PURIFICATION OF STAPHYLOCOCCAL ALPHA-HEMOLYSIN

MORTON A. MADOFF AND LOUIS WEINSTEIN

Department of Medicine, Tufts University School of Medicine, and Infectious Disease Service of the Pratt Clinic—New England Center Hospital, Boston, Massachusetts

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ABSTRACT

MADOFF, MORTON A. (New England Center Hospital, Boston, Mass.) AND LOUIS WEINSTEIN. Purification of staphylococcal alpha-hemolysin. J. Bacteriol. **83**:914–918. 1962. — Staphylococcal alpha-toxin has been purified by metal-ion precipitation, column chromatography, and continuous-flow paper electrophoresis. The resultant preparation is highly unstable. A single line is obtained on agar-gel diffusion analysis against staphylococcal antiserum. Two bands, both associated with hemolytic activity and migrating cathodally at pH 8.4, are obtained on starch-gel electrophoresis. Levels of lytic, dermonecrotic, and lethal toxin activity maintain a constant relationship in the crude and purified materials, suggesting the unity of these effects.

Hemolytic, leukocidic, dermonecrotic, and lethal activity as well as various physiological and anatomical changes have been attributed to the action of alpha-toxin of *Staphylococcus aureus* (Elek, 1959). However, because of the unavailability of highly purified material, it has been impossible to ascribe specific function to a single entity or to determine the unity or distinction of various biological effects. Numerous attempts at purification have met with only partial success (Wittler and Pillemer, 1948; Turpin et al., 1954; Butler, 1959; Robinson, Thatcher, and Montford, 1960).

It is the purpose of this paper to describe a method of purification of alpha-toxin which yields a preparation of high specific activity showing a single line on agar double-diffusion analysis.

MATERIALS AND METHODS

Analytical procedures. Hemolytic activity against rabbit erythrocytes was determined by measurement of 50% hemolysis at 541 mμ in a Coleman Junior spectrophotometer. Equal volumes of 1% red blood cell suspensions and doubling dilutions of toxin were incubated for 30 min at 37 C in phosphate-buffered saline (pH 6.9). Unhemolyzed cells were removed by cold centrifugation, and the optical density of 1 ml of the supernatant fluid in 6 ml of 1% sodium carbonate was determined. The hemolytic unit was defined as the reciprocal of the final dilution producing 50% hemolysis, when compared to a prepared standard.

Two rabbits were used as the source of erythrocytes throughout the study. Their cells yielded nearly identical hemolytic titers and were stable when tested prior to each group of determinations. Blood was obtained by cardiac puncture and mixed immediately with an equal volume of Alsever's solution. Samples were washed four times in buffered saline (pH 6.9) immediately prior to testing and adjusted to a 1% solution by determination of microhematocrit. Fresh cells were obtained weekly.

Dermonecrotic activity was determined by injecting 0.1-ml samples of doubling dilutions of the test material endermally into the shaved backs of normal rabbits. The units of activity were expressed as reciprocals of the final dilution causing unequivocal necrosis in 4 to 7 days.

Lethal activity was measured by the intraperitoneal injection of 0.5-ml samples of doubling dilutions of toxin into groups of three mice. The end point was considered to be the final dilution which killed the entire group within 18 hr.

Controls of test toxin heated at 80 C for 1 hr were used for both the dermonecrotic and lethal activity measurements. Staphylococcal coagulase activity was assayed by the method of Blobel, Berman, and Simon (1960), hyaluronidase by the method of Tolksdorf et al. (1949), and fibrinolysin by the method of Lack and Wailing (1954).

A modification of the Folin-Ciocalteau phenol reaction (Lowry et al., 1951) and determination of the ratio of optical density (OD), 260 to 280 mμ, in a Beckman model DU spectrophotometer with a 1-cm light path were used to measure protein. A preparation of crystalline bovine plasma

albumin (Armour and Co., Chicago, Ill.) was employed as the reference standard.

Starch-gel electrophoresis was performed by the method of Ferguson and Wallace (1961). The gel was then cut in a horizontal plane. The lower half was stained for protein with 1% nigrosin (Consolidated Laboratories, St. Louis, Mo.), and sections of the upper half were applied directly to a rabbit-blood agar plate to detect hemolytic activity.

Agar double-diffusion analyses of the Ouchterlony type were carried out in flat-bottomed petri dishes coated with silicone. First, 12 ml of 1% (w/v) Ionagar no. 2 (Oxo) in 0.155 M NaCl were allowed to harden; six holes, arranged around a central wall, were removed with a Feinberg agar-gel cutter (Consolidated Laboratories). Then, varying quantities of toxin preparations were tested against dilutions of staphylococcal horse antiserum (900 units/ml) in a moist chamber and examined daily for 14 days for the development of antigen-antibody lines.

Production and purification of toxin. An overnight culture (200 ml) of the Wood 46 strain of *S. aureus* was added to 12 liters of enriched Casamino acids (Difco) medium (Madoff, Eylar, and Weinstein, 1960) and incubated at 37 C for 96 hr in a constant atmosphere of 30% CO_2 and 70% O_2. The bacterial bodies were removed by high-speed centrifugation at 4 C.

Zinc acetate (1.0 M) was added dropwise to the clear supernatant fluid (pH 6.5) until a final concentration of 20 mM was reached. The precipitate was removed by centrifugation for 30 min at 2,000 rev/min and gradually resuspended in 50-ml samples of 0.2 M disodium and trisodium ethylenediaminetetraacetate (pH 5.8) until no further activity was obtained; 100-ml portions of this material were passed through a 25 × 200 mm column of Sephadex G-25 (Pharmacia) equilibrated in barbital buffer (pH 8.6; ionic strength, 0.01 at 4 C). The active fractions were pooled and passed through a similar column of Sephadex G-75 and 5-ml samples were collected in a constant-volume fraction collector (Buchler Instruments). The hemolytic activity was found in 110 to 120 ml of eluate recovered immediately after the void volume of the column.

The active material was applied above drip point 25 at a flow rate of 3.6 ml per hr to a Spinco Model CP paper-curtain electrophoresis at 750 v, 65 ma, 2 C. The same barbital buffer was used as background electrolyte. Migration was cathodal and the principal activity was recovered in drip points 9 to 13.

The fractions with the highest specific activity were pooled, concentrated by pressure ultrafiltration (Madoff et al., 1960), and dialyzed for 18 hr against 0.005 M phosphate buffer (pH 7.4); 3 ml (1.2 mg.) of the dialyzed concentrated material were applied to a 0.1-g diethylaminoethyl cellulose (DEAE-SF Bio Rad) column prepared as described by Sober et al. (1956) and equilibrated in the above buffer at 4 C. Samples of 1 ml were collected. The activity was not retained under these conditions and appeared in the 2 to 3 ml of effluent immediately after the void volume of the column.

RESULTS

A summary of the purification procedure is shown in Table 1.

Despite the selection of actively hemolytic staphylococcal colonies, initial titers of hemolysin were low. This probably reflected the modest growth of organisms in the semisynthetic medium which was used to avoid the presence of large amounts of protein.

Zinc precipitation was found to be the most satisfactory single method for initial concentration and purification. Precipitation between 50 and 70% saturation with ammonium sulfate at pH 6.0 resulted in concentration, but minimal purification, with 40 to 60% loss of activity. Similar results were obtained with cold-methanol fractionation. Zinc was found to exert a marked inhibitory effect upon hemolysin activity.

TABLE 1. *Purification of staphylococcal alpha-toxin*

Material	Activity*	Protein	Specific activity	Recovery (percentage initial activity)
		mg/ml		
Crude broth	86	1.26	68	—
EDTA-zinc supernatant fluid (dialyzed)	1220	0.72	1694	83
'Sephadex'	956	0.37	2584	78
Curtain electrophoresis	724	0.085	8515	46
DEAE-cellulose	460†	0.011	41818	33

* Hemolytic units/ml (50%) after 30 min incubation at 37 C with 1 ml of 1% rabbit erythrocytes.

† Units/ml equivalent to activity prior to concentration by pressure ultrafiltration.

Dialyzed material (barbital buffer, as above) revealed a 25-fold increase in specific activity. By further adjustment of the crude material to pH 6.8 with 10% Na_2CO_3 and the addition of 10 mM of zinc acetate, it was possible to increase the recovery of hemolysin of low specific activity by 7 to 10%.

Gel diffusion in Sephadex resulted in a 1.5-fold increase in specific activity. The G-25 column step was used primarily to remove zinc and contributed little, if any, to the actual purification. Recoveries of 150 to 300% of the hemolytic activity applied to the column were frequently found, reflecting the loss of the inhibitory activity of the zinc. The actual purification probably resulted from passage through G-75 Sephadex.

The increase in specific activity afforded by paper-curtain electrophoresis varied from 1.3, if all active fractions were combined, to 7.4 if only the most active materials from the leading edge of the hemolysin were included. The latter resulted in 60 to 80% loss of total toxin activity in this single step. Therefore, all fractions showing a minimum of a twofold increase in specific activity were combined. This resulted in an average increase of approximately three to four fold. Recycling of the material resulted in only slight further purification with sizeable loss of total activity.

DEAE-cellulose column chromatography at pH 7.4 (0.005 M phosphate) did not adsorb the active material and yielded an additional three to fivefold purification. The adsorbed material could be eluted from the column by addition of 0.6 M NaCl, and demonstrated an absorption maxima at OD_{260}, suggesting that this step was primarily responsible for the removal of nucleic acids.

Materials from the sequential purification steps were concentrated to approximately the same protein content (0.4 mg/ml) by dialysis against solid polyethylene glycol (Carbowax '4000', Union Carbide). When subjected to agar diffusion analysis against staphylococcal antiserum, the number of precipitation lines decreased with each purification. Precipitation lines did not develop when toxin was tested against normal horse serum. Crude broth showed five distinct lines and several areas of hazy precipitation. The material from the curtain electrophoresis revealed one broad and one fine line. The purified material from DEAE-cellulose passage resulted in a single agar-diffusion line showing a reaction of identity with the fine line obtained with the curtain electrophoresis material.

Starch-gel electrophoresis of the purified material at pH 8.4 revealed two bands, both migrating weakly toward the cathode. Each was associated with distinct hemolysis when cut sections were applied to rabbit blood agar plates. Hemolysis was not detected in any other area. Hemolysis of human and horse blood agar was not demonstrable. These bands were not subjected to agar-diffusion studies.

The active materials from the several steps, when concentrated to the same level of hemolytic activity (5400 units), also showed constant levels of 640 and 320 units, respectively, of dermonecrotic and lethal toxin effect. These activities were detected only in fractions associated with measurable rabbit red-cell hemolytic activity. Hemolytic activity against sheep erythrocytes was not separable from rabbit cell hemolysis by the procedures used. The purified material was free of measurable coagulase, hyaluronidase, fibrinolysin, and delta lysin.

Toxin obtained after initial Sephadex G-25 passage could be adsorbed onto carboxymethylcellulose (0.01 M phosphate, pH 4.8) and eluted with an increasing salt gradient (NaCl, 0.4 M) with a single symmetrical peak of activity yielding a two to fourfold increase in specific activity. The purified material adsorbed and eluted in this fashion showed no increase in specific activity.

The final purified material was markedly unstable. Both slow and rapid freezing resulted in immediate loss of activity. Deterioration appeared to begin after 24 hr at 4 C and was not delayed by maintenance in vacuo or under nitrogen. It was recently noted that further stability was afforded by the addition of 0.3 to 0.5% sodium chloride. This and other methods for stabilization are under investigation to enable further characterization of the purified alpha-toxin.

DISCUSSION

The unity of the alpha-hemolysin, dermonecrotoxin, and lethal toxin of the staphylococcus has been the subject of considerable discussion (Elek, 1959; Butler, 1959). The demonstration in the present study that these activities are maintained at equivalent levels during the course of a

substantial purification process suggests that they are attributable to a single substance. No attempt has been made to correlate these effects with leukocidin activity.

Because of problems of instability, it has thus far been impossible to process sufficient active material for Tiselius electrophoresis or analytical ultracentrifugation. The suggestion that purity has been achieved rests entirely in the finding of a single line developed in agar against potent staphylococcal antiserum. It is recognized that this does not represent ultimate proof of purity. It is further possible that the antiserum employed, although prepared against the Wood 46 strain (Barr, *personal communication*), does not contain antibodies to components produced by the specific organism used in this study.

Two bands, closely oriented on the cathodal side of the origin but nonetheless distinct, were observed on starch-gel electrophoresis. Multiple bands may arise from a variety of physicochemical alterations of homogeneous materials subjected to this procedure and are apparently not unusual (Ferguson and Wallace, 1961). Each band was associated with a distinct area of hemolytic activity against rabbit-blood agar. The location of the hemolytic effect is apparently the same as that noted by Bernheimer and Schwartz (1961). They described, however, only a single band associated with hemolytic activity.

The techniques employed in the present study have been used for the successful purification of a variety of biological materials (Blobel et al., 1960; Sober et al., 1956; Spivack and Karler, 1958; Hausler and Dick, 1960). The final recovery of 33% of the initial activity would be entirely adequate, in view of the ease with which crude material can be obtained, if a means of stabilization of the final product were developed. It is possible that substitution of a second chromatographic procedure (for example, adsorption and elution on carboxymethyl-cellulose) for the cumbersome paper-curtain electrophoresis could result in similar purification with greater yield. Studies of this nature are in progress.

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