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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF STAPHYLOCOCCAL ENTEROTOXIN B

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SUMMARY

Size-exclusion, ion-exchange, and reversed-phase chromatography were investigated for use in purification and characterization of Staphylococcal enterotoxin B (SEB), a 28,000 M_r protein associated with Staphylococcal food-borne intoxication. In all approaches chromatography of crude or purified SEB yielded one or more components which displayed toxin activity and contained the 28,000 M_r SEB protein plus other lower-molecular-weight protein species. Reversed-phase chromatography of detergent-treated SEB permitted resolution of the 28,000 M_r protein from lower-molecular-weight components.

INTRODUCTION

Staphylococcal food-borne intoxication, one of the most common forms of food-borne disease, is caused by protein enterotoxins elaborated by certain strains of *Staphylococcus aureus*. There are at least six immunologically distinct enterotoxins, and multiple forms of individual enterotoxins have been observed by ion-exchange chromatography[1,2], isoelectric focusing[2–5], and polyacrylamide gel electrophoresis[6]. The heterogeneity of staphylococcal enterotoxins has hindered the analysis of these proteins and development of accurate and sensitive assays for the enterotoxins in foods.

Our interest has been in developing a rapid method of purifying enterotoxins for use in the development of immunological assays. We have explored the use of high-performance liquid chromatography (HPLC) for isolation of staphylococcal enterotoxin B (SEB), and have investigated several chromatographic approaches,

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including size-exclusion chromatography, anion- and cation-exchange chromatography, and reversed-phase chromatography.

Ion-exchange chromatography of crude or purified SEB yielded multiple components, one or more of which contained the 28,000 M_R SEB protein plus other species. Reversed-phase chromatography of pure SEB yielded a single peak, which again contained SEB plus additional proteins. Incubation of the SEB preparation with detergent apparently disrupted association of the 28,000 M_r SEB with these components and permitted its resolution as a single polypeptide by reversed-phase chromatography.

MATERIALS AND METHODS

Crude and purified preparations of SEB were isolated as described by Casman and Bennett[7].

Chromatographic conditions

Size-exclusion chromatography was performed using a MicroPak TSK Model M6000 pumps with a model 660 programmer and Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a Varichrom variable-wavelength UV-Vis detector (Varian Instruments, Walnut Creek, CA, U.S.A.). Reversed-phase chromatography was carried out using a Model 5020 Liquid Chromatograph equipped with a Model UV-100 programmable UV-Vis detector (Varian Instruments) and a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.)

Size-exclusion chromatography was performed using a MicroPak TSK 3000SW 300 × 8 mm I.D. column (Varian Instruments) with a mobile phase of 0.067 *M* potassium phosphate plus 0.1 *M* potassium chloride at pH 6.8 and a flow-rate of 1.0 ml/min. Cation-exchange chromatography was carried out using a MicroPak TSK IEX 530CM 300 × 4 mm I.D. column (Varian Instruments) operated at 0.5 ml/min. Buffer A consisted of 0.005 *M* ammonium acetate (pH 5.7) and Buffer B was 1.0 *M* ammonium acetate (pH 6.5). The elution program consisted of initial isocratic elution with Buffer A for 16 min, followed by a linear gradient to 40% Buffer B in 60 min.

Reversed-phase chromatography was performed using a MicroPak C_{18}-Protein 300 × 4 mm I.D. column (Varian Instruments) operated at 1.0 ml/min. Solvent A was 0.1% trifluoroacetic acid (TFA) in water and Solvent B was 0.1% TFA in water–l-propanol (40:60). The elution program consisted of a 30-min linear gradient from 0 to 100% B. Detection was by absorption at 277 or 280 nm.

Electrophoresis

SDS-polyacrylamide gel electrophoretic (PAGE) analysis was performed according to the method of Laemmli[8] as follows:

Fractions isolated by size-exclusion and cation-exchange chromatography were lyophilized and dissolved in 0.0625 *M* Tris (pH 6.8)–2% SDS–10% glycerol–5% β-mercaptoethanol–0.001% Bromphenol Blue and applied to a 6% stacking gel (pH 6.8), then resolved in a 15% 0.5-mm gel at pH 8.8. Following electrophoresis, proteins were stained with 0.25% Coomassie Blue G-250 in 45% methanol–9% acetic acid and destained with 5% methanol–7.5% acetic acid.

Fractions isolated by reversed-phase chromatography were concentrated and applied to a 12.5% 0.75-mm gel with Bromphenol Blue as the tracking dye. Following electrophoresis, proteins were silver-stained according to the method of Morrissey[9].

Serological analysis and analysis of biological activity

Serological analysis was performed using the Ouchterlony microslide gel double-diffusion assay, developed by Casman and Bennett[10]. Lyophilized samples were dissolved in physiological saline containing 10% brain–heart infusion broth and applied directly to the microslide using known reference enterotoxin as control. Identity of the sample precipitin line with the reference enterotoxin antigen–antibody precipitin line was considered positive identification. Analysis of biological activity was performed using the kitten test as described by Hammon[11].

RESULTS

Size-exclusion chromatography of a crude preparation of SEB on a MicroPak TSK 3000SW column revealed approximately six components with a major peak, corresponding to 23,000–25,000 M_r (Fig. 1A), which by the Ouchterlony microslide test contained SEB. Analysis of this peak by PAGE (Fig. 1B) indicated that it contained an enterotoxin-sized component of 28,000 M_r as well as several smaller pro-

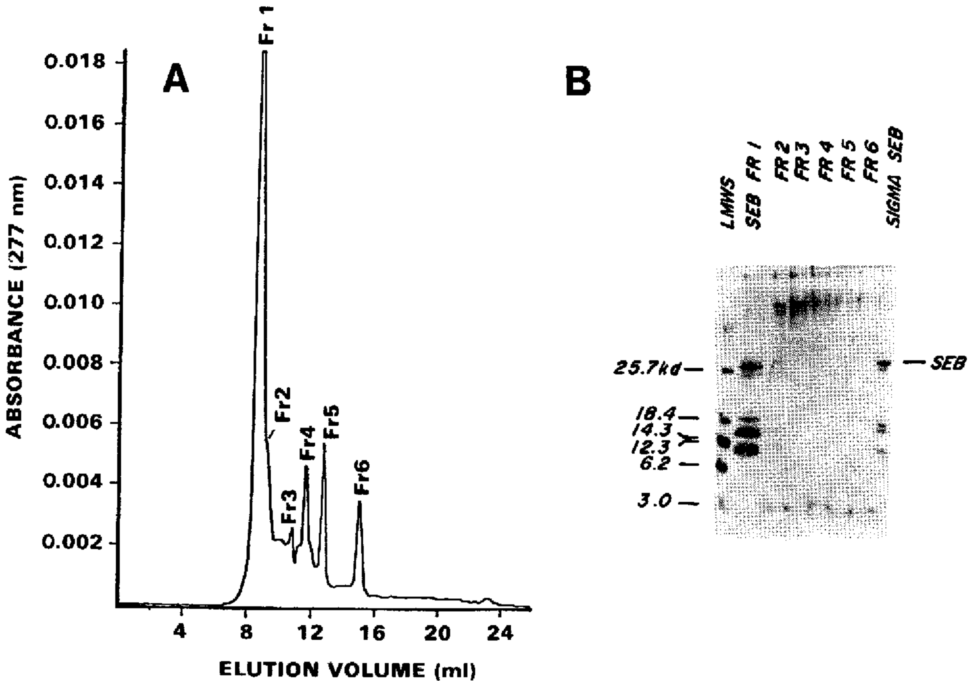

Fig. 1. Size-exclusion chromatography of a crude preparation of SEB (A) and PAGE analysis of collected fractions (B).

teins in the 12,000–18,000 M_r range. Note that the pure SEB used as marker (Lane 8) also contained these low-molecular-weight impurities.

Size-exclusion chromatography of crude SEB with a mobile phase of high ionic strength or in the presence of 6 *M* guanidine hydrochloride also failed to resolve the 28,000 M_r protein from low-molecular-weight contaminants. It was concluded that size-exclusion chromatography could not provide adequate resolution of SEB for our purpose.

Both anion-exchange and cation-exchange chromatography were investigated as possible approaches for SEB purification. Anion-exchange chromatography using a SynchroPak AX-300 Column (Synchrom, Lafayette, IN, U.S.A.) with sodium chloride or ammonium acetate gradients was unsuccessful in separating SEB from contaminants. Even at low solvent strengths, SEB was eluted as an unretained peak with several other components and tailed into later eluting peaks. This is not unexpected, considering the basic nature of staphylococcal enterotoxins.

Retention of SEB could be achieved by cation-exchange chromatography. Using a MicroPak TSK IEX 530CM weak cation exchanger with an ammonium acetate gradient, crude SEB was resolved into *ca.* sixteen components (Fig. 2A). Analysis of collected fractions by PAGE indicated that several peaks eluted between 0.19–0.23 *M* ammonium acetate (Fractions 14–16) contained enterotoxin proteins of *ca.* 28,000 M_r as well as two smaller proteins of 12,500 and 14,000 M_r (bands X and Y in Fig. 2B). Analysis of fractions by the Ouchterlony microslide test indicated that Fractions 13–16 contained material with antigenic identity to reference SEB. The Ouchterlony microslide test results for Fraction 14 are depicted in Fig. 3. Results for other fractions and those described above for size-exclusion chromatography were identical. In all cases where a positive result in the microslide test was obtained and sufficient material was available a positive reaction in the kitten test was observed.

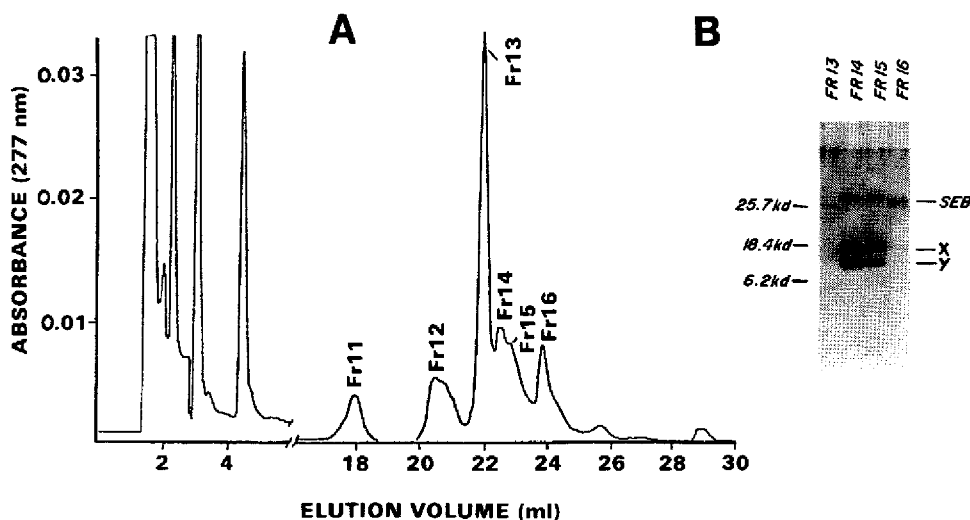

Fig. 2. Cation-exchange chromatography of a crude preparation of SEB (A) and PAGE analysis of collected fractions (B).

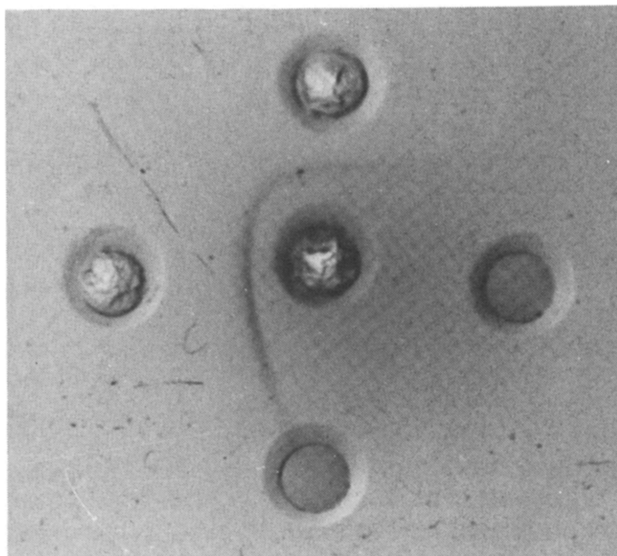

Fig. 3. Ouchterlony microslide gel double-diffusion assay of Fraction 14, isolated from the crude preparation of SEB by cation-exchange chromatography, shown in Fig. 2. Top well contained reference SEB; left well contained Fraction 14; center well contained reference SEB antiserum.

Cation-exchange chromatography of purified SEB also revealed multiple peaks during gradient elution which contained an SEB-sized protein plus low-molecular-weight components, showing antigenicity in the Ouchterlony test.

Cation-exchange separation of crude and purified SEB into several components with SEB-sized proteins and SEB antigenicity is consistent with previous reports of multiple forms of staphylococcal enterotoxins. The occurrence of low-molecular-weight species in every fraction containing SEB suggests that the toxin may be associated with two or more proteins which migrate independently under PAGE conditions.

When purified SEB was subjected to reversed phase chromatography using a MicroPak Protein-C_{18} column with TFA–propanol gradient elution, a single peak eluted by 35% propanol was observed (Fig. 4A). Analysis by PAGE (Fig. 4B) indicated that, as in previous HPLC separations, an SEB-sized protein of 28,000 M_r was eluted with several low-molecular-weight components (in this case *ca.* seven other species were observed in the ranges 24,000–26,000 and 16,000–17,000 M_r).

Occurrence of low-molecular-weight contaminating proteins in PAGE could be explained as (A) proteins eluted along with SEB; (B) degradation of SEB during chromatography; (C) proteins non-covalently associated with SEB which are released under PAGE conditions. The first explanation is unlikely, considering the great selectivity differences between size-exclusion, ion-exchange, and reversed-phase HPLC. The second explanation is unlikely, since SEB is a heat-stable protein consisting of a single polypeptide chain. It is conceivably that low-molecular-weight peptides could become convalently associated with SEB via disulfide bonds or arise from proteolytic cleavage products generated during preparation, which remain associated through disulfide bonds; in either case treatment with mercaptoethanol should release the

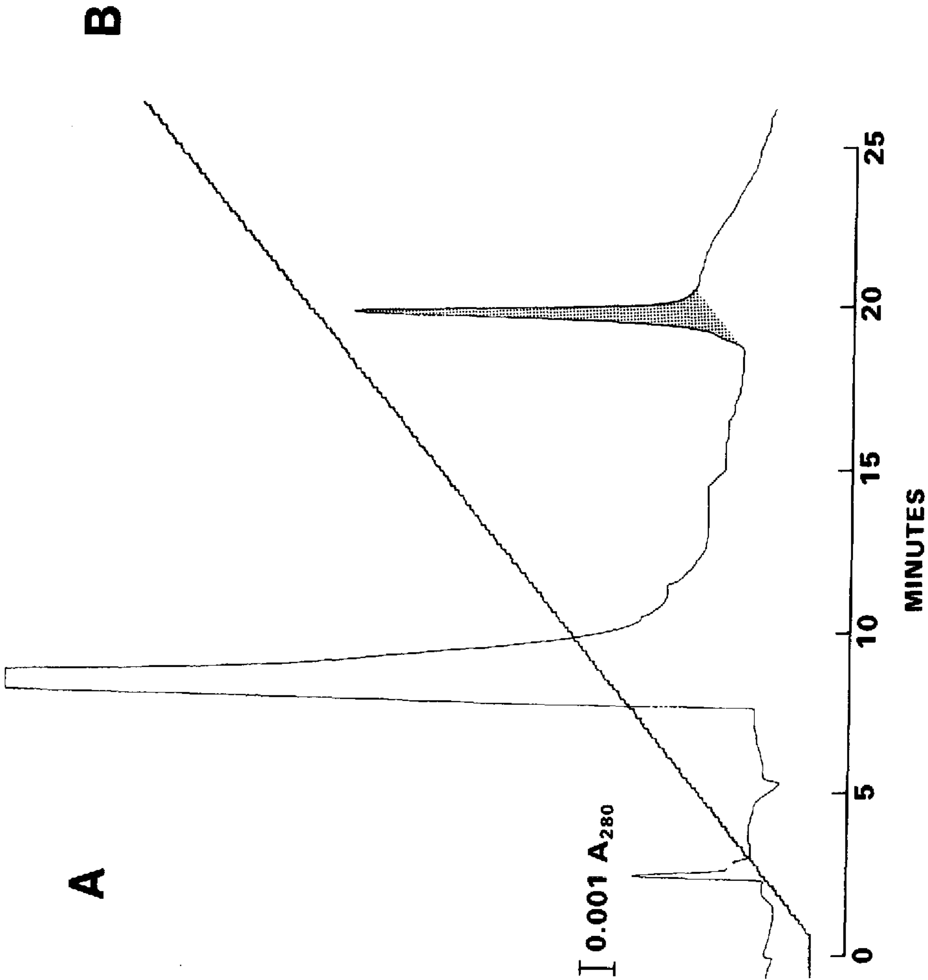

Fig. 4. Reversed-phase chromatography of a purified SEB preparation (A) and PAGE analysis of collected fractions (B). The peak eluted after 9 min is a solvent impurity.

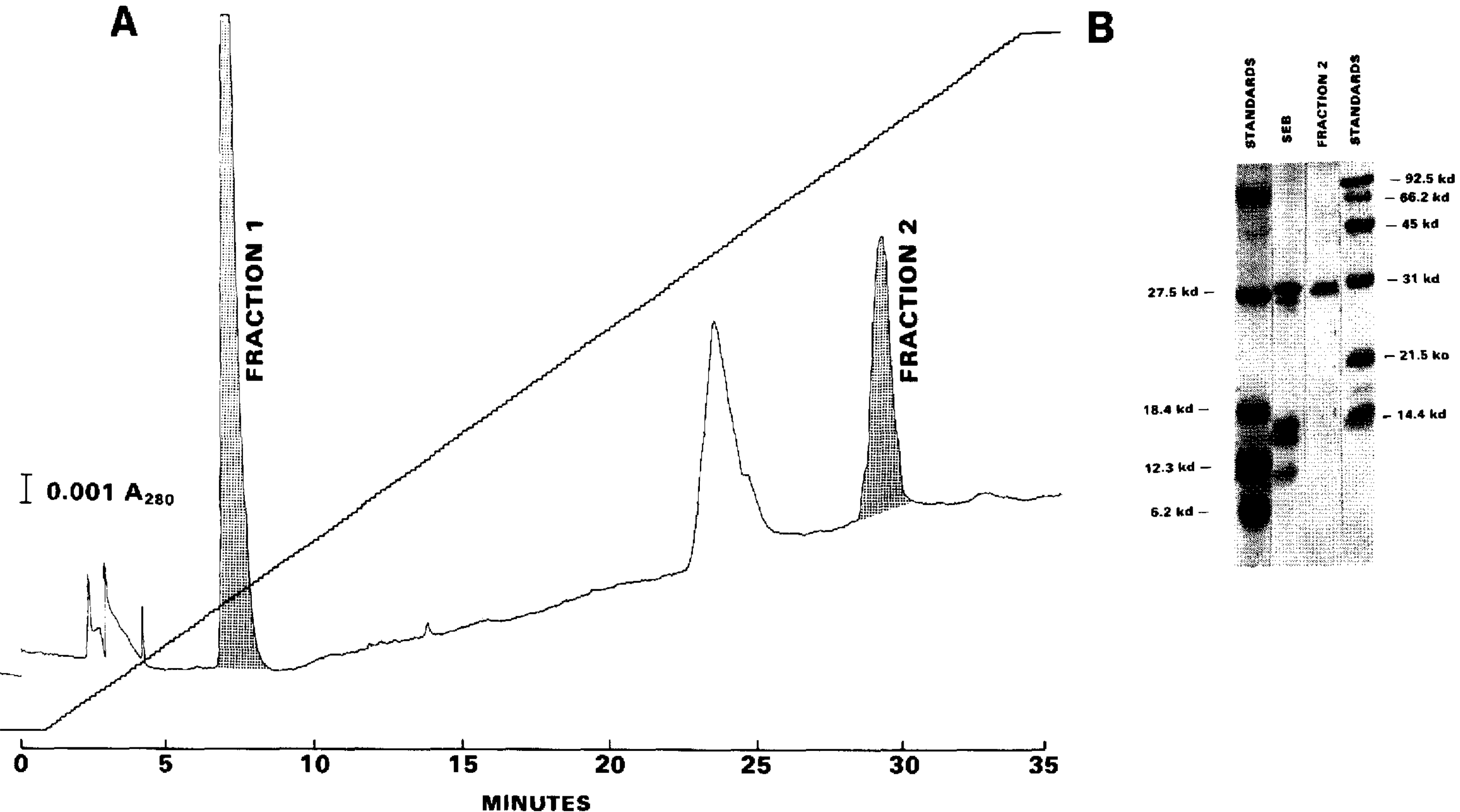

Fig. 5. Reversed-phase chromatography of purified SEB treated with PAGE buffer (A) and PAGE analysis of collected fractions (B). The peak eluted after 7 min is a solvent impurity and the peak eluted after 24 min represents UV-absorbing components in the SDS buffer.

associated peptides. However, SEB contains only two cysteinyl residues, which form a single internal disulfide bond, located close to the center of the polypeptide[12]. Thus, it is unlikely that the peptide contaminants arose from cleavage of covalently attached peptides from the 28,000 M_r protein.

To test the third possibility, purified SEB was incubated in the PAGE SDS–glycerol–mercaptoethanol buffer and chromatographed under the same conditions used previously. In this case (Fig. 5A), a single peak eluted late in the gradient by 60% propanol contained only the 28,000 M_r SEB-sized protein (Fig. 5B). The material in this peak was negative in both the Ouchterlony microslide test and the kitten test.

CONCLUSIONS

In the chromatography of crude and purified SEB preparations, an enterotoxin-size protein was eluted with high- and/or low-molecular-weight contaminants; fractions containing this 28,000 M_r protein were shown to possess antigenic identity with SEB by the Ouchterlony microslide assay. Cation-exchange chromatography resolved multiple peaks containing the SEB-sized protein and low-molecular-weight species, while the 28,000 M_r protein and contaminants were eluted as a single peak in size-exclusion and reversed-phase chromatography. Incubation of purified SEB with SDS enabled the 28,000 M_r protein to be separated chromatographically from contaminating species by reversed-phase HPLC. The absence of serological or biological activity of the 28,000 M_r protein recovered from this step probably reflects the strong denaturing effects of the SDS incubation procedure.

We interpret these results as suggesting that SEB in crude or purified preparations is closely associated with two or more proteins which are not separated from SEB in HPLC but are separated under denaturing conditions in SDS PAGE analysis or by pre-incubation with SDS. Existence of such complexes and heterogeneity in the associated proteins may account for multiple peaks in ion-exchange chromatography and may in part explain observations of multiple forms of staphylococcal enterotoxins.

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