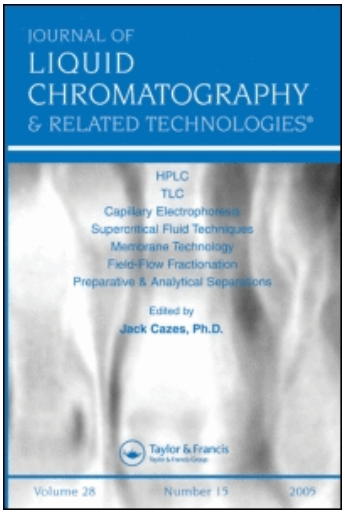

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PEPTIDE PROFILES OF SURFACE-LABELED PROTEINS SHED OR RELEASED BY ENZYME DIGESTION FROM *STAPHYLOCOCCUS AUREUS* DETERMINED BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The surface proteins of Staphylococcus aureus were radiolabeled and subsequently characterized by autoradiography and reverse phase-high performance liquid chromatography. Approximately 25% of the surface-labeled proteins were shed from nongrowing cells; the shed proteins contributed to the labeled-protein pool obtained by protease digestion of whole cells. The elution profiles of ^{125}I-labeled peptides allowed direct comparisons of shed surface proteins (untreated or protease digested) with surface proteins cleaved from the cells by protease treatment.

INTRODUCTION

Staphylococcus aureus is the most common cause of severe infection in the noncompromised patient including osteomyelitis,

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arthritis, endocarditis, bacteremia, toxic-shock syndrome, and pneumonia, particularly during epidemics of influenza (1). The organism produces a variety of extracellular enzymes which probably participate in its pathogenicity and the toxins it produces are 'legion' (2). In the case of S. aureus, the biochemistry of its cell walls is well-documented; peptidoglycan comprises about 40 to 60% by weight of the cell wall with teichoic acid as the other major component (3). Teichoic acid has been implicated in the binding of staphylococci to buccal mucosal cells (4), and quantitation of antibodies against teichoic acid has proven to be a useful diagnostic tool for clinicians treating patients with bacteremia (5).

Many cell wall preparations contain substantial proportions of proteins which may account for up to 30% of the weight of the wall (3). Some of these cell surface proteins are known to be receptors for some types of human proteins. For example, protein A interacts with the Fc region of immunoglobulins (6), clumping factor reacts nonenzymatically with plasma fibrinogen (7), and an 18 kd staphylococcal protein reacts with fibronectin (8). Other proteins have been studied extensively in an attempt to develop a clinically useful serologic typing system which is needed to identify differences among strains of staphylococci; at least 30 type-specific antigens have been recorded thus far (9). The number of antigens and their quantities show great variation from strain to strain and because they occur in very small amounts in the cell wall their isolation is obviously difficult (9).

These protein antigens have come to our attention recently because it appears they may be involved in mediating the binding of S. aureus to influenza A virus-infected cells (10). To date only an immunological approach has been used to identify these proteins. In the present study we were interested in determining if reverse-phase HPLC could be used to develop a characteristic profile of staphylococcal surface proteins from one clinical strain. The ultimate goal would be to use this technique as an alternative method for comparing strains of clinical importance.

During the course of the study, we discovered that considerable amounts of surface proteins were shed from the cells without the use of exogenous proteolytic enzyme, and these shed proteins were found to have a characteristic profile by HPLC.

MATERIALS AND METHODS

Iodination of Bacteria.

S. aureus Ci-1, a fresh clinical isolate of low passage history, was grown in static culture in M199 Medium (GIBCO Laboratories, Grand Island, N.Y.) by seeding 500-ml vol of M199 with 1-2 x 10^8 colony-forming units of bacteria and incubating at 37°C for 18 hr. Staphylococci were harvested by centrifugation, washed in H_2O, and freeze-dried. Fifty mg (dry wt) batches of Ci-1 were washed 1X in phosphate buffered saline (PBS), pH 7.2, and surface proteins were labeled with 1 mCi of ^{125}I by the chloramine T method (11). The surface-labeled staphylococci were washed 2X in PBS before use.

Enzyme Treatment.

Optimum amount of enzyme needed to release ^{125}I-labeled proteins from the staphylococci was determined as follows: to 8.3 mg (dry wt) pellets was added 1.5 ml of PBS only or PBS containing 0.1, 0.3, 1.0, 5.0 or 10 units of insoluble Streptomyces griseus protease (0.1 units/mg protease-bound beaded agarose; Sigma Chemical Co., St. Louis, Mo.). The suspensions were vortexed and incubated at 37°C for 3 hr with intermittent agitation. The bacteria and insoluble enzyme were pelleted by centrifugation and the amount of radioactivity present in the supernatant fluids was determined with a Packard Auto-Gamma scintillation spectrometer.

Materials for characterization by SDS-PAGE, autoradiography and HPLC were obtained as follows: A 200-mg (dry wt) amount of Ci-1 was surface-labeled and washed 2X in a 15-ml vol of PBS. To 50 mg pellets of the washed cells was added 6 ml of PBS only

(three tubes) or 6 ml of PBS containing 1 unit of insoluble enzyme/ml (one tube). The suspensions were vortexed. In one tube containing bacteria and PBS only, the bacteria were pelleted by centrifugation and the pellet was processed as will be described. The three remaining bacterial suspensions were incubated at 37°C for 3 hr with intermittent agitation. The tube containing bacteria and enzyme was centrifuged, the pellet saved for processing, and the supernatant fluid dialyzed exhaustively against triple deionized H_2O (m.w. cutoff of 6-8,000) in the cold. This procedure was repeated for one tube containing bacteria in PBS only. In a second tube containing bacteria and PBS only, the pellet was saved for processing; to the 6 ml of supernatant fluid was added 6 units of insoluble enzyme, the mixture was vortexed and further incubated at 37°C for an additional 2 hr with intermittent agitation. The enzyme-treated supernate was dialyzed as described. Before dialysis, each supernate was filtered (0.45 μm) and a 50-μl vol tested for radioactivity.

Following dialysis, the supernates were concentrated by ultrafiltration (YM2 filter, m.w. cutoff of >1,000, 40 psi [Amicon Corp., Danvers, MA.]). A 10-μl vol of each concentrate was tested for radioactivity, total protein was determined by a modified Lowry technique (12), and the remainder was aliquoted into a 1-ml vol and stored freeze-dried. Specific activity of the enzyme digest was 3.4×10^4 cpm/ng protein/ml; specific activity of the buffer extracts was 5.8×10^3 cpm/ng protein/ml.

The staphylococcal pellets were processed as follows: The bacteria were washed in buffer (50 mM tris HCl, 10 mM EDTA, pH 8.0), pelleted by centrifugation and resuspended in 4 ml of sample buffer (0.125 M tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue [Sigma]). The tube was placed on ice, and the bacteria were sonicated for 90 sec with a model W140D Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, L.I., N.Y.) with a setting at the micro tip limit. Suspensions of broken cells were heated at 100°C for 90 sec, a 50-μl vol tested for radioactivity, and the remainder stored at -70°C until needed.

SDS-PAGE and Autoradiography.

Bacterial sonicates were heated at 100°C for 10 min and freeze-dried bacterial supernates were solubilized in sample buffer and heated at 100°C for 5 min. A 30-µl vol of each test sample was subjected to electrophoresis (120 v constant current) on a 5% stacking and 10% resolving SDS-polyacrylamide gel according to the method of Laemmli (13). Protein profiles were detected by staining with Coomassie brilliant blue R. A duplicate set of electrophoresed samples were electroeluted out of the gel onto untreated nitrocellulose paper (NCP) by electrophoresis at a constant current of 20 mA for at least 48 hr in transfer buffer (14% glycine, 0.3% tris, 20% methanol). The NCP were dried in air and exposed to Kodak X-Omat AR film with a Du Pont Quanta-III CK intensifying screen (Du Pont Co., Wilmington, DE) at -70°C for 4 hr to 4 days. Standard molecular weight markers were included in slab gels and were visualized on the NCP after staining with Ponseau S.

HPLC.

One-ml vol of the reconstituted supernates were subjected to reverse-phase HPLC on a Zorbax Bio Series PEP-RP1 column (6.2 mm id by 8 cm; Du Pont Co.) in conjunction with a Du Pont gradient liquid chromatography system including the series 8800 gradient controller, an 860 absorbance detector and the 8800 chromatographic pump module. The supernates were eluted under the following conditions: 60 min gradient (exponent = 1) from 5% to 60% solvent (CH_3CH, 0.1% trifluoroacetic acid and 0.1% morpholine); flow rate of 1 ml/min. Fractions (750-µl vol) were collected and assayed for radioactivity.

RESULTS AND DISCUSSION

In the initial experiment, increasing concentrations of insoluble protease were added to suspensions of S. aureus Ci-1 to

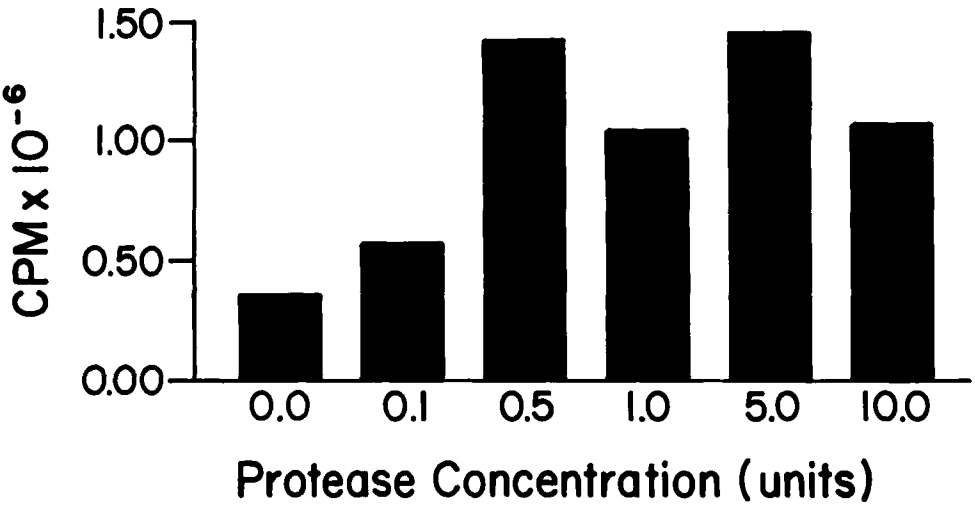

FIGURE 1. Release of ^{125}I-labeled surface proteins from S. aureus Ci-1 incubated in buffer only or in buffer containing increasing concentrations of insoluble protease.

determine the concentration needed to yield maximum cleavage of the ^{125}I-labeled surface proteins. Results, seen in Fig. 1, indicated that a concentration of $\geq$0.5 units of enzyme/1.5 ml/8.3 mg (dry wt) of staphylococci released maximum amounts of the surface proteins. However, we also noticed that ~25% of the radioactivity present in enzyme digests could be accounted for by release of surface-labeled proteins into the buffer in the absence of enzyme. It has been reported that actively growing strains of S. aureus turn over their old walls at constant rates of 15 to 30% per generation (14-16). Using electron microscopy, Giesbrecht et al. (17) showed that one strain of S. aureus, when cultured in the presence of chloramphenicol, thickened their cell walls by building up completely new wall layers underneath the old ones. When the antibiotic was removed, the bacteria released old wall material from all over their surface and reestablished the normal cell wall thickness. In cultures that are not actively growing, the old cell wall is preferentially released in buffer presumably due

to the action of autolysin (18). Obviously, a release of old cell wall material should also result in a shedding of the proteins associated with the cell wall surface, and this phenomenon may account for some, or all, of the shed proteins present in our test suspensions. To our knowledge no one has studied these surface proteins nor their potential clinical significance.

Radioactivity was determined on a 50-μl vol of each staphylococcal sonicate derived from cells that had: 1) received no treatment, 5.5 x 10^5 cpm, 2) been incubated in PBS only, 4.0 x 10^5 cpm, or 3) been digested with enzyme, 1.5 x 10^5 cpm. Cells shed 27% of the total counts when incubated in buffer compared to a release of 73% of the total counts following enzyme treatment. The latter percentage was not appreciably increased when the cells were incubated in the presence of enzyme for 24 hr instead of the 3 hr time used in the test system. These sonicates were subjected to SDS-PAGE and stained for protein patterns (Fig. 2A). No difference was seen between sonicates from untreated cells (lane 1) and cells incubated in PBS (lane 2), each of which had ~34 visible bands; a striking difference was seen between these two sonicates and the sonicate from cells that had been enzyme-treated (lane 3). Seven bands were still visible at molecular weights between 29 and 205 kd which undoubtedly represented polypeptides that were either resistant or inaccessible to the enzyme. Autoradiographs of the three sonicates can be seen in Fig. 2B. Again, no difference was noted between sonicates from untreated cells (lane 1) and cells incubated in PBS (lane 2); ~16 bands were visible ranging in molecular weights from <14 kd to 116 kd. Only one faint band (<14 kd) was visible in enzyme-treated sonicates, indicating the successful removal of surface-labeled proteins.

We used autoradiography (Fig. 3) and RP-HPLC (Fig. 4) to characterize the buffer extracts and enzyme digests obtained from Ci-1. Profiles of shed surface proteins are depicted in Fig. 3, lane 1 in which ~16 bands are visible by autoradiography with molecular weights ranging between <14 kd to 136 kd. By RP-HPLC (Fig. 4A) there are at least 9 definite peaks. It was obvious

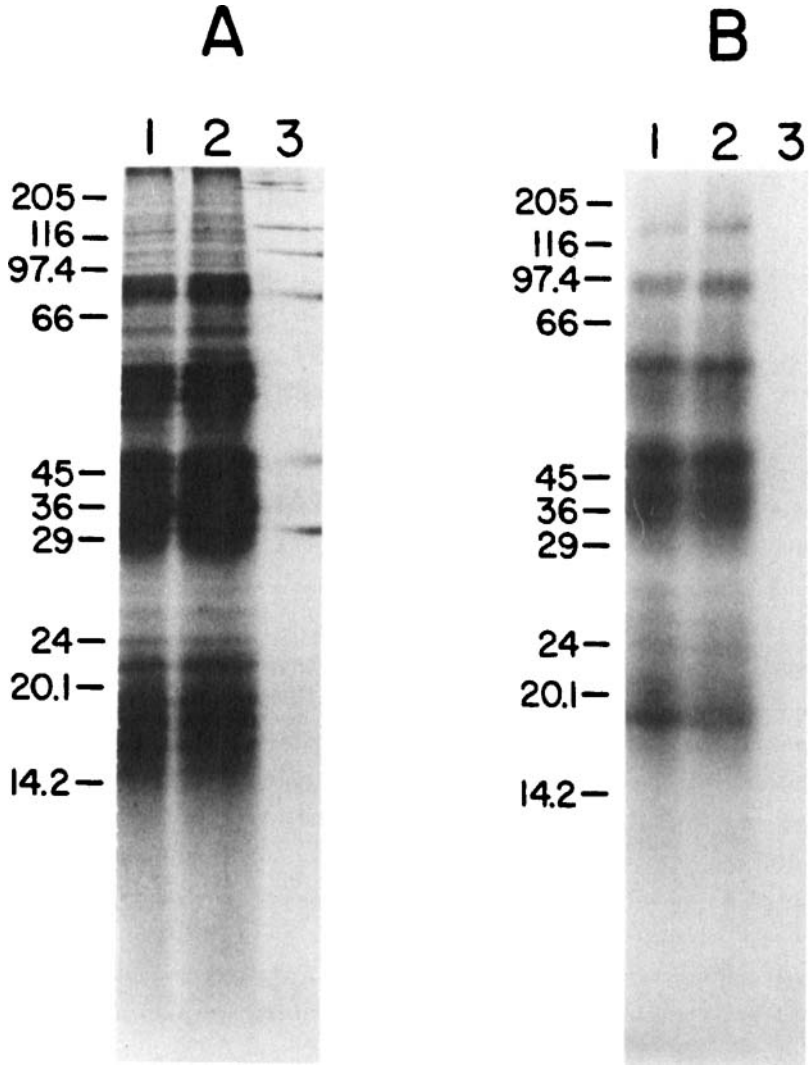

FIGURE 2. Polypeptides of S. aureus sonicates separated by SDS-PAGE and stained with Coomassie brilliant blue R (A) or electro-eluted onto nitrocellulose paper for the identification of ^{125}I-labeled surface proteins by autoradiography (B). Sonicates were obtained from cells that were untreated (lane 1), incubated in PBS only (lane 2), or digested with protease (lane 3). Molecular weight standards (x 10-3 daltons) are shown.

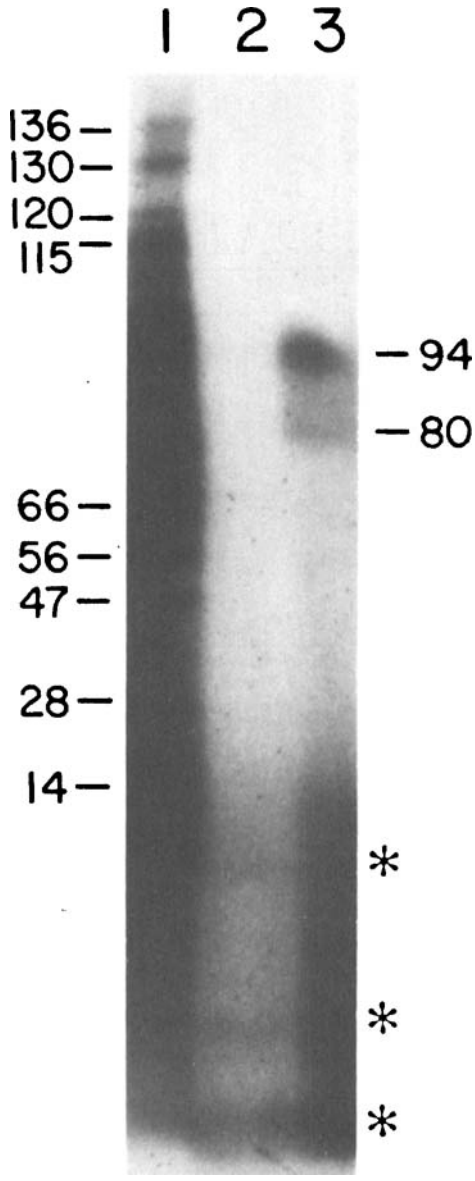

FIGURE 3. Autoradiograph of ^{125}I-labeled surface proteins of S. aureus that were either: shed from the cells and left untreated (lane 1); shed from the cells then digested with protease (lane 2); or, released from the cells by protease treatment. Molecular weights (x 10-3 daltons) of some bands are designated. The * identifies bands with molecular weights <14 kd.

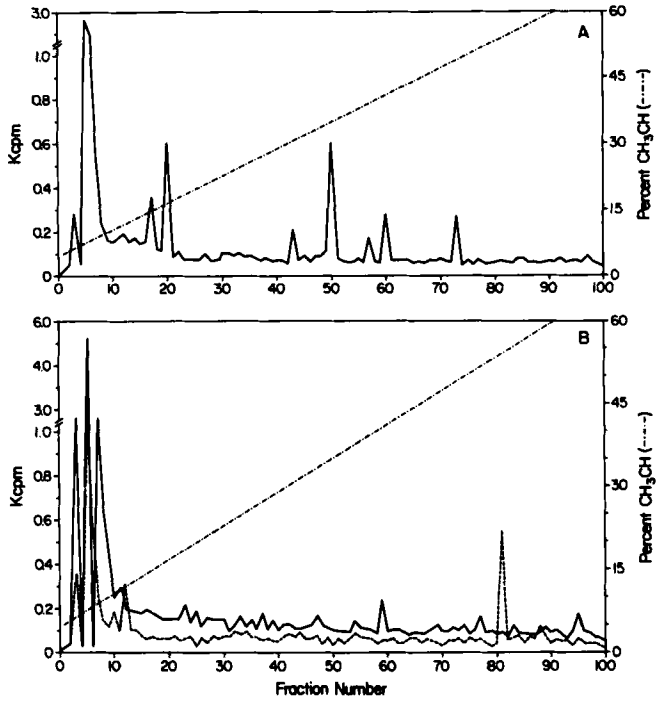

FIGURE 4. RP-HPLC elution profiles of ^{125}I-labeled surface peptides of S. aureus that were: (A) shed from the cells and left untreated; (B) shed from the cells then digested with protease (----), or released from the cells by protease treatment (——).

that shed proteins would contribute to the total number of surface-labeled proteins present in protease digests; therefore, a control was included that consisted of proteins shed into the buffer subsequently digested with enzyme. By autoradiography the enzyme-treated shed proteins (Fig. 3, lane 2) showed three bands with molecular weights <14 kd that co-migrated with bands in the enzyme digest of Ci-1 (Fig. 3, lane 3); the latter also had two additional high molecular weight bands (94 and 80 kd). When the enzyme digest of Ci-1 was subjected to RP-HPLC, three major peaks and numerous minor peaks (≥21) that were in relatively low concen-

trations were seen (Fig. 4B). The contribution of enzyme digested, shed proteins to the HPLC elution profile is also seen in Fig. 4B. The patterns obtained by both SDS-PAGE and RP-HPLC have been shown to be reproducible using different batches of surface-labeled Ci-1 processed on different days.

Several different approaches, other than the classic serological approach, have been used to compare difference between strains of S. aureus including peptidoglycan fingerprinting of protein-free cell wall by thin-layer chromatography and SDS-PAGE (19) and pattern analysis of solubilized cytoplasmic membrane proteins by two-dimensional gel electrophoresis (20). In the present work, we have shown the potential usefulness of RP-HPLC in analyzing the surface proteins of S. aureus. This technique can be applied in determining differences (the disappearance of peaks, or the appearance of new peaks) within a single strain, e.g., comparing the effects of different growth conditions, or in comparing differences between strains of clinical importance. It would be possible to customize the gradient conditions to allow better separation and recovery of proteins and peptides of particular interest for use in following the host's immune response during infection, as well as, for production of monoclonal antibodies to follow specific surface markers on the staphylococci during pathogenesis.

ACKNOWLEDGEMENT

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