Intrinsic and Chemically Produced Microheterogeneity of
Staphylococcus aureus Enterotoxin Type C

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Staphylococcus aureus enterotoxin (SEC), and C2 (SEC), produced from
50-liter quantities of crude culture supernatants were purified chromatographi-
cally in a neutral or acid milieu. Microheterogeneity of SEC was markedly
increased by treatment of the purified toxin with alkalai, and new, more acidic
charged species appeared. SEC was more heterogeneous than any of the other S.
aureus enterotoxins and was affected only slightly by treatment with alkali.
Prolonged incubation of the organism during production of the SEC-produced
changes in charged species that may be related to a bacterial deamidase, since
similar changes were not seen with alkaline treatment of the purified toxim.
Although SEC and SEC2 showed complete identity immunologically, they are
separate, distinct toxins, and alkalii treatment of SEC, did not produce SEC.

The heterogeneity of Staphylococcus aureus enterotoxins was first described by Baird-
Parker and Joseph (2). Confirmation of this heterogeneity was reported by Schantz et al.
(11) for S. aureus enterotoxin B (SEB) by means of electrophoresis on starch gel. By using
isoelectric focusing, Metzger et al. (8) demonstrated that SEB consisted of four species. The
two major components were stable at neutral pH at 4°C. Spero et al. (13) studied the effects of
an alkaline milieu on SEB and found that there was progressive amide hydrolysis at 37°C lead-
ing to a loss in the alkaline components with concomitant development of the more acidic
ones. The several species formed were one charge apart.

In the original description of the isolation of S. aureus enterotoxin C1 (SEC1), Borja and
Bergdoll (3) found two components by starch gel electrophoresis that were attributed to either
buffer interaction or dimerization. Extreme heterogeneity was observed with a preparation of S.
aureus enterotoxin C2 (SEC2) obtained by purifi-
cation from a culture that had been incubated for 72 h at 37°C (6). S. aureus enterotoxin
production, however, has been reported to be complete by 10 to 18 h (17, 9). The toxin was thus
exposed unnecessarily for a long period to an alkaline milieu and possible bacterial deam-
dases before purification. Furthermore, some methods of purification of SEC1 (3) and SEC2,
(11) utilize a period of initial concentration of the alkaline culture filtrate that could affect qualit-
avitively and quantitatively the homogeneity of the toxins before purification.

In this report, we describe methods for purifi-
cation that promptly remove the toxin to a neutral or acid pH. The effects of alkali at 37°C
on SEC1 and SEC2 purified in this manner are compared with untreated purified toxins. In
addition, SEC, purified from a 72-h fermentation was studied for possible differences in
isoelectric composition.

MATERIALS AND METHODS

S. aureus strains. Strain 137-H-2 was utilized for production of SEC1, strain 3611 was utilized for
production of SEC2. All cultures were maintained in lyophilized form, and a new ampule was used for
each experiment.

Fermentation. All studies used a 70-liter fermentor (Fermentation Design, Allentown, Pa.). Controlled
settings consisted of 400 rpm agitation, 10 liters/min of air sparge, and 37°C temperature. All fermentations
were carried out for 18 h except where noted.

Medium. All fermentations were carried out in 50
liters of medium containing 4% NAR (Sheffield
Chemical Co., Norwich, N.Y.), 1% yeast extract
(Difco, Detroit, Mich.), and 0.2% glucose (w/v).

Centrifugation. After fermentation the culture
was centrifuged at 10,000 rpm by using a continuous-
flow head (Lourdes, Old Bethpage, N.Y.).

Demineralization. All crude bacterial superna-
tants were partially desalted by passing through a
demineralizer cartridge (Harnstead, Boston, Mass.).

Chromatography. CG 50 (Mallinckrodt, Millville,
N.J.) was activated by alkali and acid treatment.

After activation, the resin was equilibrated at the
appropriate pH with phosphate buffer. The washed
resin was stirred into the diluted culture supernatant
The resin was allowed to settle and was then poured
into a chromatography column. Carboxymethylcel-
lulose (CM-cellulose) (Bio-Rad, Richmond, Calif.)
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was equilibrated with phosphate buffer at the appropriate pH and then washed with distilled water. The CM-cellulose was stirred into the diluted crude toxin, allowed to settle, and then poured into a chromatography column. Elution characteristics are given under each purification scheme.

**Isoelectric focusing.** Isoelectric focusing in sucrose gradients was carried out as recommended by LKB (Stockholm, Sweden). Isoelectric focusing in gels containing pH 3 to 10 ampholines was done at 17°C according to the method of Wrigley (11). Antiserum. Anti-SEC, was prepared by repeated intramuscular injections of the major component of purified SEC, isolated by electrofocusing. The preparation was mixed with complete Freund adjuvant (Difco for injection into goats.

**Ouchterlony (10) double diffusion.** One percent agar (15 ml) dissolved in pH 8.3 borate buffer was layered onto glass plates (8 by 10 cm). Three-millimeter holes were punched at 5-mm intervals in a circular pattern with a well in the center. 5 μl of antigen or antiserum was placed in each well.

**Purification of SEC.** The culture supernatant from strain 137-H-2 was diluted 1:5 with distilled water and the pH was adjusted to 6.2 with phosphoric acid. CG-50 (350 g) equilibrated at pH 6.2 with 0.01 M phosphate was added to the diluted culture supernatant. The toxin was eluted from the column with 0.5 M phosphate buffer containing 0.25 M NaCl (pH 6.8). The toxin peak was dialyzed at 4°C against distilled water to reduce the salt concentration. The dialyzed toxin was diluted 1:10 with distilled water; 100 g of CG-50 equilibrated at pH 6.8 was added. The column was washed with distilled water and the column was eluted with 0.15 M NaHPO₄. The toxin peak was dialyzed against 0.01 M phosphate buffer (pH 6.2); 200 g of CM-cellulose equilibrated at pH 6.2 was added to the dialyzed toxin. The toxin was eluted from the column by a linear gradient (0.01 to 0.07 M phosphate buffer, pH 6.2 to 6.8). The toxin-containing fractions were combined, dialyzed against 0.01 M phosphate buffer (pH 7.0), and lyophilized.

**Purification of SEC.** The supernatant from a culture of strain 361 was prepared as for SEC, except that the pH was adjusted to 5.6. CG-50 (350 g) was equilibrated to pH 5.6 with 0.05 M phosphate. Elution of toxin was accomplished with 0.5 M phosphate buffer plus 0.5 M NaCl at pH 6.2. The toxin-containing eluate was dialyzed first against distilled water and then against 0.01 M phosphate buffer at pH 6.0. CM-cellulose (200 g) equilibrated at 0.01 M, pH 6.0, was added to the dialyzed toxin. The toxin was eluted from the column by using a linear gradient (0.01 to 0.05 M phosphate buffer, pH 6.0 to 6.8). The toxin-containing fractions were combined and dialyzed against 0.01 M phosphate buffer, pH 5.7. Hydroxylapatite (150 g) was swollen in 0.03 M phosphate buffer at pH 5.7. A column (50 by 90 cm) was poured at 4°C. The column was further equilibrated with buffer at 4°C. The column was washed slowly through the column and then washed with the equilibrating buffer. Elution was accomplished by using a linear gradient (0.01 to 0.4 M phosphate, pH 5.7). The toxin peak was dialyzed against 0.01 M phosphate buffer (pH 7.0) and then lyophilized.

**RESULTS**

SEC, purified by the described chromatographic method contained three components by electrofocusing (Fig. 1) in a sucrose gradient analytical instrument. The two major components had isoelectric points of 9.19 and 8.83, which were determined at 4°C. Three bands were also demonstrated by gel electrophoresis (Fig. 2A). The most alkaline species by both procedures was present in only trace amounts. The major components were in a ratio of approximately 1:10:1.

**Fig. 1.** Isoelectric focusing of staphylococcal enterotoxin C, using pH 7 to 10 ampholine sucrose gradient. Electrophoresis was performed at 4°C. The pH values were determined at 4°C.
in the gel (estimated pI ~ 8.3). The former components were greatly diminished after exposure of the purified toxin to pH 9.0 at 37°C for 10 days (Fig. 2D).

SEC (72 h) had two major components with only trace amounts in the more alkaline region of the gels. The densitometric scan illustrated how markedly the relative concentrations of the several components were altered. In addition, a definite new acidic species was demonstrable (Fig. 2E and 4).

All toxin preparations showed lines of complete identity when examined by the Ouchterlony technique using ant-SEC, antiserum. Composite immunoelectrophoretic (Fig. 5) of the five preparations reveals that SEC, treated with alkali had decreased cathodic movement.

![Fig. 2. Isoelectric focusing of enterotoxin preparations in polyacrylamide gel. using pH 3 to 10 amphotolines. (A) SEC, (B) SEC, treated with alkali; (C) SEC, (D) SEC, treated with alkali, (E) SEC, (72 h).](image)

![Fig. 3. Isoelectric focusing of staphylococcal enterotoxin C, using pH 3 to 10 amphotolines-sucrose gradient. Electrophoresis was performed at 4°C. The pH values were determined at 4°C.](image)

![Fig. 4. Densitometric scan of electrophoresed gels. SEC, (---) and SEC, (72 h) (-----).](image)
compared with untreated SEC. Alkaline treatment of SEC did not appear to change the average charge. The more acidic nature of SEC (72 h) was demonstrated by its slight anodic movement.

Sodium dodecyl sulfate-acrylamide electrophoresis revealed that all C-type enterotoxin preparations co-migrated with purified SEB; therefore, the molecular weight is between 28,000 and 29,000.

**DISCUSSION**

SEC, consists of three components and has comparable microheterogeneity to *S. aureus* enterotoxins A (12) and B (8). In addition, the two major components of SEC, have isoinic points approximately 0.4 pH units apart, similar to the difference in isoinic points seen with the major components of *S. aureus* enterotoxins A (12) and B (8) and consistent with a single charge difference between isolectric species. The behavior of SEC, treated with alkali is similar to that reported for SEB (13), i.e., a sequential conversion from more to less alkaline forms and the appearance of new, more acidic species. It is noteworthy, however, that SEC, is considerably more altered than SEB.

SEC, demonstrates more isolectric pauci-

dispersity than the other staphylococcal enterotoxins. Surprisingly, however, it is the most resistant variety to chemical deamidation induced by exposure to pH 9.0 and 37°C. Only the most alkaline species appeared to be affected, and the average charge, as evidenced by immunoelectrophoresis, was unchanged. A much greater change was brought about by prolonging the incubation of the SEC, culture to 72 h before isolation. The shorter time period suggests strongly that the change was produced enzymatically, presumably by a deamidase.

Two preparations of SEC have been examined isolectrically by Dickie and co-workers (5, 6). Both were isolated after 72 h of incubation and by a procedure involving a preliminary concentration by dialysis against polyethylene glycol. In one instance the component present in highest concentration had a pl of 7.35 and in the other a pl of 6.50 (Their pl values were obtained by measurement of pH of the samples at 25°C. The values cited here were corrected to 4°C, our temperature of measurement, by the van't Hoff equation, assuming that the amino groups of the ampholines have ΔH of 10,000 cal/mol.) The 7.35 component probably corresponds to our 7.1 component, and the composition of the preparation, lacking our 8.4 component, is comparable.
to our SEC₁ (72 h) material. The other preparation was considerably more deamidated and contained as its most alkaline species a fraction with a pI of 7.25, again comparable to our 7.1 component. It was thus devoid of both the 8.1 and 8.4 isoelectric species found in our preparations. It is apparent that considerable care must be taken in the isolation of the enterotoxins if one is to avoid degrading the proteins, and it would be well to characterize individual preparations by their isoelectric focusing patterns. It was found that when the initial steps of the isolation were not carried out promptly, SEC preparations were badly nicked. The extent of nicking is readily determined by sodium dodecyl sulfate-polyacrylamide electrophoresis in the presence and absence of β-mercaptoethanol.

The data support the report of Avena and Bergdoll (1) that SEC₁ and SEC₂ are different enterotoxins with identical immunological reactions. The gel isoelectric focusing patterns obtained after prolonged exposure of SEC, at pH 9.0 bore little resemblance to those of SEC₁.

LITERATURE CITED