THE PRODUCTION, CONCENTRATION, AND PARTIAL CHARACTERIZATION OF STREPTOLYSIN O¹

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Received for publication August 6, 1954

Streptolysin O is the oxygen-labile hemolysin present in broth filtrates after the growth of hemolytic streptococci. Neill and Mallory (1926) studied the effect of exposure of this lysin to oxygen and reported that, depending on the age of the culture and other factors, Streptolysin O existed in broth filtrates in two forms. These were the reduced hemolytically active form and a reversibly oxidized inactive form which could be reactivated by the addition of a suitable reducing agent. They showed that under some conditions oxidation of the lysin proceeded to such a degree that it was no longer reversible, and under these circumstances its hemolytic activity was completely lost.

A partially successful method of concentration of Streptolysin O by means of ammonium sulfate fractionation was reported by Smythe and Harris (1940). No record of their recoveries was given, but undoubtedly some purification was achieved. Analyses of their preparations were those typical of proteins. Sulfur was present to the extent of 1.9 per cent, partly as SH groups, partly as sulfate sulfur, and partly in an unidentified form. These authors, as well as others, have postulated that the reversible oxidation-reduction properties of the lysin could be accounted for in terms of a reversible sulfhydryl system.

Todd (1932a,b; 1938a,b) demonstrated the antigenic properties of the lysin as well as other characteristics which clearly distinguished it from Streptolysin S. Herbert and Todd (1941) published a method for its isolation and concentration which again depended on fractionation with

ammonium sulfate. The product obtained was a protein. An optimum pH of 6.5 at 37 C was found for hemolytic activity with very little activity occurring at 0 C. Reducing agents activated the lysin, and iodoacetic acid inactivated it. These authors suggested that Streptolysin O was probably an enzyme but that no substrate for it was known.

The present report describes a method of production and concentration of this lysin which obviates the necessity of ammonium sulfate fractionation. It has proven satisfactory for three different preparations and is in essence a modification of the Herbert and Todd procedure.

MATERIALS AND METHODS

The culture. *Streptococcus pyogenes*, strain C203U, was obtained from Dr. A. W. Bernheimer. This strain was reported by him to produce only Streptolysin O and no Streptolysin S (Bernheimer, 1949) and was reputedly a nonhemolytic variant which had been isolated after growth of a hemolytic strain of C203 on his semisynthetic culture medium. It has been found here, as well as by others (Slade and Knox, *personal communication*), that strain C203U largely reverts to the hemolytic variety on subculture to blood agar plates. Only a few remaining nonhemolytic colonies can be identified.

The culture was lyophilized by the method of Fry and Greaves (1951) and was so stored. When a growing culture was desired for inoculation purposes, lyophilized material was transferred to broth (Todd and Hewitt, 1932) and grown at 37 C for 16 hr. It was then streaked on a blood agar plate and incubated at 37 C overnight. From this plate nonhemolytic colonies were picked and transferred to Todd-Hewitt broth and grown for 24 hr. This 24 hr culture, in which growth was light, was used for inoculating large volumes of broth for the production of Streptolysin O.

Isotonic buffer. Sodium chloride, 4.5 g; potassium acid phosphate, 9 g; disodium phosphate,

¹ Part of the material here reported was used by the senior author in a dissertation submitted to the Department of Biochemistry, Northwestern University Medical School, in partial fulfillment for the Degree of Doctor of Philosophy.

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dodecahydrate, 23.3 g; made to 1 L with water. This is slightly modified from the isotonic saline described by Bernheimer (1946) to give a pH of 6.4-6.6.

Methods for measuring Streptolysin O. Two methods for measuring hemolytic activity have been used. The first of these was that originally used by Todd (1938a). Activation of all Streptolysin O solutions was accomplished in the following manner: Just before use a 2 per cent solution of cysteine hydrochloride was made in isotonic buffer solution, and to each 2 ml of Streptolysin O solution, 0.1 ml of the cysteine hydrochloride solution was added. The mixture was incubated for 15 min at 37 C. If dilutions of the lysin were required, these were made with isotonic buffer immediately after the incubation period. If repeated assays had to be made of the same solution, activation of the undiluted material was always carried out just prior to use and fresh dilutions made for each assay. A 3 per cent suspension of rabbit red blood cells was used in place of the 5 per cent recommended by Todd. The antistreptolysin O which the method requires was obtained from the Serum Institute, Carshalton, Surrey, England. This test is rapid and required little attention and was used wherever an approximate determination of titer would suffice. When it has been used in the experiments to follow, the titer will be reported in Todd Units (TU).

When it was required to obtain lytic activity in more precise terms, the method of Herbert (1941), as modified by Slade and Knox (1950), was used. The following procedure was adapted for routine use: A suspension of laked red blood cells was standardized to read 0.70 at a wavelength of 550 m μ in a Coleman Junior spectrophotometer. This was accomplished by first making a 10 per cent suspension of triple washed red blood cells (rabbit) in isotonic buffer and laking 2.3 and 2.5 ml, respectively, of this suspension with 50 ml of distilled water. The casts were centrifuged down and the supernatants read at 550 m μ . These readings usually fell between 0.67 and 0.72. The proper amount of the 10 per cent suspension was then estimated so that a reading of 0.70 would be obtained. This amount was added to 25 ml of isotonic saline. One ml of this suspension was added to each tube for assay. Occasionally the readings of laked cells did not fall in the above range, in which case different

amounts of the 10 per cent suspension had to be laked and a reestimate made. Activation of Streptolysin O solutions was carried out as for the Todd assay. When this was the method used for measuring hemolytic activity, the results were recorded in Herbert Units (HU).

Inoculation of the Todd-Hewitt broth. It was found in this study that the best preparations of Streptolysin O were obtained when an inoculum of 5 ml of a 24 hr Todd-Hewitt broth culture was used per 3,500 ml of culture medium. The most active preparations were obtained when growth was stopped at 14 to 15 hr.

Removal of hemolytic streptococci after growth. Since it was known that exposure to a large surface of Berkefeld filter candle removed some of the Streptolysin O (Slade and Knox, 1950), the following procedure was adopted for the removal of the bacteria after growth. The culture was first centrifuged for 1 hr at 2,000 rpm at room temperature. The supernatant was decanted and filtered through a small (2.5 in), well washed, Berkefeld candle (pore size N) also at room temperature. If a large volume of broth had to be cleared, two such candles were used. This procedure resulted in no detectable loss of hemolytic activity as measured by the Todd method.

All dialyses were carried out at 4 C for periods of 20 hr unless otherwise stated.

RESULTS

Procedure for the Production of Streptolysin O

Starting material. 3,589 ml of broth filtrate. This was obtained from a 14.5 hr culture, having a final pH of 7.0. It contained 10 TU/ml. Total: 35,890 TU.

Step I. To the above were added 14.5 g of cysteine hydrochloride in 200 ml of water (neutralized to pH 7.1). After stirring, 85 ml of $\frac{M}{2}$ phosphate buffer, pH 6.95, were added. This was followed, after mixing, by the dropwise addition of 47 ml of M calcium acetate. The pH was adjusted to 6.4 with 10 per cent acetic acid. The whole operation was conducted at room temperature, and the mixture was allowed to stand this way for 30 min with occasional stirring. It was then centrifuged, decanted, and the supernatant assayed for hemolytic activity. The supernatant was found to have retained no hemolytic activity.

The calcium phosphate precipitate was eluted

with 544 ml of eluting fluid composed of the following: 97.5 ml of saturated ammonium sulfate, 2.5 ml of concentrated ammonium hydroxide, 860 mg of cysteine hydrochloride, 300 ml of water, and glacial acetic to pH 7.1. This eluting fluid was filtered if necessary before use. Trituration was carried out at room temperature simply by the addition of the eluting fluid to the precipitate and stirring until an even suspension had been achieved. The suspension was allowed to stand for 30 min with occasional stirring. It was then centrifuged and the supernatant decanted and assayed. The precipitate was again triturated with 500 ml of eluting fluid, and after 30 min the suspension was centrifuged and the supernatant decanted.

The first and second eluates were combined and dialyzed against 5 L of $M/15$ phosphate buffer at pH 7.2. The mixture was then dialyzed against slowly running deionized water, and finally against 15 L of distilled water and lyophilized. Yield: 1.753 g; Activity: 11.9 TU/mg; Total: 20,860 TU.

A third and fourth trituration of the calcium phosphate precipitate yielded no further activity.

The product obtained from the above treatment served as a supply of crude stable material from which more highly concentrated lysin was prepared. The second step of the procedure may be carried out directly on the solution obtained from Step I without previous lyophilization or on the dry product in the following manner.

Step II. 1.75 g of material from Step I were dissolved in 500 ml of distilled water. Any undissolved material was removed by filtration through glass wool. To the clear brownish solution were added 2 g of cysteine hydrochloride dissolved in 20 ml of water, having the pH adjusted to 7.2. This was followed by the addition of 44 ml of $M/2$ phosphate buffer, pH 7.0, and, after mixing, the dropwise addition of 17 ml of M calcium acetate. The pH was adjusted to 6.4 with 10 per cent acetic acid, and the suspension allowed to stand at room temperature for 20 min with occasional stirring. It was then centrifuged, decanted, and the supernatant assayed. The supernatant was negative for hemolytic activity and therefore discarded.

The calcium phosphate precipitate was triturated with 200 ml of eluting fluid for 20 min at room temperature as before. The suspension was centrifuged, and the clear, reddish supernatant

decanted. Trituration of the calcium phosphate was repeated once more. The hemolytically active supernatant solutions were stored separately at 4 C for 16 hr. They were dialyzed separately against 5 L of $M/15$ phosphate buffer, pH 7.15. A small precipitate which formed was removed by centrifugation and filtration through glass wool. Each solution was then dialyzed against running, deionized water, again separated from any precipitate, and lyophilized. Yield from first trituration: 364 mg; Activity: 39 TU/mg, 812 HU/mg; Yield from second trituration: 150 mg; Activity: 10 TU/mg, 166 HU/mg.

Step III. A solution having similar activity to that obtained from the first trituration in Step II was treated in the following manner:

To 140 ml containing 176 mg of material were added 560 mg cysteine hydrochloride, pH 6.9, contained in a volume of 15 ml. The solution was mixed, and 12 ml of $M/2$ phosphate buffer, pH 7.0, were added. After mixing, this was immediately followed by the dropwise addition of 4 ml of M calcium acetate. The pH was adjusted to 6.4, and the mixture was allowed to stand with occasional stirring for 20 min at room temperature. It was then centrifuged, decanted, and the precipitate triturated with 50 ml of eluting fluid. The eluate was dialyzed against 5 L of $M/15$ phosphate buffer and against running, deionized water. It was then lyophilized. Yield from Step III: 72.3 mg; Activity: 55 TU/mg, 1,300 HU/mg.

The portion of active solution used in Step III represents roughly $\frac{3}{4}$ of the total yield of similar preparations at the end of Step II. Applying a correction factor of 2.5 to the yield recorded for Step III would give an expected total yield of 181 mg, thus representing a total recovery of 9,955 TU.

Characteristics of Streptolysin O Prepared by the Procedure Outlined

Stability and solubility. The stability of the hemolysin in solution was studied. For short periods of one to two weeks it was found to maintain all of its activity in isotonic buffer at a concentration of 0.1 per cent. Over longer periods the hemolytic activity gradually decreased. A 3 per cent solution, stored at 4 C for a three month period, with no preservative or wax seal added, was observed to have lost one-half of its activity.

The lyophilized form, stored at 4 C over anhydrous calcium chloride, showed no loss of activity over a period of two years.

The maximum solubility of Streptolysin O was at pH 5.7. Its hemolytic activity was most stable between pH 5.7 and 7.0. The maximum stability was at pH 7.0, and above pH 7.5 hemolytic activity was rapidly destroyed.

Hemolytic activity was neutralized by cholesterol and by antistreptolysin O in a typical manner (Todd, 1938a; Hewitt and Todd, 1939).

Chemical characteristics. Nitrogen and sulfur analyses were done on several preparations of Streptolysin O to allow correlation studies with hemolytic activity. On the most active product obtained (called IIIb), carbon and hydrogen analyses were done as well. These are listed in table 1.

The method of Flesch and Kun (1950) was used to analyze for sulfhydryl groups. They were present to the extent of 3.7 μ g per mg (average of values obtained on Preparations no. 3 and IIIa).

Carbohydrates, as measured by the method of Seifter *et al.* (1950), showed a value of less than 0.1 per cent. A negative Molisch test was obtained on all preparations tested.

A 300 mg pool was made of various lyophilized preparations of Streptolysin O ranging in activity from 3 to 15 TU/mg. This was hydrolyzed with 20 per cent trichloroacetic acid and extracted with ether in the Soxhlet for 6 hr. The yield of ether soluble substances was negligible.

Electrophoretic studies to be reported separately showed the better preparations of the lysin to consist primarily of a single component when the analysis was carried out in a reducing medium. On this basis, the more concentrated preparations were considered homogeneous enough to justify amino acid analyses. Such analyses yielded the following results.

Amino acid content of Streptolysin O. Amino acid analyses of an hydrolyzate of the Streptolysin O (Preparation IIIb) were carried out by means of paper chromatography. The hydrolysis was done as described by Block (1950). Identification of the amino acids present was made by means of two dimensional chromatography using, first, 80 per cent phenol as the solvent and, second, butanol-acetic acid. The amino acids found to be present in largest amounts were glutamic acid, aspartic acid, glycine, alanine,

TABLE 1
Chemical analyses

	C	H	N	Total S	Hemolytic Activity
	%	%	%	%	HU/mg
Prep. no. 1			14.6		108
Prep. no. 2			14.1	1.64	400
Prep. no. 3			13.7	1.82	840
Prep. IIIa			13.2	4.87	925
Prep. IIIb	42.2	7.5	12.7	3.10	1,300

and norleucine and in smaller amounts serine, lysine, threonine, arginine, tyrosine, methionine, valine, proline, phenylalanine, and cysteic acid. With the above combination of solvents there was some doubt as to the presence of serine, but its presence was confirmed by using, as the first solvent, a mixture of 15 per cent water, 15 per cent propionic acid, and 70 per cent diethylene glycol monoethyl ether and, as the second solvent, 80 per cent phenol.

DISCUSSION

The procedure outlined for the concentration of Streptolysin O is relatively simple and combines a minimum of manipulation with good reproducibility and satisfactory yields. The chief differences between this and other published methods are the addition of a reducing agent to all solutions and the direct adsorption of the lysin onto calcium phosphate from the broth filtrate, thus avoiding any ammonium sulfate fractionation. These modifications, combined with careful observation of pH during growth so that it was not allowed to fall below 7, have proven efficacious.

The procedure recorded shows a recovery of 27 per cent of the original hemolytic activity. The reduction in the amount of solids from those present in the original broth filtrate to those present at the end of Step III varied from preparation to preparation, but this reduction usually was of the order of 80 per cent. Such a recovery of activity coupled with a relatively large reduction in solid material provides a fairly satisfactory concentration procedure. Occasionally, the method has yielded as high as 50 per cent recovery of the original activity.

The long periods of dialysis have been shown to concentrate the lysin. This is due either to the presence of a contaminating enzyme, responsible

for proteolysis, or to spontaneous hydrolysis, which is characteristic of the lysin itself. A detailed report of this phenomenon will be the subject of another paper.

SUMMARY

A modified method for the production and concentration of Streptolysin O has been described. It has been possible to effect satisfactory concentration of the lysin without the use of ammonium sulfate fractionation procedure. The addition of an excess of reducing agent (cysteine) to the lysin at each step in the concentration was found to be essential for satisfactory recoveries of the hemolysin.

The chemical evidence indicates that Streptolysin O is a protein containing neither carbohydrate nor fat moieties. It does contain sulfhydryl groups, and a tentative amino acid content is reported.

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