

Preparation and Characterization of a Slime Layer Material Produced by *Staphylococcus aureus*¹ (36964)

RICHARD D. EKSTEDT AND JANET M. BERNHARD

Department of Microbiology, Northwestern University Medical School, Chicago, Illinois 60611

Previous studies in this laboratory (1) have shown that most strains of *Staphylococcus aureus* when cultured under appropriate conditions on a modified staphylococcus 110 medium synthesize large amounts of slime layer material. Organisms grown in this manner were shown to be more virulent for mice, and some strains were capable of absorbing protective antibody from hyperimmune rabbit antisera prepared against the Smith diffuse strain of *S. aureus* (2).

Further purification and chemical characterization of the slime layer material as well as some of its immunochemical properties are presented here.

Materials and Methods. Organisms. Slime layer has been prepared and purified from several recently isolated strains, and from laboratory strains of *S. aureus* described by other investigators in the past. The results have in all cases been essentially identical, therefore only those observed using the recently isolated 439 strain of *S. aureus*, and the Copenhagen strain obtained from Dr. J. Strominger, Harvard University, Cambridge, MA are presented here. Other strains used as vaccines in the preparation of antisera have been described (3). The cultures were maintained by monthly transfer on brain heart infusion (BHI, Difco, Inc., Detroit, MI) agar slants stored at 4° between transfers.

Preparation of slime layer. The slime layer material was separated and purified from organisms which had been cultured on a dialysate of Staphylococcus medium No. 110, modified by reducing the NaCl concentration to 3.0% as previously described (1). The or-

ganisms were grown first in 100 ml volumes of modified 110 broth at 37° for 18–20 hr without shaking. Petri plates (90 mm diameter) containing approximately 30 ml of modified 110 agar were then inoculated with 5–7 ml of the broth culture and incubated at 37° for 18 hr. Sufficient broth must be used to maintain the agar surfaces under fluid during the period of incubation. If the surface dries out, the slime layer material synthesized by the organisms is greatly reduced. Following incubation, the cells and slime layer material were loosened from the surface of the agar by gently agitating the plates, and removed with a pipette. The slime layer was separated from the cells by mixing them in a Waring blender for 15 min at 4°. The cells were sedimented by centrifugation at 3000 rpm (1700g) for 30 min in a refrigerated centrifuge. The supernatant was dialyzed against distilled water for 48 hr and lyophilized. This was designated the *crude slime layer* material.

Further fractionation of this material was carried out by trichloroacetic acid (TCA) and ethanol precipitations as outlined in Fig. 1. The final 3 vol ethanol precipitate was designated *partially purified slime* and was stored dry at –20°.

The partially purified slime was further separated into three fractions by column chromatography on Sepharose 4 B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). Fifty milligrams of partially purified slime were dissolved in 2–3 ml of distilled water, placed on a 2 × 70 cm column previously equilibrated with water, and eluted with water. Three milliliter fractions were collected at the rate of approximately 1 ml/15 min, and the absorption was read at 220 nm wavelength.

Analytical chemical methods. Total hexose

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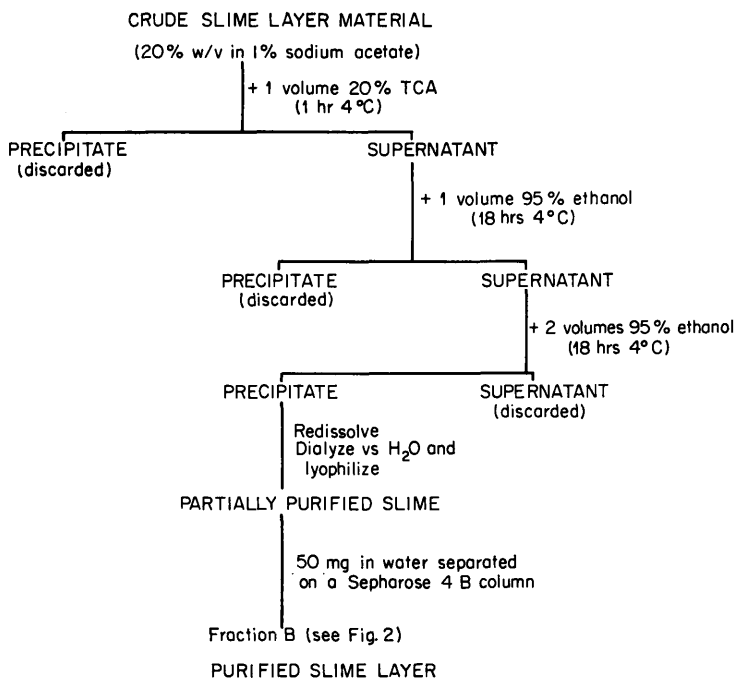


FIG. 1. Steps in the purification of slim layer from *S. aureus*.

was determined by the anthrone reaction using galactose as the standard (5). Total uronic acid was determined by the Dische carbazole method (6). Galacturonic acid was determined by the method described by Dische (7). Total phosphorus was determined by the method described by Matsumo and Slade (8). The nucleic acid and protein content was estimated by ultraviolet absorbancy at 260 and 280 nm using the calculations originally described by Warburg and Christian (9).

Gas chromatographic analyses were done on 10 mg samples hydrolyzed in 1.0 ml 2 *N* HCl at 100° for 3 hr. The samples were then evaporated to dryness *in vacuo* at room temperature. The hydrolysates were redissolved in 1.0 ml of silylating reagent which consisted of 1.5 mEq/ml *N*-trimethylsilylimidazole in silylation grade pyridine (Tri-Sil "Z," Pierce Chemical Co., Rockford, IL). Two microliters of the silylated sample were injected into the gas chromatograph (Varian Aerograph Model 1400) at a column temperature of 180° and run isothermally. The column was a 3% SE-30 100/120 (Varaport 30) prepacked by the manufacturer. All standards

were hydrolyzed, silylated, and run under the same conditions as the samples to be analyzed.

Immunochemical methods. Gel diffusion analyses were done in 5 cm plastic petri dishes with tight fitting lids (Falcon No. 1006) containing 5.0 ml of 1.0% agarose in 0.01 *M* phosphate buffered saline (PBS). The plates were allowed to react at room temperature for 48 hr, and the resulting precipitin bands were photographed unstained with an immunodiffusion camera (Cordis Corp., Miami, FL).

A quantitative precipitin curve was determined for the 439 purified slime layer and homologous hyperimmune rabbit antiserum using standard methods (6). Following equilibration and washing, the precipitates were analyzed for protein by the method of Lowry *et al.* (4). Hapten inhibition studies were done by the method described by Reeder and Ekstedt (10).

All antisera were prepared by repeated intravenous injection of rabbits with washed, autoclaved suspensions of the designated strains of *S. aureus* which had been grown for 18–20 hr at 37° in BHI broth. The vaccines were adjusted to contain approximately 10⁹

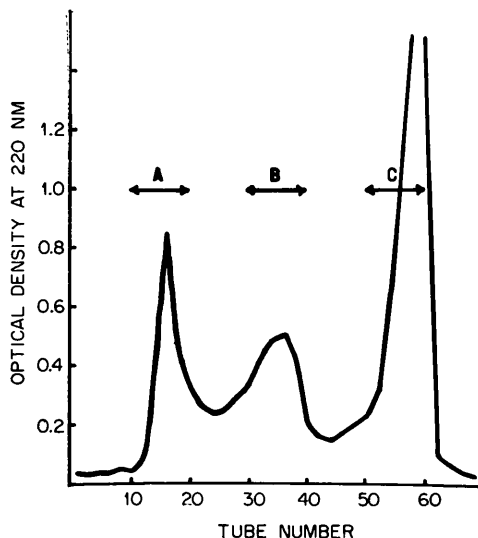


FIG. 2. Elution pattern of partially purified slime from the 439 strain of *S. aureus* on Sepharose 4B.

organisms/ml and were injected on 3 consecutive days each week for a period of 3 wk. The sera, separated 7–10 days after the last injection and at approximately weekly intervals for the next 6–8 wk, were stored at -20° in screw capped vials.

Results. Figure 2 shows a typical elution pattern of the 439 partially purified slime layer material from a Sepharose 4B column. The fractions designated as A, B, and C were lyophilized, redissolved in PBS and reacted

at various concentrations ranging from 10 to 1000 $\mu\text{g/ml}$ in a capillary precipitin test with homologous antiserum and with antisera prepared against the Cowan Type 1 and Foggie strains of *S. aureus*. Only fraction B reacted. The yield, on a dry weight basis, of fractions A, B, and C was 20, 75, and 5%, respectively. Since fraction B was the only fraction which was reactive immunologically, this fraction was designated *purified slime layer*, and all subsequent work was done with this fraction. It should be made clear that this designation was used for convenience only, and as shown below, does not imply that the material is of one molecular species both chemically and immunologically.

The purified slime layer from the 439 strain of *S. aureus* showed 32.6% hexose; 5.7% hexuronic acid; and 0.02% phosphorus. An ultraviolet absorption curve for a 0.1% aqueous solution gave optical densities at the indicated wavelengths (1 cm light path) as follows: 220 nm, 0.79; 240 nm, 0.36; 260 nm, 0.26; 280 nm, 0.20; 300 nm, 0.16. These data would suggest that there was less than 0.01% protein and essentially no nucleic acids present in this preparation.

Thin layer chromatography of acid hydrolysates indicated that there were several amino acids associated with purified slime layer, and this was confirmed by analyses on an automatic amino acid analyzer (Beck-

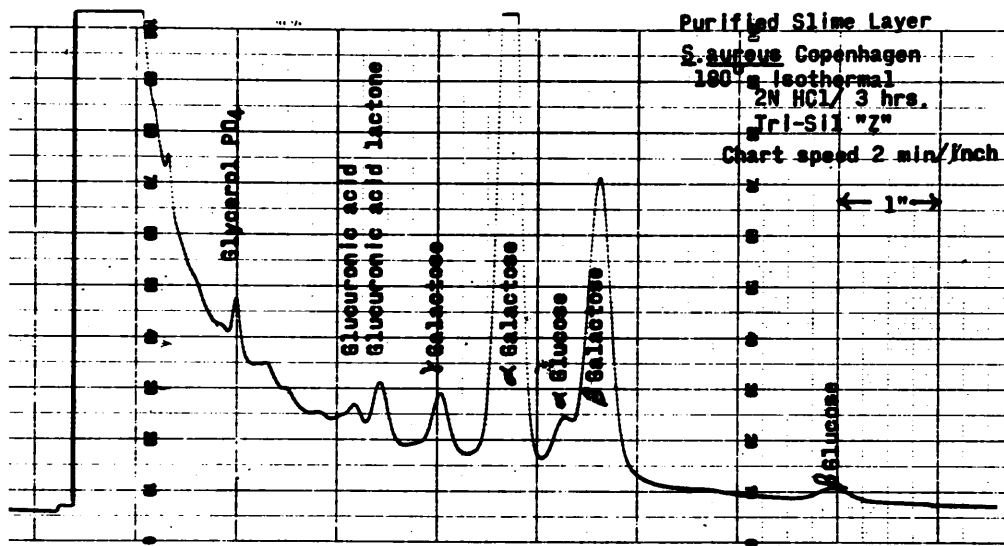


FIG. 3. Gas chromatographic pattern of purified slime from the Copenhagen strain of *S. aureus*.

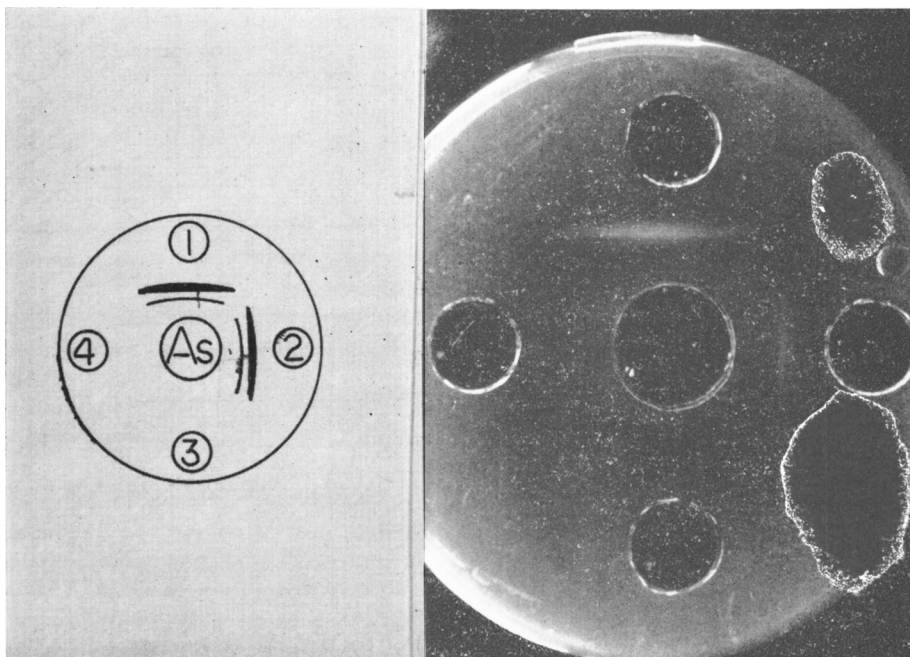


FIG. 4. Gel diffusion analysis of purified slime from the 439 strain of *S. aureus*. Wells 1, 2, and 3 contained slime at 1000, 100, and 10 $\mu\text{g/ml}$, respectively. Well 4 contained saline, and the center well contained hyperimmune rabbit antiserum against the homologous strain.

man Model 116). A sample of the 439 strain purified slime layer was hydrolyzed with 6 *N* HCl for 14 hr at 100°, lyophilized, and analyzed by standard methods. Glycine, proline, glutamic acid, and alanine were present in significant amounts, and setting alanine at 1, a ratio of approximately 3.3:1:1:1 was found. On a weight basis the values were 0.43, 0.13, 0.13, and 0.13 $\mu\text{M/mg}$, respectively, for the above amino acids. No glucosamine could be detected in this preparation under the conditions described.

Gas chromatographic analysis. Gas chromatographic analyses of the 439 and Copenhagen purified slime layer material revealed that galactose was the sugar consistently found in highest concentration. Glycerol phosphate was also present in significant amounts, while glucose and glucuronic acid were also identified in varying amounts in the samples, depending upon the preparations. A typical gas chromatographic pattern of the Copenhagen slime layer is shown in Fig. 3. The 439 pattern was essentially identical. No attempt was made to quantitate these data.

Further characterization of the uronic acid

component. In view of the significant amount of uronic acid found in the purified slime layer material from both the 439 and Copenhagen strains of *S. aureus*, it was considered important to further identify this component. Utilizing the cysteine-sulfuric acid method, developed originally by Dische (7), which distinguishes qualitatively between glucuronic and galacturonic acids, it was found that these slime layer materials also contained galacturonic acid. The typical rise in optical density between 540 and 600 nm wavelength for both strains' slime layer, which is characteristic of galacturonic acid, was observed. The difference between the optical density at 600 and 540 nm is positive for galacturonic acid and negative for all other hexuronic acids and polyuronides (7). In the case of the purified slime layer materials from the 439 and Copenhagen strains of *S. aureus*, these values were +0.42 and +0.46, respectively.

Immunological properties of purified slime. The 439 purified slime was reacted at 1000, 100 and 10 $\mu\text{g/ml}$ in gel diffusion analyses with antisera prepared against the homologous strain, and against the Cowan Type

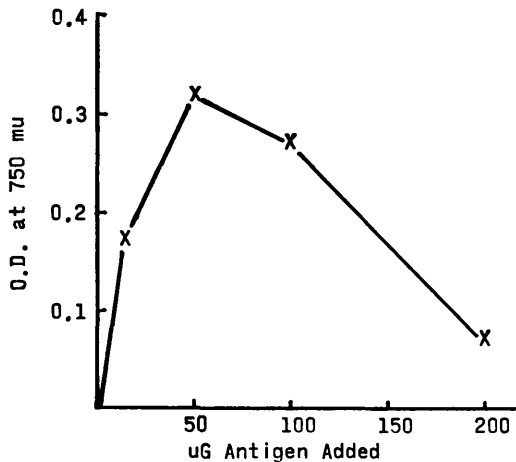


FIG. 5. Quantitative precipitin curve with purified slime from the 439 strain of *S. aureus* and homologous rabbit antiserum.

1, Copenhagen, Foggie, and Smith diffuse strains of *S. aureus*. One major precipitin band formed with the Cowan Type 1 and Foggie antisera, while 1 major and 1 minor band formed in the interaction with the homologous, Smith diffuse, and Copenhagen antisera. The reaction observed with the homologous antiserum is shown in Fig. 4. Figure 5 shows the typical quantitative precipitin curve obtained by reacting the purified 439 slime with rabbit antiserum to the homologous strain. Hapten inhibition studies with the 439 purified slime and homologous rabbit antiserum using several concentrations of galactose, glucuronic acid, galacturonic acid, and glucose did not reveal the immunodominant group in this antigenic material. Studies to further characterize the slime layer from *S. aureus* and to assess its protection inducing activity against staphylococcal infection are in progress.

Discussion. It is clear from the results presented that the slime layer material produced by strains of *S. aureus*, is a complex polysaccharide. The slime was shown to contain galactose, glucuronic and galacturonic acid, glycerol phosphate, and glucose. It also contained glycine, alanine, glutamic acid, and proline in significant amounts.

The presence of galactose and particularly galacturonic acid in the slime material was of considerable interest, since these sub-

stances have not been previously reported to be contained in any surface or extracellular products produced by *S. aureus*. Galacturonic acid has been reported, however, to be a constituent of the capsular polysaccharide of *Diplococcus pneumoniae* Type 1 (6). Antiserum to this microorganism did not cross react with slime layer preparations from several strains of *S. aureus* in a capillary precipitin test.

Unfortunately there are no convenient methods available for the quantitative determination of galacturonic acid derived from natural polysaccharides. The detection of glucuronic acid by gas chromatography is possible by virtue of its ability to readily form the lactone during acid hydrolysis of the polymer. Because of steric considerations, in the case of galacturonic acid, lactonization is not possible, since furanoid rings are difficult to accommodate, and the conditions necessary to break the uronosyl link results in the degradation of the galacturonic acid as it is released (11).

McDonald and Karakawa (12) recently reported on the immunochemical analysis of a uronic acid polymer from a strain of *Staphylococcus epidermis*. They found that the acidic polysaccharide was composed of D-glucuronic acid and glucosamine in equimolar amounts. It was antigenic, but hapten inhibition studies with D-glucose, D-glucuronic acid, N-acetylglucosamine, cellobiose, and maltose did not reveal the immunodominant structure. These investigators suggested that the antigenic specificity could depend on the linkage of the two sugar residues and not on the individual components. The lack of glucosamine in our slime material, as determined by the automatic amino acid analyzer, indicates it is different in its chemical composition than the polymer described by McDonald and Karakawa.

Glucuronic acid has been shown to be a constituent of the staphylococcal polysaccharide antigen (SPA) (13) and probably also of the Smith surface antigen (SSA) (14). These antigens have been shown to induce protective antibody in mice against challenge with the Smith diffuse strains of *S. aureus*. Previous studies (2) have shown that protective rabbit antisera against the Smith dif-

fuse strain of *S. aureus* when absorbed with slime layer from several non-Smith strains loses its protective activity. These results suggest that the slime layer material contains protection inducing antigens related to SPA or SSA. These experiments were undertaken as a result of the suggestion made some time ago by Rogers (15) that all strains of *S. aureus* may have the potentiality for synthesizing SSA when growing in the tissues of the host, but lose this capacity rapidly during subculture in laboratory media. It is conceivable that our method of growing the organisms in modified staphylococcal 110 medium has allowed for the induction of the latent capacity of all strains of *S. aureus* to synthesize SSA.

Summary. The preparation, purification, and partial chemical and immunochemical characterization of a polysaccharide slime layer material synthesized by strains of *Staphylococcus aureus* when cultured on a modified staphylococcus 110 medium has been described. The polysaccharide was found to be composed of galactose, glucuronic acid, galacturonic acid, glycerol phosphate, and some glucose. Glycine, alanine, glutamic acid, and proline were also associated with highly purified preparations of the polysaccharide. Reaction of the purified slime layer material with antisera prepared against several strains of *S. aureus* revealed 1 or 2 antigens depending upon the serum used. A classical precipitin curve could be demonstrated, but hapten

inhibition studies did not reveal the immunodominant determinant.

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