INFECTION AND IMMUNITY, Dec. 1981, p. 900-907
0019-9567/81/120900-08$02.00/0

Vol. 34, No. 3

# Purification and Partial Characterization of a Putative Precursor to Staphylococcal Enterotoxin B†

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Received 24 June 1981/Accepted 18 August 1981

A putative precursor to staphylococcal enterotoxin B (SEB) has been identified as a component of purified membranes from *Staphylococcus aureus* S6. Agarose gel immunodiffusion analysis of the solubilized membranes demonstated an immunoreactive protein that formed complete lines of identity with purified extracellular SEB. This putative precursor (pSEB) also had a different electrophoretic mobility from that of extracellular SEB when analyzed by immunoelectrophoresis. When membrane proteins from S6 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose sheets and probed with I-125 labeled, affinity-purified anti-SEB, the pSEB band was identified. The pSEB was approximately 3,500 daltons larger than extracellular SEB. This component was purified by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two-dimensional peptide maps of the putative SEB precursor revealed that most of the tryptic peptides were identical to those of mature extracellular SEB. When purified membranes of other $SEB^+$ (DU4916 and 10-275) and $SEB^-$ (RN450, RN451, S6R, and FRI100) *S. aureus* strains were analyzed by the nitrocellulose blot procedure, only the $SEB^+$ strains contained this putative SEB precursor on their membranes.

Staphylococcal enterotoxin B (SEB), an extracellular toxin produced by many strains of *Staphylococcus aureus* (15), is the causative agent of staphylococcal gastroenteritis (2). The structure of SEB has been extensively investigated (11-13, 20, 31, 32). Sequence studies by Huang and Bergdoll (11-13) have shown SEB to contain 239 amino acid residues and to have a molecular weight of approximately 28,500. Although the physical characteristics of SEB have been investigated, few studies have addressed the transport of this protein.

Blobel and Sabatini (4) proposed a model for protein secretion based on the presence of an additional amino terminal sequence that would function to initiate the transfer of proteins across a membrane. Independent of Blobel's prediction, Milstein et al. (22) found an amino terminal extension present on immunoglobulin light chains synthesized in vitro. This model is supported by the results of recent investigations in both procaryotes (7, 14, 19, 24, 26, 27, 30) and eucaryotes (9, 10, 17, 18, 28, 29). In every case but one, ovalbumin (25), these extracellular proteins have been synthesized as larger membrane-associated precursors. The precursors sequenced to date (35) have shown a peptide extension to be present at the amino terminus, composed of a core of hydrophobic amino acids usually 15 to 30 amino acids long. Recent evidence suggests that this "extension" or "signal sequence" functions to initiate protein transport (6), although it appears that the extension itself may not be sufficient for the entire transport process (23). The mature form of the peptide is subsequently obtained by a proteolytic cleavage that removes the leader sequence (33).

The staphylococcal enterotoxins (SEB in particular) may be secreted via a similar mechanism. Altenbern (1) proposed that a proteolytic event was necessary for enterotoxin transport when he observed that protease inhibitors prevented SEB accumulation in the medium. However, this work did not directly address the existence of a cell membrane-associated toxin precursor. This study presents evidence that a larger precursor to extracellular SEB exists on the membrane of $SEB^+$ *S. aureus*.

## MATERIALS AND METHODS

**Bacterial strains.** Various $SEB^+$ and $SEB^-$ strains of *S. aureus* were used in this study. *S. aureus* S6, DU4916, and 10-275 (an S6 mutant that produces high levels of SEB) are $SEB^+$ strains, and S6R, RN450(8325-4), RN451(8325-4[$\phi$11]), and FRI100 are $SEB^-$ strains.

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**Purification of cytoplasmic membranes.** Cultures were grown in broth containing 3% N-Z Amine (Humko-Sheffield, City, State), 3% protein hydrolysate powder (Mead Johnson, Evansville, Ind.), and 0.075% minimal essential medium vitamins (GIBCO, Grand Island, N.Y.). An overnight culture was diluted 1:4 in fresh broth and grown at 37°C for 4 h. The cells were harvested and washed once in 10 mM $Na_2HPO_4$, pH 6.5, containing 20 mM $MgSO_4$ (hypotonic buffer). The typical cell yield was 20 g (wet weight) per 3 liters of broth culture. Purification of the cytoplasmic membranes was performed according to Theodore et al. (34) with the following modifications. The cell paste (20 g, wet weight) was suspended in 200 ml of hypertonic buffer (hypotonic buffer plus 3.5 M NaCl) for protoplast formation. For the induction of protoplasts, 15 μg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) per ml was added, and the cells were incubated at 37°C for 90 min. Conversion to protoplasts was greater than 95% after 60 min at 37°C as determined by microscopy.

**Affinity purification of anti-SEB immunoglobulin.** Approximately 30 mg of purified SEB (a gift of J. Metzger, Ft. Detrick, Md.) was immobilized on 5 ml of Affigel-10 (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions provided by the manufacturer. After coupling, the gel was washed first with 200 ml of 0.1 M borate, pH 8.0, containing 0.2 M NaCl (buffer A) and subsequently with eluting buffer (buffer B), 0.2 M glycine-hydrochloride, pH 2.7, to remove any unbound SEB. After reequilibration of the gel in buffer A, goat immune serum directed against SEB was equilibrated with the SEB affinity matrix for 1 h. The gel was then washed with buffer A until the absorbancy at 280 nm of the effluent was less than 0.005. The affinity gel was then drained of buffer A and equilibrated in buffer B for 30 min to elute specifically bound antibody. The eluted antibody was renatured by dialysis against buffer A.

**AGID and IEP of solubilized membranes.** Gels were prepared in buffer C (0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0, containing 0.5% deoxycholate, 2.0% Nonidet P40, and 1.5 M urea). This buffer was used to solubilize the membranes and to prepare SEB standards for both agar gel immunodiffusion (AGID) and immunoelectrophoresis (IEP). IEP was carried out in 1% agarose for 8 h at 60 V. AGID was performed according to the procedure of Spero et al. (31). The gels were finally dried and stained with Coomassie blue B made up in 7.5% acetic acid and 20% methanol.

**SDS-PAGE and nitrocellulose blot analysis of membrane proteins.** A modification of the discontinuous SDS-PAGE procedure of Blattler et al. (3) was used for the separation of the membrane proteins. The resolving gel was composed of 17.5% acrylamide and 0.4% bisacrylamide. It was necessary to use a high level of cross-linker to achieve separation of the putative precursor from the extracellular SEB. All samples were solubilized in sample buffer (36 mM sodium phosphate buffer, pH 7.2, 4% sodium dodecyl sulfate, 35% glycerol, and 0.1% phenol red) and boiled for 5 min. Electrophoresis was carried out at 30 mA until a cytochrome C marker reached the bottom of the resolving gel. After electrophoresis, the proteins were passively blotted to nitrocellulose sheets by the procedure of Bowen et al. (5) for 24 to 36 h at room temperature in the following "blot" buffer: 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.0, containing 2mM ethylenediaminetetraacetate, 50 mM NaCl, and 0.1% NaI.

After transfer of the proteins from the gel to the nitrocellulose, additional protein-binding sites were blocked by incubating the blot for 3 h at 37°C in blocking buffer (blot buffer containing 3% bovine serum albumin and 10% heat-inactivated preimmune goat serum). After this blocking, affinity-purified anti-SEB immunoglobulin G (IgG), labeled with I-125 by the chloramine-T method of Krause and McCarty (16), was made up in the solution used for the blocking reaction. The specific activity of the iodinated antibody was $10^6$ cpm/μg of IgG. The nitrocellulose blot was incubated in 50 ml of the blocking buffer containing the labeled anti-SEB ($5 \times 10^5$ cpm/ml; usually sufficient for a 15- by 15-cm blot) for 8 h at room temperature. In the above buffers, the preimmune serum was included not only to block additional protein-binding sites but also to block any IgG binding by protein A and to lower any nonspecific interactions. The NaI was added to compete with any free I-125 still present in the labeled antibody. This was necessary because some free I-125 remained even after passage of the labeled antibody through a 1.5- by 30-cm column of Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). We found that free I-125 bound to the nitrocellulose blot, which raised the background upon exposure to the X-ray film and thus lowered sensitivity.

After incubation with the radioactive probe, the blot was washed five times for 5 min each time with 200 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.0, containing 1% NaCl to remove the unbound antibody. The nitrocellulose sheet was then dried at 90°C for 10 min and wrapped in cellophane. Visualization of the protein bands recognized by labeled anti-SEB was accomplished by exposing the blot to Kodak X-Omat AR X-ray film for 12 to 36 h at −70°C. Ultimate sensitivity of this assay, in our hands, is about 5 ng per band after 36 h of autoradiography.

**Purification of the putative SEB precursor.** Purification of the putative precursor to SEB (pSEB) was achieved in a single step by immunoprecipitation. Approximately 700 to 800 units of absorbancy at 660 nm purified membranes from *S. aureus* 10-275, a high SEB producer, were pelleted and solubilized in 0.5 ml of buffer C. Affinity-purified goat anti-SEB IgG (100 μg) was added to the solubilized membranes and incubated for 24 h at 4°C. The antibody complex was coprecipitated with affinity-purified rabbit anti-goat IgG immobilized on nylon beads (AMF/Biological and Diagnostic Products Co., Seguin, Tex.) for 24 h at 4°C. The immunoprecipitate was collected by centrifugation and washed five times with 10 ml of buffer C, followed by two 10-ml washes with distilled water. The antigen-antibody complex was then disrupted by boiling the immunoprecipitate for 10 min in 0.2 ml of 10 mM tris(hydroxymethyl)aminomethane, pH 7.0, with 4% sodium dodecyl sulfate. The nylon beads were removed by centrifugation, and the supernatant con-

taining the putative precursor and the primary antibody was labeled with I-125 and prepared for SDS-PAGE. After electrophoresis, the gels were dried and exposed to Kodak X-Omat AR X-ray film for visualization of the labeled proteins.

**Peptide mapping.** To prepare the pSEB and SEB for peptide mapping, the I-125 labeled derivatives were isolated from SDS-PAGE gels according to the following procedure. After electrophoresis, the gel was washed for 6 h in a solution of 25% isopropanol and 10% methanol and dried onto 3MM filter paper (Whatman, Inc., Clifton, N.J.). The labeled bands of the pSEB and SEB were located by autoradiography and excised, and most of the paper backing was removed. Each gel slice was soaked in 4 ml of 50 mM ammonium bicarbonate (pH 7.8) containing 0.05 mg of trypsin (2× crystallized; Millipore Corp., Bedford, Mass.) per ml.

After incubation at 37°C for 24 h, the solution containing the tryptic peptides was withdrawn and concentrated in a Speed Vac (Savant Instruments, Inc., Hicksville, N.Y.). The tryptic peptides were then suspended in electrophoresis buffer (acetic acid-formic acid-water, 3:1:16 [vol/vol]) for two-dimensional peptide mapping. Approximately 50,000 cpm of each digest was spotted onto a 20- by 20-cm thin-layer cellulose plate (Kodak Chromagram, no. 6064). Electrophoresis was carried out at 50 V/cm at 10°C until the tracking dye (0.1% Pyronine Y) migrated 14 cm. After electrophoresis, the cellulose plate was dried and chromatographed in the second dimension in pyridine-butanol-acetic acid-water, 25:32.5:5:20 (vol/vol). The tryptic peptides were located by autoradiography. Common tryptic peptides were determined by performing a mixed peptide map that contained the peptides from both the pSEB and SEB. Each spot was counted, and the number of counts per spot was compared with that found in the individual tryptic maps.

## RESULTS

**AGID and IEP analysis of *S. aureus* membrane proteins.** To analyze the membrane proteins of *S. aureus*, they had to be solubilized under conditions that would allow the antigen-antibody complex to form. Nonionic detergents such as Triton X-100, Nonidet P40, and Tween 80 at concentrations of up to 2% were completely ineffective in solubilizing the immunoreactive component. The use of urea (1.5 M) or deoxycholate (2.0%) also resulted in little or no solubilization of this component. A combination of urea (1.5 M), Nonidet P40 (2%), and deoxycholate (0.5%) effectively solubilized the pSEB component and interfered minimally with the antigen-antibody reaction.

When analyzed by AGID (Fig. 1), membrane proteins from *S. aureus* S6 ($SEB^+$) demonstrated a component that reacted with anti-SEB IgG and formed complete lines of identity with purified extracellular SEB. No reaction was observed when this assay was repeated with preimmune globulin (data not shown). This membrane component was not contaminating extracellular SEB adhering to the membranes, as subsequent experiments proved. Analysis by IEP revealed an immunoreactive component (Fig. 2, well 3) that was found to possess an electrophoretic mobility different from that of extracellular SEB. By comparison, extracellular SEB migrated very little (Fig. 2, well 1).

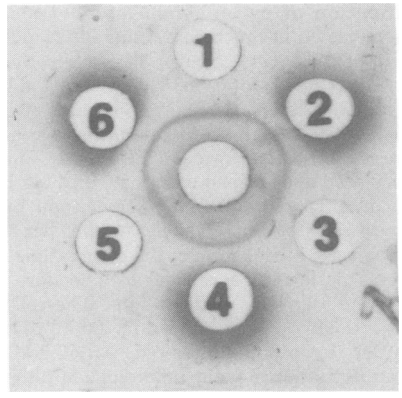


FIG. 1. *Agar gel immunodiffusion analysis of solubilized S. aureus S6 membranes. Wells 1, 3, and 5 contain 10 μl of a 100-μg/ml solution of purified SEB. Wells 2, 4, and 6 contain 10 μl of solubilized S6 membranes (approximately 50 to 70 units of absorbance at 660 nm). The center well contains 10 μl of affinity-purified anti-SEB (2.1 mg/ml).*

These results suggested that we were dealing with a membrane-bound protein that shared antigenic determinants with extracellular SEB but was electrophoretically different. This prompted us to investigate the possibility that this protein was actually a membrane-bound precursor to extracellular SEB. According to present evidence, such a precursor was likely to be larger by virtue of the hydrophobic signal sequence.

**SDS-PAGE and nitrocellulose blot analysis of membrane proteins from *S. aureus*.** To determine whether the immunoreactive component identified by AGID and IEP on *S. aureus* S6 membranes was different in molecular weight from extracellular SEB, we utilized a nitrocellulose blot procedure after SDS-PAGE. This allowed us to probe specific membrane proteins transferred to nitrocellulose with an affinity-purified iodinated antibody to SEB. By using this method, we were able to analyze the membrane proteins resolved by SDS-PAGE for the pSEB and screen the membrane proteins of several strains for this putative precursor fairly rapidly. Since our antibody was affinity purified, we were reasonably sure that nonspecific reactions due to contaminating antibodies would be minimal. This is important since *S. aureus* is a common infectious agent in wounds and the animals from

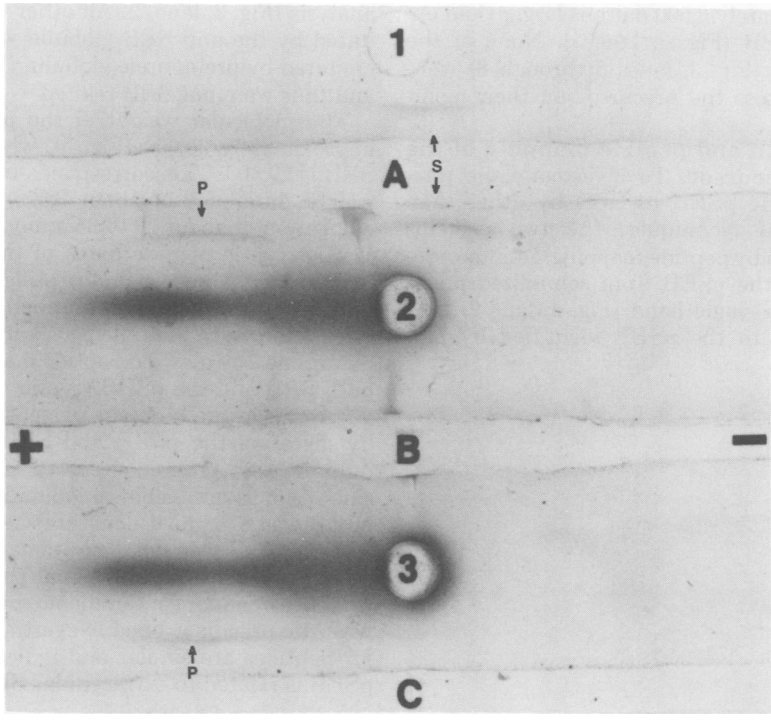


FIG. 2. *Immunoelectrophoretic analysis of solubilized membranes from S. aurues S6. Well 1, 10 μl of a 100-μg/ml solution of purified SEB; well 2, mixture of solubilized membranes from S. aureus S6 and purified SEB; well 3, solubilized membranes from S6. P and S indicate the precipitin lines for pSEB and SEB, respectively. Troughs A and C, affinity-purified anti-SEB (2.1 mg/ml); trough B, preimmune IgG (2.1 mg/ml).*

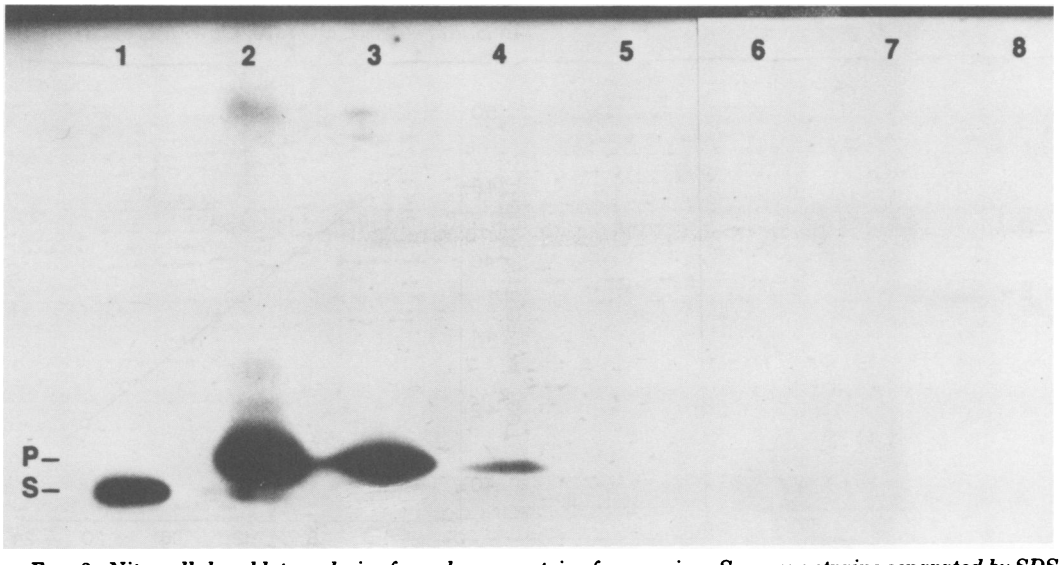


FIG. 3. *Nitrocellulose blot analysis of membrane proteins from various S. aureus strains separated by SDS-PAGE. Lane 1, purified SEB (2 μg); lane 2, 10-275; lane 3, S6; lane 4, DU4916; lane 5, RN450; lane 6, RN451; lane 7, S6R; lane 8, FRI100. P and S denote positions of pSEB and SEB, respectively.*

which the immune serum was obtained (goats) could have had antibody titers against other *S. aureus* antigens.

Membranes were purified from SEB$^+$ (S6, 10-275, and DU4916) and SEB$^-$ (RN450, RN451, S6R, and FRI100) strains and analyzed by this procedure (Fig. 3). The SEB$^+$ strains (Fig. 3, lanes 2 through 4) all contain a membrane spe-

cies approximately 3,500 daltons larger than extracellular SEB (Fig. 3, lane 1). None of the $SEB^-$ strains (Fig. 3, lanes 5 through 8) were found to possess the precursor on their membranes.

**Purification and peptide mapping of the putative precursor.** To investigate the relationship of the pSEB to SEB by other than immunological techniques, the two proteins were analyzed by peptide mapping. Immunoprecipitation of the pSEB from solubilized membranes gave a single band (Fig. 4, lane 2) that corresponded to the pSEB identified by blot analysis (Fig. 3, lane 2). All other bands precipitated by the anti-SEB globulin were also precipitated by preimmune globulin (Fig. 4, lane 3) and thus were not SEB related.

The molecular weight of the precursor was approximately 33,000 (Fig. 5), whereas that of SEB is 29,500. This corresponds to a molecular weight difference of about 3,500 or a peptide elongation of about 23 to 35 amino acids.

The tryptic peptide maps of the pSEB and SEB (Fig. 5) show several peptides common to both proteins (peptides 1 through 6). The peptides common to both the pSEB and SEB wsere determined by a mixed peptide map (Fig. 5C) of both SEB and the pSEB tryptic peptides. The difference in the numbers of peptides obtained by digesting the pSEB and SEB may reflect differences in accessible trypsin sites or in tyrosine residues accessible to iodination (since the pSEB was SDS-heat denatured before labeling and the SEB was not), additional tryptic peptides resulting from the signal peptide, or the presence of minor contaminants that comigrated with the precursor band. Nevertheless, the major peptides are similar and indicated that the pSEB is related to extracellular SEB (Fig. 5).

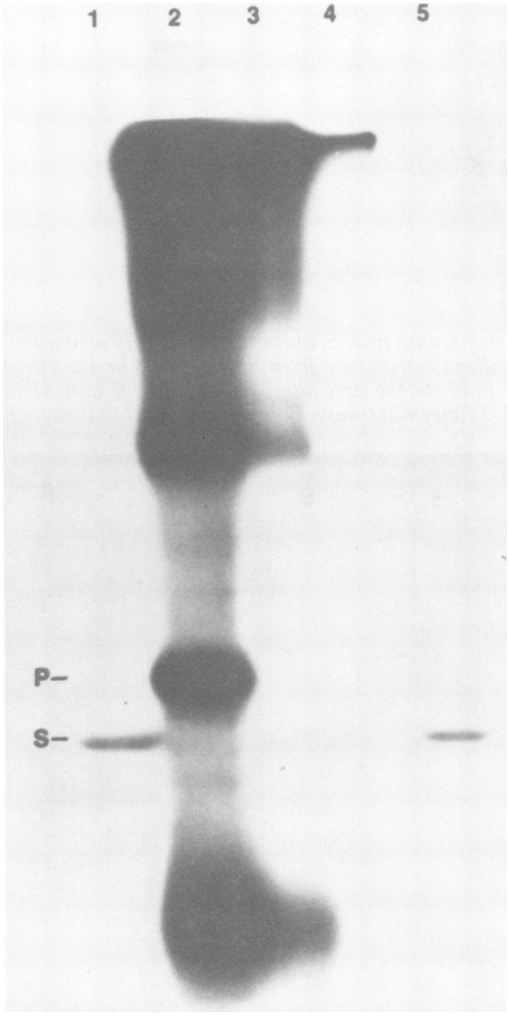


FIG. 4. *SDS-PAGE of immunoprecipitated p'ɔEB from the membrane of S. aureus 10-275. Lanes 1 and 5, purified SEB; lane 2, anti-SEB immunoprecipitate from the membrane of S. aureus 10-275; lane 3, preimmune IgG immunoprecipitate from the membrane of S. aureus 10-275; lane 4, IgG. P and S denote positions of pSEB and SEB, respectively.*

## DISCUSSION

Earlier studies have attempted to demonstrate cell-associated SEB in *S. aureus* but have yielded conflicting results. Forsgren et al. (8) were unable to demonstrate cell-associated SEB in *S. aureus* S6, DU4916, Cowan I, or Wood 46

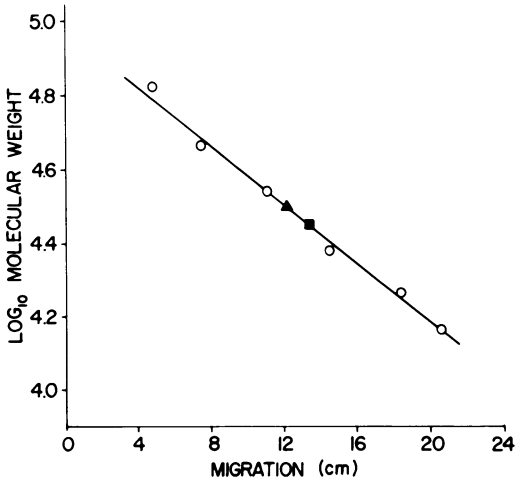


FIG. 5. *Molecular weight plot of pSEB and SEB from gel shown in Fig. 4. From left to right, the circles indicate the following markers: bovine serum albumin (66,000 dal), egg albumen (45,000 dal), pepsin (34,700 dal), trypsinogen (24,000 dal), beta-lactoglobulin (18,400 dal), and lysozyme (14,300 dal). ●, pSEB; ■, SEB.*

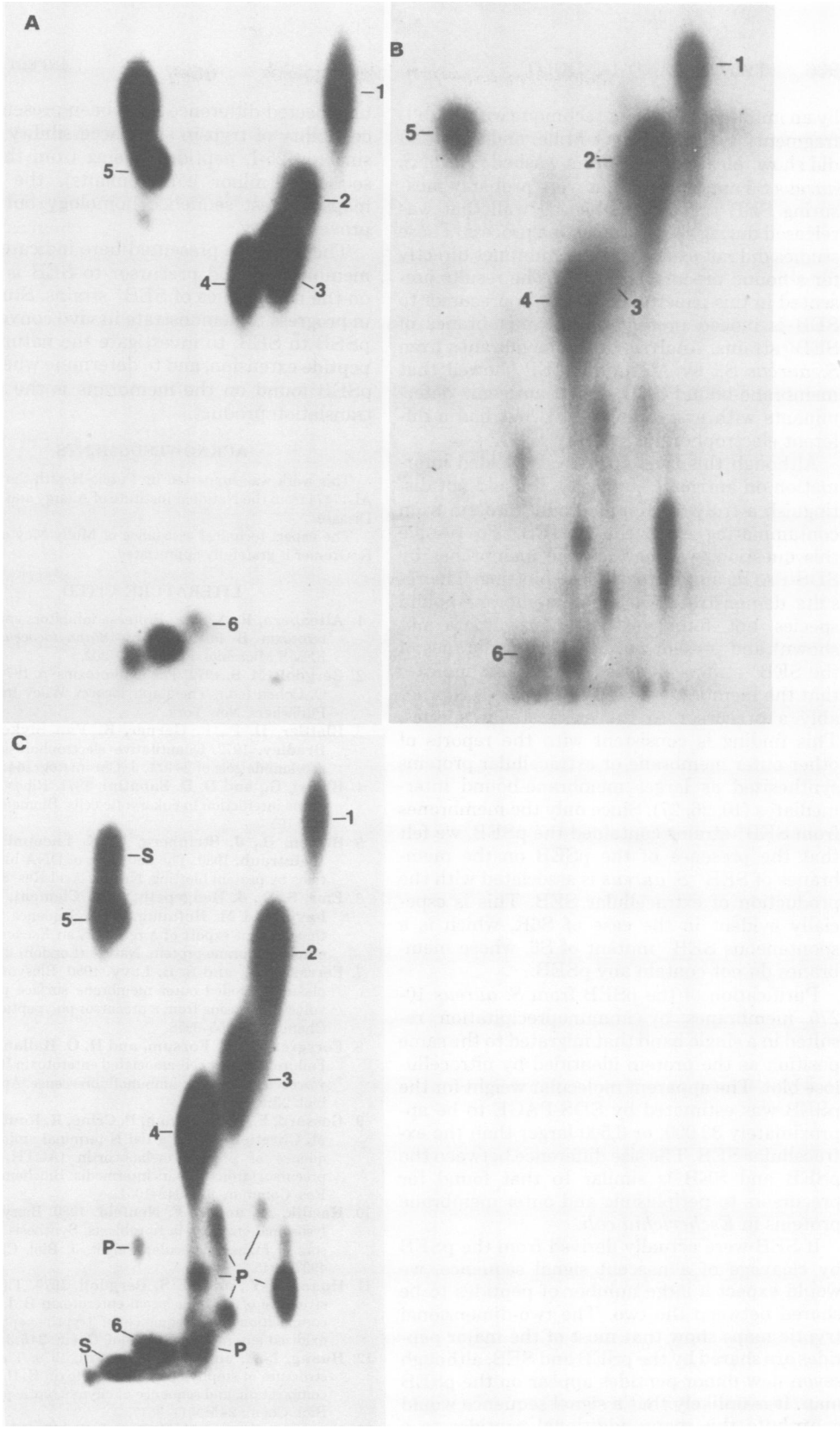


FIG. 6. *Two-dimensional tryptic peptide maps of pSEB and SEB. (A) SEB; (B) pSEB; (C) mixture of peptides from both SEB and pSEB. Peptides common to both SEB and pSEB are denoted by the numbers 1 through 6; peptides unique to SEB or pSEB are designated by S and P, respectively. Electrophoresis was from left to right (+ to −), and chromatography was from bottom to top.*

by an immunofluorescent technique with $F(ab')_2$ fragments of anti-SEB IgG. Miller and Fung (21) did show release of SEB from washed cells of *S. aureus* S6 and DU4916 but were probably measuring SEB trapped in the cell wall that was released during the protoplasting process. These studies did not examine the membranes directly for a bound precursor to SEB. The results presented in this paper indicate that a precursor to SEB is indeed present on the membranes of $SEB^+$ strains. Analysis of the membranes from *S. aureus* S6 by AGID and IEP showed that membrane-bound SEB shared antigenic determinants with extracellular SEB but had a different electrophoretic mobility.

Although this type of analysis yielded information on antigenic similarity, it could not distinguish a truly membrane-bound protein from contaminating extracellular SEB. To resolve this question, we analyzed the membranes by SDS-PAGE and nitrocellulose blotting. The results demonstrated a larger membrane-bound species not found extracellularly (data not shown) and present only on the membranes of the $SEB^+$ strains analyzed. These data indicate that the membrane-bound SEB species is probably a precursor to the extracellular species. This finding is consistent with the reports of other outer membrane or extracellular proteins synthesized as larger membrane-bound intermediates (10, 26, 27). Since only tbe membranes from $SEB^+$ strains contained the pSEB, we felt that the presence of the pSEB on the membranes of $SEB^+$ *S. aureus* is associated with the production of extracellular SEB. This is especially evident in the case of S6R, which is a spontaneous $SEB^-$ mutant of S6, whose membranes do not contain any pSEB.

Purification of the pSEB from *S. aureus* 10-275 membranes by immunoprecipitation resulted in a single band that migrated to the same position as the protein identified by nitrocellulose blot. The apparent molecular weight for the pSEB was estimated by SDS-PAGE to be approximately 33,000, or 3,500 larger than the extracellular SEB. The size difference between the pSEB and SEB is similar to that found for precursors to periplasmic and outer membrane proteins in *Escherichia coli*.

If SEB were actually derived from the pSEB by cleavage of a nascent signal sequence, we would expect a large number of peptides to be shared between the two. The two-dimensional tryptic maps show that most of the major peptides are shared by the pSEB and SEB, although seven new minor peptides appear on the pSEB map. It is unlikely that a signal sequence would contribute this many additional peptides to a precursor molecule. Although reasons for this unexpected difference have been presented (accessibility of trypsin sites, accessibility of tyrosine to 125-I, peptides arising from the signal sequence, minor contaminants), the peptide maps suggest sequence homology but do not prove identity.

The evidence presented here indicates that a membrane-bound precursor to SEB is present on the membranes of $SEB^+$ strains. Studies are in progress to demonstrate in vivo conversion of pSEB to SEB, to investigate the nature of the peptide extension, and to determine whether the pSEB found on the membrane is the primary translation product.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-17474 from the National Institute of Allergy and Infectious Disease.

The expert technical assistance of Molly May and Kathy R. Gromer is gratefully appreciated.

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