Production, Purification, and Chemical Characterization of Staphylococcus aureus Exfoliative Toxin

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Methods for the production and isolation of exfoliative toxin are described. Fermentation conditions were established under which large quantities of the crude material can be produced. Column chromatography methods, including carboxymethyl cellulose and hydroxypatite, were utilized to purify the toxic protein. The pure toxin had a molecular weight of 26,000 as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The pure toxin is a simple protein composed of 17 amino acids. Tests for carbohydrate and for alpha- and beta-hemolysin were negative. The mean effective dose of the purified toxin was 0.5 μg per newborn mouse.

An exoprotein of Staphylococcus aureus, phage type II, has been suggested as the etiological agent of an epidermal exfoliative disease. Although the toxic activity has been described most commonly in children (Ritter’s disease), it has also been reported in adults (Lyell’s disease) (10). Melish and Glasgow described a suitable animal model for the identification and assay of the biological activity of the exfoliatin in newborn mice (11).

Different methods for the production of toxin have been reported, ranging from production in dialysis bags implanted in the peritoneum of the rabbit (12) to in vitro production in shaking flasks (8). However, cultural conditions utilized for producing adequate amounts for the development of purification methods or biochemical characterization have not been described.

This investigation was undertaken to study toxin production under controlled conditions of fermentation and, in addition, to define methods of purification permitting the isolation of gram amounts from a single fermentation. In this report, we describe the major biological and chemical properties of the exfoliatin, including a complete amino acid analysis.

MATERIALS AND METHODS

Bacterial strains. S. aureus strains TA and SA were supplied by Richard Warren. Strain TA was selected for the purification of the exfoliative toxin. It produces alpha-hemolysin and coagulase and is of phage group II.

Medium. The liquid medium consisted of 17 g of Trypticase (Baltimore Biological Laboratory, Cockeysville, Md.), 10 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g of NaCl, and 2.5 g of K2HPO4 per liter of distilled water. The pH of this medium was 7.1 and was used without further adjustment. Cultures were maintained as lyophillized ampules. Overnight growth of bacteria was harvested from tryptic soy agar (Difco) plates and lyophilized in 0.1-ml portions. A separate ampule was used for each experiment. Shake flasks contained 50 ml of medium in a 300-ml Erlenmeyer flask.

Fermentation conditions. A 50-liter portion of the Trypticase-yeast extract medium was used in a 70-liter fermenter (Fermentation Design, New Brunswick, N.J.). The temperature was maintained at 37 C; agitation was set at 400 rpm. Sparging was maintained at a rate of 10 liters of a gas mixture consisting of 90% air and 10% CO2 per min. An overnight culture (100 ml) propagated in shake flasks was used as the inoculum. After a fermentation of 20 h, the culture was centrifuged at 4 C (at 18,000 rpm) with a continuous-flow rotor (Lourdes, Old Bethpage, N.Y.). The culture supernatant was concentrated at 4 C with an Amicon TC3E system, using three UM-10 membranes (180 mm in diameter). After a 10-fold concentration which required about 24 h, the material was dialyzed against distilled water and lyophilized. This process yielded 14.5 g of crude toxin. Calculating from an activity per milligram basis, the crude material thus contained 1.45 g of pure toxin.

Chromatography. Microgranular carboxymethyl cellulose (Whatman CM52) was equilibrated in 0.01 M sodium phosphate, pH 6.0. Hydroxypatite (BioRad Laboratories, Richmond, Calif.) was equilibrated in 0.03 M sodium phosphate, pH 5.7.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Electrophoresis in 0.1% sodium dodecyl sulfate at pH 7.0 was performed in 8% polyacrylamide gels, as suggested by Weber and Osborn (16). Prior to electrophoresis, protein was denatured by incubation at 100 C for 10 min in a solution of 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, and 4 M urea. Gels were stained with Coomasie brilliant blue and destained by diffusion. Molecular weight standards were staphyloccocal enterotoxin B (28,500), chymotrypsinogen (25,700), and myoglobin (17,000).

Analyses. Nitrogen was determined by the micro-
Kjeldahl method. Carbohydrate was estimated by the method of Dubois et al. (4) by using phenol sulfuric acid. As an additional, more sensitive method for carbohydrate, the toxin was analyzed on a gas chromatograph by a modification of the method of Bhatti et al. (2).

Assay for exfoliation. Mice from strain CD-1 were utilized when 1 to 2 days old; older mice were variable in their reaction. Test solutions diluted in phosphate-buffered saline (0.01 M, pH 7.1) were injected (0.1 ml) subcutaneously at the nape of the neck to groups of 5 to 10 mice per test point.

One unit was defined as the amount of toxin causing a positive Nikolsky sign (peeling off of skin caused by slight rubbing) in 50% of the test animals at 4 h. Samples known to contain hemolytic activity were mixed with the appropriate amount of antiserum to neutralize the hemolysin prior to testing.

Assay for alpha-hemolysin. Test samples were diluted in buffer containing 0.15 M NaCl in a microtiter plate. An equal amount of a 2% solution of washed, rabbit erythrocytes was added. The titer is expressed as the reciprocal of the highest dilution causing complete lysis of the erythrocytes after 30 min at 37 C.

Production of antiserum. Antiserum to alpha-hemolysin was purchased commercially from Wellcome Research Laboratories, Beckenham, England. Antiserum to the exfoliative was prepared from the purified material isolated from hydroxyapatite columns. New Zealand rabbits were used for production of antiserum.

The first injection was 1 mg of toxin contained in 0.5 ml mixed with 0.5 ml of Freund complete adjuvant. Subsequent injections of 1 mg each were given at weekly intervals without the adjuvant. Test bleeding was done 7 days after the third inoculation.

Amino acid analysis. For the amino acid analysis, samples were hydrolyzed in sealed tubes in 6 N HCl under vacuum at 110 C for 24 h. The amino acid content was determined by the automated system of Evelleigh et al. (6). Elution was achieved with a sequence of lithium citrate buffers by a slight modification of the method of Benson et al. (1). Serine and threonine were corrected for decomposition assuming 89.5 and 94.7% recoveries, respectively (14). Tryptophan was measured spectrophotometrically in 6 N guanidine hydrochloride by the method of Edelhoch (5). An integral number of amino acid residues was calculated statistically by the method of Delaage (3).

C-terminal amino acid determination. Carboxypeptidase A and B were utilized by the method of Guidotti (7). The incubations for both enzymes were done at 37 C. The substrate-enzyme ratio was 10:1.

RESULTS

Isolation of toxin from crude material. A 2-g portion of the crude lyophilized toxin was dissolved in 200 ml of 0.01 M phosphate buffer, pH 6.0, and then dialyzed against the same buffer to insure equilibration. A column (2.5 by 40 cm) was filled with carboxymethyl cellulose and equilibrated with the same buffer for 24 h at room temperature at a flow rate of 1 ml/min. The toxin was run slowly onto the column (1 ml/min), and the column was washed with the equilibrating buffer until the optical density at 280 nm of the effluent return to near zero. The toxin was eluted from the column with 0.05 M sodium phosphate, pH 6.8, at a flow rate of 1 ml/min. Fractions of 10 ml each were collected. The peak eluted from the column contained alpha-hemolysin activity, as well as exfoliative activity (Fig. 1).

The peak fractions from the carboxymethyl cellulose column were dialyzed against 0.03 M sodium phosphate, pH 5.7. A column (1.5 by 30 cm) was filled with hydroxypatite equilibrated in the phosphate buffer at pH 5.7. The toxin sample was applied to the column at a flow rate of 0.5 ml/min. The column was washed with 0.03 M phosphate (pH 5.7) until 2 column volumes had passed through. A linear gradient elution was set up from 0.2 M phosphate (pH 5.7) to 0.4 M phosphate (pH 5.7). A 200-ml portion of each buffer was used to make the gradient. The flow rate of the column was 0.5 ml/min. Two distinct peaks were eluted from the column (Fig. 2). The first peak contained the exfoliative activity and was completely free from any hemolytic activity. The second, smaller peak contained the alpha-hemolysin and was completely free from any exfoliative activity.

![fig.jpg](http://iai.asm.org/)  
**Fig. 1. Carboxymethyl cellulose chromatography of crude exfoliative toxin.** Lyophilized material (2 g) was applied to a column (2.5 by 40 cm) equilibrated in 0.01 M sodium phosphate, pH 6.0. Elution was achieved with 0.05 M phosphate, pH 6.8. Fractions were 10 ml each. Flow rate was 1 ml/min.
activity. A summation of the purification is presented in Table 1.

Properties of purified toxin. In sodium dodecyl sulfate gels, the pure toxin showed only a single band (Fig. 3). From migration on these gels, the molecular weight of the toxin was determined to be 26,000 by using standard markers.

The Kjeldahl nitrogen content of the toxin was 16%. It had a maximum absorption of 278 nm and an extinction (E$_{278}$) of 8.4.

Tests for carbohydrate by the method of Dubois and by gas chromatography were negative. Tests for alpha- and beta-hemolysin were negative.

In Ouchterlony double diffusion (13), the pure toxin showed a single line of precipitate with its specific antiserum. The antiserum prepared to the exfoliative toxin did not neutralize the effects of reference alpha-hemolysin, nor did it form a precipitin line in Ouchterlony diffusion. Commercially available antiserum to the alpha-hemolysin did not neutralize the effectiveness of the exfoliative toxin, nor did it form a precipitin line with the toxin in Ouchterlony diffusion.

The complete results of the amino acid analysis are presented in Table 2. A 30-min incubation of 100 nmol of the exfoliatin with carboxypeptidase B revealed that the C-terminal amino acid is lysine. Extensive incubation with carboxypeptidase A yielded no free amino acids.

DISCUSSION

The demonstration that a single protein is responsible for the exfoliation of newborn mice supports the findings of other investigators in this field (8, 12). By defining methods for the

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**Table 1. Summation of exfoliative activity through purification procedure**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total material (mg)</th>
<th>Total toxin (mg)</th>
<th>Activity (U/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilized crude toxin</td>
<td>2,000$^a$</td>
<td>200</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>132</td>
<td>110</td>
<td>1,000</td>
<td>55</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>68</td>
<td>68</td>
<td>2,000</td>
<td>62</td>
</tr>
<tr>
<td>Overall yield</td>
<td></td>
<td></td>
<td></td>
<td>34</td>
</tr>
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</table>

$^a$ Although 14.5 g of material was harvested from 50 liters of culture supernatant, 2.0-g portions were processed individually to facilitate handling.
production of large amounts of this protein, future studies can readily be undertaken, such as toxoid preparation and further chemical characterizations of the protein.

Our results from gas chromatographic analysis indicating the absence of any monosaccharides or disaccharides are in opposition to the data previously reported by Rogolsky et al. (15), which stated that the toxin is a glycoprotein composed of 9% carbohydrate. We feel that the carbohydrate present in their preparation is a contaminant, probably from the glycerol gradient used in the electrofocusing step of purification. An incomplete separation of the toxin from the supportive density gradient material (glycerol) would cause false positive results in the Dubois carbohydrate reaction. Tests performed in our laboratory show that the pure toxin is negative in this analysis, but that the addition of only a trace amount of glycerol produced a positive result.

In addition, Rogolsky et al. (15) reported an interaction of the exfoliatin with concanavalin A as substantiation for the presence of a sugar moiety in his product. We have duplicated this result by producing a precipitate when the two substances are reacted at room temperature at concentrations of 10 mg of concanavalin A per ml and 200 µg of exfoliatin per ml. However, we feel that this is a nonspecific reaction because of the excess amount of concanavalin A required to achieve precipitation. Similar results were obtained by mixing concanavalin A (10 mg/ml) with recrystallized bovine serum albumin (200 µg/ml). The same solution of bovine serum albumin gave a negative reaction in the Dubois carbohydrate test.

The exfoliative toxin, like alpha- and beta-hemolysin, has no cysteine. All of the staphylococcal enterotoxins have a half-cysteine in their molecular structure.

Earlier reports of purification methods have relied on either disc electrophoresis (9), starch block electrophoresis (12), or isoelectric focusing (9, 12) as a final necessary step in separating the exfoliative toxin from contaminating amounts of alpha-hemolysin. These procedures, by nature, are restrictive in the sample size that can be applied. The purification method described in this paper is virtually unlimited in the quantities of toxin that can be isolated.

ACKNOWLEDGMENTS

We wish to thank James Gilford for his advice and encouragement and Richard Dinterman for the amino acid analysis.

LITERATURE CITED


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**Table 2. Amino acid analysis of exfoliative toxin**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mol%</th>
<th>Residues/molecule</th>
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<tbody>
<tr>
<td>Lys</td>
<td>8.96</td>
<td>21</td>
</tr>
<tr>
<td>His</td>
<td>2.94</td>
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</tr>
<tr>
<td>Arg</td>
<td>3.54</td>
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<tr>
<td>Asp</td>
<td>14.25</td>
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<tr>
<td>Thr</td>
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</tr>
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<td>Ser</td>
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</tr>
<tr>
<td>Glu</td>
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</tr>
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</tr>
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</tr>
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<td>Tyr</td>
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<tr>
<td>Phe</td>
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<td>9</td>
</tr>
<tr>
<td>Trp</td>
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<td>1</td>
</tr>
<tr>
<td>Total</td>
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<td>231</td>
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<tr>
<td>Mol wt</td>
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<td>25,776</td>
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* Determined separately by the method of Edelhoth (5).