

fractions with SEC_1 specificity were eluted: a minor fraction (P1) at pH 8.6, two major fractions (P2 and P3) at pH 8.44 and 8.28, respectively, and three minor fractions (P4 to P6) at pH 8.11, 8.0, and 7.91 as determined by immunodiffusion.

Analysis of fractions after chromatofocusing. The three SEB fractions (P1 to P3) isolated by chromatofocusing were examined by isoelectric focusing on polyacrylamide gels, using the measured linear part of the pH gradient for pI determinations.

As illustrated in Fig. 4, SEB fraction P1 (lane a) revealed a single component at pH 8.85. In SEB fraction P2 (lane b), a major component was seen at pH 8.44 with two minor bands at pH 8.85 and a more acidic species which focused at pH 8.1. SEB fraction P3 (lane c) showed an increased heterogeneity, with a major component at pH 8.1 and two minor bands at pH 8.44 and 8.25. Thus, the essential features of the distribution of SEB determined by chromatofocusing could be confirmed by isoelectric focusing.

For comparison, highly purified SEB from M. S. Bergdoll (Food Research Institute, Madison, Wis.) and SEB purchased from Sigma were analyzed by isoelectric focusing. As illustrated in Fig. 4 (lane d), SEB from M. S. Bergdoll revealed, in addition to the major component at pH 8.85, a more basic species, whereas SEB from Sigma (lane e) consisted of several components focusing in the region between pH 8.44 and 7.9 but lacked the pH 8.85 component completely.
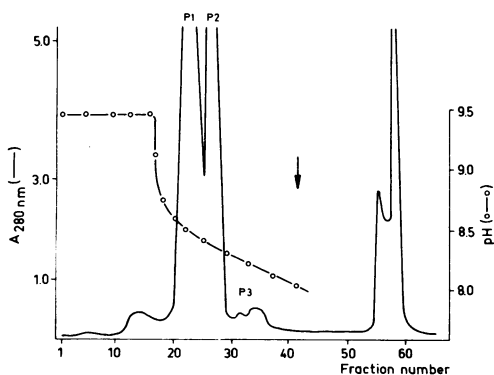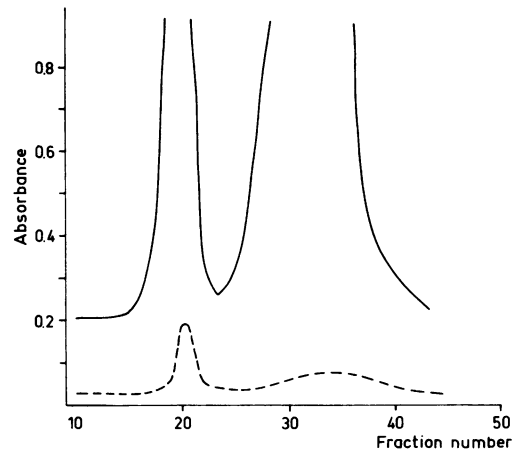

FIG. 2. Representative elution profile from a chromatofocusing column. SEB free of alpha-toxin was applied to the Polybuffer exchanger column (1 by 40 cm) and eluted with Pharmalyte, pH 7.7, in fractions of 2.4 ml at a flow rate of 15 ml/h. A_{280nm}, absorbance at 280 nm. The start of elution with 1 M NaCl is marked by an arrow.
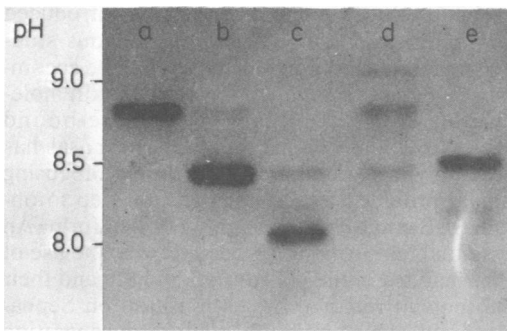
FIG. 4. Isoelectric focusing of SEB in polyacrylamide gels using pH 7 to 10 Ampholines. Focusing was performed at 4°C, and the pH values were determined at 4°C. Lane a, SEB P1; lane b, SEB P2; lane c, SEB P3; lane d, SEB (Bergdoll); lane e, SEB (Sigma).

The microheterogeneity of SEC_1 observed in chromatofocusing studies could be confirmed by isoelectric focusing (not shown), showing six components between pH 8.85 and 8.1. In general, SEC_1 exhibited a more acidic nature than SEB.

TABLE 1. Amino acid composition of SEB fractions P1 and P2

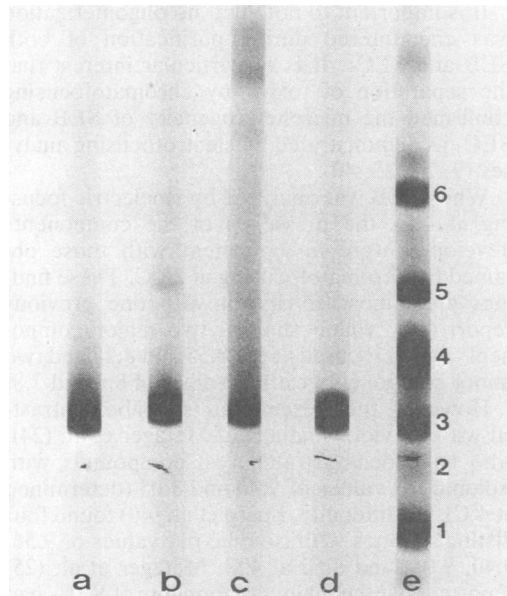| Amino acid	No. of amino acid residues or amide N per 28,400 kilodaltons		
	P1	P2	Composition determined in reference 18
Alanine	5.46	5.28	5
Arginine	5.21	5.23	5
Aspartate	43.70	43.09	44
Cysteine	2.03	1.95	2
Glutamate	20.33	20.64	20
Glycine	9.91	10.07	9
Histidine	6.15	6.05	5
Isoleucine	8.46	8.36	9
Leucine	16.87	16.56	16
Lysine	34.24	34.33	33
Methionine	6.47	7.71	8
Phenylalanine	11.79	11.29	12
Proline	6.03	6.05	6
Serine	13.61	13.70	14
Threonine	13.46	13.24	13
Tryptophan	1.00	1.00	1
Tyrosine	20.33	19.51	21
Valine	15.84	15.55	16
Amide N	28.9 (1.42%)	27.6 (1.36%)	30

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel analysis of purified SEB and SEC_1 in the presence of 2-mercaptoethanol. The gels were aligned according to the migration distance of bromphenol blue (marked with India ink). Lane a, SEB (P1 to P3); lane b, SEB (Bergdoll); lane c, SEB (Sigma); lane d, SEC_1 (P1 to P6); and lane e, standard proteins (Pharmacia). The standard proteins used were: (1) α-lactalbumin (14,000), (2) trypsin inhibitor (20,100), (3) carbonic anhydrase (30,000), (4) ovalbumin (43,000), (5) bovine serum albumin (67,000), (6) phosphorylase (94,000).

As revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, both SEB (P1 to P3) and SEC_1 were purified to homogeneity (Fig. 5), showing single bands at 34,000 and 30,000 daltons, respectively, as compared to marker proteins. SEB from M. S. Bergdoll also showed 60,000-dalton dimers, which usually occur during storage, whereas SEB from Sigma revealed several components with molecular weights above 30,000. The sedimentation coefficients of purified fractions P1 and P2 were determined as 2.6×10^{13} at 0.85×10^{-2} g/ml of phosphate-buffered saline, pH 7.2. Both the amino acid compositions and the amide nitrogen contents of P1 and P2 are given in Table 1, and the values obtained are quite similar and comparable to those derived from sequence data (18).

Tests for impurities revealed that SEB fractions P1 to P3 were free of any of the contaminating activities tested for, e.g., DNase, alpha-, beta-, gamma-, and delta-toxin, staphylokinase, protease, and protein A. The SEB obtained from M. S. Bergdoll did, however, contain 32 HU of alpha-toxin, 12 HU of beta-toxin, and 8 HU of gamma- and delta-toxin, but was devoid of protease, DNase, staphylokinase, and protein A. The SEB from Sigma did not contain any detectable impurities. Tests for exoproteins in the SEC_1 fractions (P1 to P6) were completely negative. When samples from the P1 to P3 fractions of SEB or pooled fractions of SEC_1 after chromatofocusing were examined for their antigenic

specificity by using monospecific antisera, all of the components showed precipitin lines of identify by gel diffusion analysis if reacted with the homologous antiserum and gave no indication of impurities. Assays of the SEB P1 fraction in rhesus monkeys by oral intake resulted in illness characterized by vomiting and diarrhea at a dose of 5 μg/kg of body weight.

DISCUSSION

Tissue culture techniques have been used for several years to investigate the biological activity of SEs on a variety of cell culture lines and strains. Attempts at studying the effects of SE in an in vitro system have, however, met with various results. Since the criteria for purity of the toxin were not always defined, it should be recognized that the contradictory reports on the toxicity of SE might be due to the presence of contaminants with rather broad substrate specificities in purified toxin preparations (1, 12, 23, 43–45, 47). Indeed, the purification of SE by biochemical methods has been hampered by the fact that several staphylococcal enzymes and toxins have molecular weights and isoelectric points close to those of SE (23, 48).

The new purification method for SEB and SEC_1 described in this paper offers several advantages over other known procedures used so far. SE is obtained in a highly purified form without any of the contaminants we tested for and in a high overall yield, which amounted to 60% of the desalted crude culture supernatant (12, 34).

The sedimentation coefficients of homogeneous fractions P1 and P2 were both 2.6×10^{-13} at 0.85×10^{-2} g/ml of phosphate-buffered saline, pH 7.2

Detailed studies of the desalting and concentration steps required during the purification procedure revealed irreversible binding of SE to artificial membranes such as dialysis tubings, with a high concomitant loss (up to 80%) of the toxin. Therefore, desalting of crude material was accomplished by gel filtration on Sephadex G-50 fine, using high elution rates. This technique is well suited to desalting large volumes of the crude toxin without requiring preconcentration and yielding high recovery (>90%) of the toxin.

All concentration steps, if necessary, were performed by rotary evaporation with a cold trap. This proved to be a rapid and effective method for avoiding any loss of SE.

Most, if not all, of the so-called highly purified toxin preparations still contained, besides other impurities, detectable levels of alpha-toxin, and it has been suggested that this toxin damages biological membranes through a tight association with the lipid bilayer (16). To remove alpha-toxin quantitatively, its irreversible binding to rabbit erythrocyte membranes was introduced as a purification step. Based on previous studies, membrane binding was thought to be accompanied by an oligomerization of the toxin molecule to form annular, membrane-bound structures, and evidence for this proposal has recently been obtained (5). Chromatofocusing finally provided a fundamental new step to obtain SEB and SEC_1 in a highly purified form. An essential feature of the procedure was the use of Pharmalytes in the pH range 6.5 to 9.0 and their subsequent removal by gel filtration on Sephadex G-50, which allowed an effective separation of the remaining impurities from SEB and SEC_1. Pharmalytes are favorable toward resolution with Polybuffer (Pharmacia), since the latter is composed of a mixture of peptide chains longer than those of Pharmalytes and its separation from both SEB and SEC_1 by gel filtration on Sephadex G-50 proved not to be as effective as that of Pharmalytes.

Alternative methods for removal of Polybuffer such as precipitation with ammonium sulfate, hydrophobic interaction, or affinity chromatography to separate SEB or SEC_1 from Polybuffer turned out to be unsuitable, since they were associated with heavy losses of the toxin.

It is important to note that no oligomerization was encountered during purification of both SEB and SEC_1. It is of particular interest that the separation of toxins by chromatofocusing confirmed the microheterogeneity of SEB and SEC_1 as demonstrated by electrofocusing analyses (9, 24, 25, 40).

When SEB was analyzed by isoelectric focusing at 4°C, the pI values of the components developed were in agreement with those obtained by chromatofocusing at 25°C. These findings are almost consistent with one previous report (10), which showed two major components which focused at pH 8.55 and 8.25 and two minor components with pI values of 8.0 and 7.8.

However, the present results can be contrasted with previous findings of Metzger et al. (24), who have demonstrated two compounds with isoionic pH values of 9.40 and 3.05 (determined at 4°C). Additionally, Spero et al. (40) found four distinct entities with isoionic pH values of 9.56, 9.40, 9.05, and 8.50 at 4°C. Metzger et al. (25) reported that the major components of SEC_1 had isoelectric points of 9.19 and 8.83, whereas the major components in our purified preparation focused in the region between pH 8.6 and pH 7.9. This report also presents evidence that the change of SEC_1 in a more acidic pattern was strikingly more drastic than that observed with SEB, indicating a much greater lability. The amino acid compositions of the SEB fractions P1 and P2 were quite similar and comparable to that obtained from sequence data (18).

In the light of these findings, it is of particular importance to recognize that sequential conversion of SEB from an alkaline form to a more acidic species may be caused by partial hydrolysis of labile amide groups (40). This is compatible with the fact that the P2 fraction of SEB had a lower isoelectric point than P1 and was consequently more deamidated as evidenced by amino acid analysis and amide nitrogen content. This might also be valid for SEC_1 (25).

As expected, the combined P1 and P2 fraction of purified SEB provoked emesis in monkeys after oral administration.

SEB and SEC_1 were purified to homogeneity as determined by ultracentrifugation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and had a sedimentation constant of 2.6×10^{-13} and apparent molecular weights of 34,000 for SEB and 30,000 for SEC_1. The molecular weights of both SEB and SEC_1 were closely related to those found originally (40, 49). The results of the amino acid sequence showed, however, that SEB contains 239 amino acids with a molecular weight of 28,400 (18), and SEC_1 contained 239 amino acids with a molecular weight of 27,500 (33).

Taken together, the experiments described were designed to establish a new procedure for the purification of SE under optimal conditions. The findings demonstrate that the newly developed chromatofocusing method is an extremely useful tool for purification of SE on a preparative scale and is markedly faster than any of the separation procedures used previously.

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