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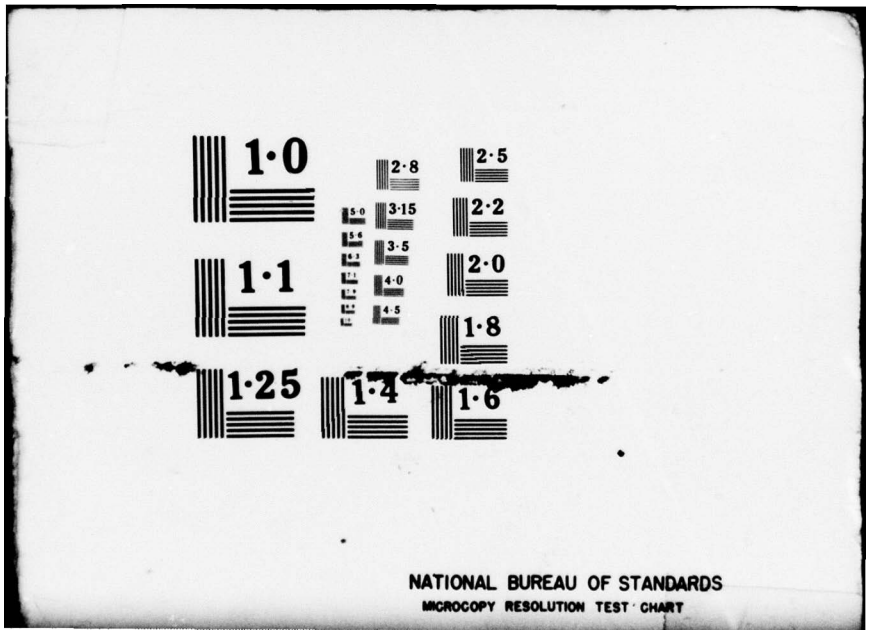
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Purification and Characterization of Different Types of Exfoliative Toxin from Staphylococcus aureus

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Running head: STAPHYLOCOCCUS AUREUS EXFOLIATINS

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

ABSTRACT

Exfoliative toxin was isolated from strain DI of Staphylococcus aureus using CM-cellulose and hydroxylapatite chromatography. This purified toxin was compared with that produced by strain TA. The specific biologic activity of the two toxins was the same, but they were serologically distinct. In addition, differences were seen in molecular weight and amino acid composition.

Staphylococcal exfoliative toxin has been implicated in clinical cases of scalded-skin syndrome (3, 4, 6, 11, 17). Symptoms of disease may include erythema, itching and localized bullae or may be characterized by widespread exfoliation covering a large portion of the body. Ritter von Rittershain (19) in 1878 first described the syndrome in children, and later Lyell (16) reported similar findings in adults. Other workers have described the same findings, and many have isolated Staphylococcus aureus from these patients. Generally these organisms belong to phage group II, although there are some toxin producers in other phage groups (14).

Melish first reported an experimental model for studying the biological action of exfoliatin (17). Injection of culture supernatants from toxin-producing staphylococci in newborn mice causes formation of a characteristic Nikolsky sign. Several groups have reported methods for purifying the product responsible for this syndrome (2, 4, 10, 13, 17, 25). Various strains have been employed for toxin production and the resulting purified preparations have been somewhat different. Distinct types of exfoliatin have been reported by Kondo et al. (13), Arbuthnott et al. (3) and Wiley and Rogolsky (25). When we initially purified exfoliatin from strain TA, its relationship to other types of exfoliatin was uncertain. We have now purified toxin from another strain (DI), and report here on some similarities as well as differences between the two toxins produced.

MATERIALS AND METHODS

Bacterial strains. S. aureus strains TA and DI were obtained from the laboratory of Dr. Rogolsky, Kansas City, Mo. (20). Both strains produce alpha-hemolysin, are coagulase positive, and belong to phage group II.

Medium. Cultures were propagated from lyophilized ampoules in liquid medium as previously described (10). Solid medium employed in these studies was trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.).

Fermentation. Large scale production of toxin was achieved by growing the bacteria in 50 liters of trypticase-yeast liquid medium in a 70-liter fermentor (Fermentation Design, New Brunswick, N.J.). Optimal conditions used were 400 rpm and a constant sparge of 10 liters/min with 90% air and 10% CO_2. Cultures were grown for 18 to 20 hr at 37°C, then centrifuged to remove bacteria. Culture supernatants were concentrated at 4°C with an Amicon TC 3E system, using UM 10 membranes, then dialyzed against several changes of distilled water and lyophilized.

Assay for exfoliatin. Culture supernatants or purified toxin preparations were assayed in newborn mice as previously described (10). Dilutions of samples were made in 10 mM phosphate buffered saline (0.85%) at pH 7.1.

Assay for hemolysin. Samples of crude culture supernatant or partially purified toxin were tested for hemolytic activity by diluting in 0.15 M NaCl in a microtiter plate, then mixing with a 2% suspension of washed rabbit erythrocytes. The hemolytic titer was expressed as the reciprocal of the highest dilution causing complete lysis of erythrocytes

after 30 min at 37°C.

Chromatography. Carboxymethyl cellulose (Whatman CM 52) was equilibrated prior to use in 10 mM phosphate buffer, pH 6.0. Hydroxylapatite (Bio-Rad) was equilibrated in 30 mM phosphate buffer, pH 5.7.

Gel electrophoresis. Seven and one-half percent polyacrylamide gels in 0.1% sodium dodecyl sulfate (SDS) were prepared by the method of Weber and Osborn (23). Slab gels were prepared by the method of Laemmli (15). Protein samples were denatured by heating to 100°C for 10 min in a solution of 1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol. Gels were stained with Coomassie brilliant blue, and destained by diffusion.

Antisera. Antisera were produced in rabbits by repeated injections of purified exfoliatin. The first injection was mixed with Freund's complete adjuvant and given intramuscularly. Subsequent injections were given with toxin alone. A total of 6 mg toxin, given over 2 months, was used to obtain antiserum for use in Ouchterlony diffusion tests.

Amino acid analyses. Samples of purified toxin in 6 N HCl were hydrolyzed in sealed tubes in vacuo at 110°C for 24 h. Analyses were carried out on a Beckman 121-MB amino acid analyzer interfaced with Beckman system AA computing integrator. Threonine and serine were corrected for destruction during hydrolysis using the factors determined by Rees (18). Tryptophan was determined spectrophotometrically by the method of Edelhoch (5).

C-terminal amino acid determination. Carboxypeptidases A and B were used to cleave C-terminal amino acids, following the procedure of

Guidotti (7). Incubations were done at 37°C using a substrate-enzyme ratio of 20:1.

N-terminal amino acid determination. Amino-terminal amino acids were determined on a Beckman 890 C sequencer using the standard Beckman fast Quadrol program. Detection of the residues was carried out by gas chromatography (8), thin layer chromatography (22) and by amino acid analysis of the hydrolyzed phenylthiohydantoins (9) on a Beckman amino acid analyzer.

Histologic examination. Sections of epidermis, as well as other organs, from control and toxin-injected mice were examined by light microscopy. Tissues were embedded in paraffin, sectioned (6 μm thick) and stained with hematoxylin and eosin.

RESULTS

Purification of toxin. Aliquots of lyophilized, dialyzed culture supernatant from strain DI were purified by column chromatography. Carboxymethyl cellulose was equilibrated at pH 6.0 in 10 mM sodium phosphate. A column 2.5 x 40 cm was filled with resin and washed with the equilibration buffer; the sample was then run onto the column at a rate not exceeding 1 ml/min. After the entire toxin sample had been applied, the column was washed with several column volumes of buffer, until the absorbance of the eluate had returned to baseline. No toxic activity could be detected in the unadsorbed material. The toxin was eluted from the column by increasing the molarity of the buffer to 50 mM at pH 6.8. A single peak was obtained, but all fractions in the peak were positive for hemolytic activity, in addition to their exfoliative activity.

In order to separate the exfoliatin from the alpha-hemolysin, this partially purified material was dialyzed against 30 mM phosphate buffer at pH 5.7. This sample was then applied to a column of hydroxylapatite previously equilibrated with the same buffer. Both exfoliatin and hemolysin bound to the column and were eluted separately with a linear gradient of sodium phosphate buffer, increasing in molarity from 30 to 400 mM. The exfoliatin eluted first (at about 250 mM) and the alpha-hemolysin followed.

Using the conditions described above, culture supernatants from strain DI usually contained 10-15 µg toxin/ml of culture supernatant. Approximately 10-12 g of lyophilized crude supernatant were obtained from each 50-liter fermentation. Recovery was about 70% from the carboxymethyl cellulose column elution. The hydroxylapatite

column yields were somewhat less, usually averaging 40-50% recovery. Starting with 2 g of lyophilized crude supernatant material, the average yield of purified toxin ranged from 20-35 mg.

Analysis of the peak fractions from the hydroxylapatite column indicated no contaminating hemolytic activity in the exfoliatin preparation. Analysis of purity by SDS gels indicated that the toxin preparation was more than 95% pure. Trace amounts of lower molecular weight components were observed in some preparations. These contaminants could be removed by rechromatography of the toxin on CM-cellulose; a linear gradient (10 to 50 mM phosphate) was utilized to elute the toxin.

These purification steps were essentially the same for both types of exfoliatin. However, minor differences in binding efficiency were demonstrated when a mixture of both types of exfoliatin was applied to a column of hydroxylapatite which had been equilibrated as described above. Using a linear gradient of phosphate buffer, the TA type exfoliatin eluted slightly ahead of the DI type toxin (Fig. 1). The presence of one or both types of toxin was confirmed serologically by testing each fraction with specific antiserum in Ouchterlony diffusion tests.

Specific activity. Using the newborn mouse model (17), we found the median effective dose (ED_{50}) of the DI toxin to be 0.5 µg. Thus, both forms of exfoliatin have the same activity (10).

Molecular weight determination. In SDS-containing polyacrylamide gels, we estimated that the DI type exfoliatin had a molecular weight of about 26,000, by comparison with known standards. This is very close to the previously reported data for TA-type toxin (10) which has a molecular weight of 26,500 daltons. In agreement with Wiley and

Rogolsky (25) we were unable to separate the two types in conventional cylindrical gels. However, when mixed samples were run in 9% SDS-slab gels, we obtained resolution of the two types. When compared with the individually run cylindrical gels, data confirmed our observation that DI-type toxin migrated slightly ahead of the TA-type, and is thus presumably somewhat smaller in molecular weight.

Amino acid composition. The calculated amino acid composition of both types of exfoliatin is presented in Table 1. The error minimization method of Katz (12) gave a single minimum with the TA toxin and was used to obtain the listed values. There are 237 amino acid residues in the molecule corresponding to a molecular weight of 26,600. This analysis is very close to the one we reported previously (10). Usable error plots versus molecular weight were however not obtained with DI toxin. Based on the assumption that DI toxin is smaller than the TA toxin and that a molecular weight difference of 10% would have permitted resolution of the two forms of exfoliatin in cylindrical polyacrylamide gels, the composition of DI toxin was calculated by an iterative process. The molecule has 228 residues and a molecular weight of 25,350 daltons. As might be predicted the amino acid composition of both toxins is similar. Neither contains cysteine; both have one residue of methionine. Significant differences were seen in some of the amino acids, notably arginine, alanine, and valine.

Incubation of both types of exfoliatin in the presence of carboxypeptidase-B revealed that the C-terminal amino acid for both types of toxin was lysine. We found that the N-terminal amino acid for the TA-type exfoliatin was glutamic acid, and for the DI type was lysine.

Antiserum reactions. Purified toxin preparations were used to

prepare specific antiserum in rabbits to each type of exfoliatin. When reacted in Ouchterlony double diffusion, each antiserum formed a single line of precipitate with 1 μg homologous antigen, indicating high specificity. However, when sufficiently high concentrations (20 μg) of heterologous toxin were reacted, each anti-serum also formed a single line of precipitate with the antigens. When reacted simultaneously, the lines of precipitate crossed, showing complete nonidentity (Fig. 2). Complementary observations to those shown in the figure for anti-TA toxin were obtained with antisera to DI toxin. These reactions could be completely eliminated by absorbing the serum with the appropriate heterologous toxin. The presence of two distinct antibody populations in each serum seemed to indicate that the antigens used for immunization were not as highly purified as desired. The trace amounts of secondary antigens were apparently present in sufficient quantity to give rise to precipitating antibodies.

Pathologic findings. Control mice, injected with normal saline showed no pathologic lesions. Animals injected with either the TA or DI exfoliatin were typified by multiple areas of separation within the stratum granulosum accompanied by subsequent elevation of the superficial layers of epidermis. These findings were first evident at 2 hr after injection of 1 μg of either form of toxin. No other lesions were observed in other organs that were examined.

DISCUSSION

Our earlier work described purification of one serologic form of exfoliative toxin from strain TA of S. aureus (10). We attempted to

eliminate toxin production in this strain by specific treatments to cure plasmids (e.g., growth at elevated temperature). Since we were unsuccessful in the experiments, we can presume chromosomal sites for synthesis of this form of toxin, agreeing with Rogolsky et al. (21). We have now used strain DI for production of a serologically and chemically distinct molecular species of exfoliative toxin. Rogolsky et al. (20) previously studied toxin production by this culture, and indicated that in this instance the controlling gene(s) are located on a 56S plasmid. Although we have referred to this form of toxin as plasmid form, we believe there may be an additional chromosomal site for its synthesis (unpublished observations).

There have been several reports dealing with serological differences among types of staphylococcal exfoliatin. Kondo et al. reported the isolation of a toxin from several strains of phage group I staphylococci and has termed this product exfoliatin B to distinguish it from the toxin produced by group II staphylococcal strains, which he termed exfoliatin A (13). Arbuthnott and Billcliffe identified two serologic forms of exfoliatin (both from strains of phage group II staphylococci) using a radial diffusion procedure (1). Others have also described serological differences in exfoliating materials derived from several strains (4, 6, 11). More recently Wiley and Rogolsky (25) serologically differentiated two exfoliating toxins synthesized under chromosomal and plasmid control.

Discrepancies have also been reported on the stability and molecular weight of some of these toxin preparations (13, 14, 24). The amino acid composition of both types of exfoliatin isolated by Kondo (4) provided clear proof that his toxins were quite different from the two

we isolated. Indeed it may be that each toxin-producing strain of S. aureus elaborates a distinct type of exfoliatin, with the products differing in both their serologic reactions and chemical composition. Thus far the only property that appears to be relatively consistent is the specific biological activity, suggesting at least some homology among the various toxic molecules.

The two forms of toxin purified in our laboratory have identical specific activities when assayed in the newborn mouse model. We have shown here that these two purified preparations are serologically distinct. We have additionally demonstrated that there are no common antigenic determinants (A. D. Johnson, L. Spero, and J. F. Metzger. Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B99, p.30) by antigen-binding capacity measurements on radiolabeled toxins. Further studies concerning the genetic control of the production of chemically and serologically different forms of exfoliative toxin are under active investigation in our laboratory.

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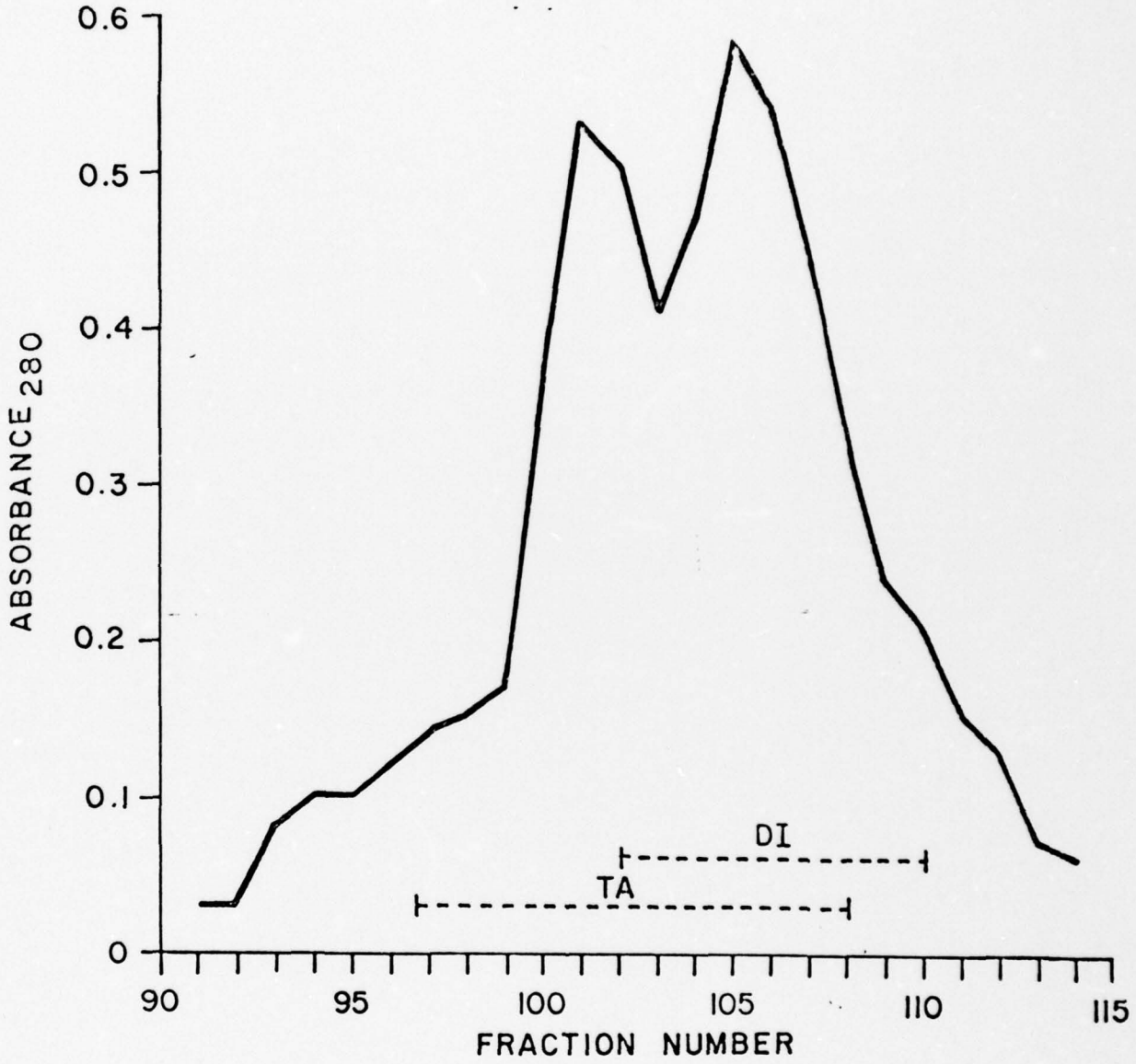
TABLE 1. Amino acid composition of two forms of exfoliatin

Amino acid	Residues/molecule exfoliatin	
	TA-Chromosomal	DI-Plasmid
Lys	22	22
His	7	5
Arg	9	5
Asp	34	29
Thr	12	12
Ser	17	17
Glu	27	26
Pro	10	12
Gly	24	21
Ala	8	13
1/2-Cys	0	0
Val	13	9
Met	1	1
Leu	15	16
Ile	17	17
Tyr	11	13
Phe	9	9
Trp	1	1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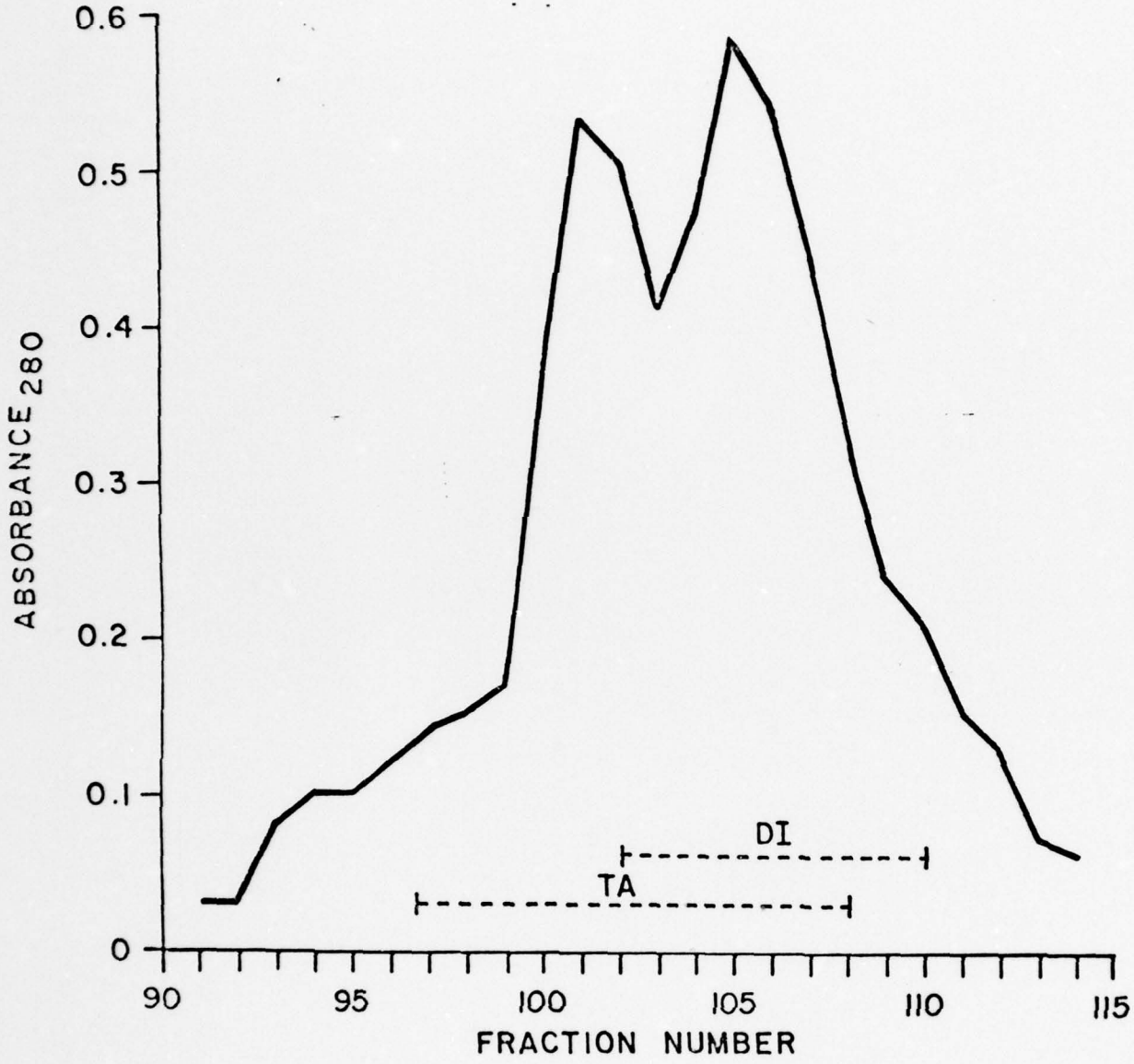
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FIGURE LEGENDS

FIG. 1. Hydroxylapatite chromatography of two forms of exfoliative toxin. A mixture of 10 mg each of TA-type and DI-type exfoliatins was adsorbed to a 1.5 x 30 cm column equilibrated with 30 mM phosphate buffer at pH 5.7. A linear gradient of increasing phosphate buffer from 30 mM to 400 mM was used to separate the two toxins. Dashed lines indicate fractions that reacted with specific antiserum in Ouchterlony diffusion tests.

FIG. 2. Double immuno diffusion of two forms of exfoliative toxin with antiserum to TA toxin. Wells labeled TA contain 1 µg TA toxin. Wells labeled DI contain 20 µg DI toxin. The center well contains 5 µl anti-TA serum. The plate was incubated for 24 h at room temperature.

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