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STAPHYLOCOCCAL δ-HEMOLYSIN

I. PURIFICATION AND CHEMICAL PROPERTIES

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SUMMARY

1. Staphylococcal δ-hemolysin, which has the capacity to hemolyze human erythrocytes, was purified by column-chromatographic technique using calcium phosphate gel and triethylaminoethyl-cellulose, and it was obtained in crystalline form.

2. The sedimentation patterns showed a high degree of homogeneity of the crystalline toxin. The sedimentation constant is 6.1 S. The intrinsic viscosity is 5.5 ml/g.

3. Complete amino acid analysis showed that the toxin is constituted of the following 609 amino acid residues: Asp, 74; Thr, 47; Ser, 40; Glu, 42; Pro, 3; Gly, 45; Ala, 39; Val, 31; Met, 25; iLeu, 59; Leu, 45; Try, 7; Phe, 48; Try, 10; Lys, 88; His, 2; Arg, 4; it has a molecular weight of 68,230.

4. The toxin is heat labile. It can be easily digested by trypsin and loses its hemolytic activity by the digestion.

INTRODUCTION

The existence of a staphylococcal hemolytic toxin, characterized by its capacity to hemolyze human erythrocytes, which are generally considered resistant to direct lysis by α- and β-lysins, has been demonstrated[1-4]. However, it has not previously been purified, and neither its chemical characteristics nor its immunological and biological properties are known.

In the present work, δ-hemolysin was crystallized from the culture medium of *Staphylococcus aureus*. Molecular weight, amino acid composition and some of the chemical properties of the toxin were elucidated.

MATERIALS

Bacterial strain: *S. aureus* strain Foggie, furnished by Dr. G. P. GLADSTONE, was used in the experiments.

Yeast extract: Oxoid yeast extract was used for preparation of the culture medium.

Trypsin: Worthington's crystalline trypsin was used.

Abbreviation: CM, carboxymethyl.

Silicic acid: Unisil (activated silicic acid) purchased from the Clarkson Chemical Company, Williamsport, Pa., was used for chromatography.

Cellulose ion exchanger: CM-cellulose and TEAE-cellulose purchased from Serva Entwicklungslabor, Heidelberg, were used for chromatography.

Calcium phosphate gel: Calcium phosphate gel (hydroxylapatite) was prepared by the method of TISELIUS, HJERTÉN AND LEVIN[5].

EXPERIMENTS AND RESULTS

Culture method

The bacteria were cultivated in the CCY medium[4] containing 20 % (v/v) of yeast diffusate prepared from oxoid yeast extract. Good δ-lysin production was observed in 1-l culture medium placed in a 3-l conical flask under gentle gyro-shaking at 37° for 18–24 h after an inoculation of 50 ml of 20-h culture. Vigorous shaking or hard aeration remarkably reduced the toxin production. The toxin could be produced also in the CCY medium containing Difco yeast extract and in Difco trypticase soy broth, but it was less in amount in these cases.

The δ-hemolysin activity in the medium increased, without lag, in association with the bacterial growth, and reached a plateau (30–60 units/ml) after cultivation for 18–20 h.

Assay of hemolytic activity

0.5 ml of 2 % suspension of washed fresh human erythrocytes in saline phosphate buffer (0.02 M phosphate buffer (pH 6.8) containing 0.04 M NaCl) was added to 0.5 ml lysin suitably diluted in the above buffer to give a total volume of 1.0 ml. After incubation for 15 min at 37°, the unhemolyzed cells were removed by centrifugation, 0.5 ml of the supernatant solution was diluted to 3.0 ml with 0.1 % sodium carbonate, and the absorbancy determined at 541 mμ on a Beckman spectrophotometer. Hemolysis was linearly related to lysin concentration, provided lysis was not greater than 70 % nor less than 35 % of total hemolysis, and hemolysis reached a plateau after incubation for 15 min at 37°.

One unit of hemolysin is defined as an amount of lysin which causes 50 % hemolysis of 1 ml suspension of 1 % human erythrocytes under the conditions of assay.

Isolation and crystallization of δ-hemolysin

It has been reported that δ-hemolysin is soluble in aqueous alcohol[3], and it was found that crude lysin is soluble in chloroform–methanol (2:1, v/v). These facts strongly suggested that the toxin might be lipid, lipoprotein or a related compound. An attempt was made to isolate the toxin by silicic acid column chromatography after previous extraction of the toxin with aqueous alcohol and chloroform–methanol (2:1, v/v). The procedures are summarized in Scheme 1.

A silicic acid column, which absorbed the crude toxin, was eluted with chloroform–methanol (75:25, v/v), chloroform–methanol (50:50, v/v) and absolute methanol successively until the effluent became colorless and, after that, it was eluted with aqueous methanol of gradually increasing water content. A typical elution pattern is shown in Fig. 1.

SCHEME 1

PURIFICATION OF δ-HEMOLYSIN

Culture medium
centrifuge, 10000 × *g* for 15 min

Supernatant
heat for 30 min at 60°
centrifuge, 10000 × *g* for 20 min

Supernatant
adjust to pH 4.0 with acetic acid
add $(NH_4)_2SO_4$ (600 g/l), hold overnight in the cold
centrifuge, 27000 × *g* for 30 min

Precipitate
dissolve in H_2O (pH 9 with NH_4OH)
dialysis against water
lyophilize

Lyophilized powder
extract with 75% aq. ethanol by sonic oscillation
(10 kc, 200 W for 30 min) in the cold
centrifuge, repeat 3 times

Extract (supernatant)
evaporate to dryness *in vacuo*

Toxin powder (aq. ethanol extract)
extract with chloroform–methanol (2:1)
centrifuge, repeat 3 times

Extract (supernatant)
evaporate to dryness under a current of air

Toxin powder (chloroform–methanol extract)
dissolve in chloroform–methanol (75:25)
fractionation by silicic acid column chromatography

Hemolytic fraction
dialysis against water in the cold
lyophilize

Toxin powder (purified through silicic acid column)
fractionation by CM-cellulose column chromatography

Hemolytic fraction
dialysis against water in the cold
lyophilize

Toxin powder (purified through CM-cellulose column)

None of the three lipid fractions which were eluted with chloroform–methanol and absolute methanol had any hemolytic activity.

The toxin peak obtained from the silicic acid column was dialyzed, lyophilized and further purified through a CM-cellulose column. The elution pattern from the CM-cellulose column chromatography is shown in Fig. 2.

Yield and specific activity of the toxin at various stages of the purification are shown in Table I.

Further purification of the toxin by a CM-cellulose column, eluting with different

conditions, is not practically useful, because the toxin is not very soluble in a buffer solution at neutral pH at this state of purification, and it deposits in the column during the elution.

Another method which was used for fractionation and crystallization of the hemolysin is shown in Scheme 2. Crude toxin was fractionated through a calcium phosphate-gel column and TEAE-cellulose column; then the toxin was crystallized from Tris buffer at pH 8.6 by dialysis.

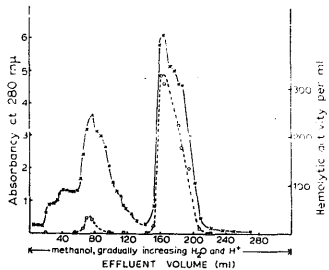

Fig. 1. Elution pattern of crude δ-hemolysin from silicic acid column. 450 mg of toxin powder (chloroform–methanol extract, 45 units/mg) was dissolved in chloroform–methanol (75:25, v/v) and placed on a silicic acid column (1 × 18 cm). The column was washed with chloroform–methanol and absolute methanol until the effluent became colorless. The toxin was eluted with aqueous methanol, gradually increasing water content with 0.01 HCl in the cold; mixing chamber 150 ml. ×—×, absorbancy; O---O, hemolytic activity.

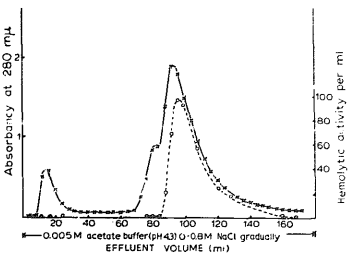

Fig. 2. Elution pattern of partially purified δ-hemolysin from CM-cellulose column. 117 mg of toxin powder (purified through silicic acid column, 90 units/mg) was placed on a CM-cellulose column (1 × 20 cm) buffered with 0.005 M acetate buffer (pH 4.3) and eluted with a salt gradient in the cold. ×—×, absorbancy; O---O, hemolytic activity.

Typical elution patterns from calcium phosphate-gel column chromatography and TEAE-cellulose column chromatography are shown in Fig. 3 and Fig. 4.

In Fig. 4 it will be seen that there are two hemolytically active peaks. The fast peak, which is $A_{280}/A_{260} = 1.4$, has higher hemolytic activity than the slower peak, which is $A_{280}/A_{260} = 0.9$. The slower peak may be nucleoproteins which are contaminated with the toxin.

TABLE I

RECOVERY AND ACTIVITY OF TOXIN IN THE PURIFICATION

Stage of purification	*Volume or weight*	*Activity (units)*	*Total activity (units)*	*Recovery (%)*
Culture medium, heated and centrifuged	1500 ml	35/ml	52500	100
Toxin powder, precipitated with $(NH_4)_2SO_4$	850 mg	30/mg	25500	49
Toxin powder, extracted with aq. alcohol	520 mg	40/mg	20800	40
Toxin powder, extracted with chloroform–methanol	450 mg	45/mg	20000	38
Toxin powder, purified through silicic acid column	117 mg	90/mg	10500	20
Toxin powder, purified through CM-cellulose column	67 mg	120/mg	8050	15

SCHEME 2

PURIFICATION OF δ-HEMOLYSIN

Supernatant of heated culture medium
dialysis against water in the cold
concentrate in dialysis sack covered by polyvinylpyrolidone
lyophilize

Lyophilized powder
fractionation by calcium phosphate-gel column chromatography

Hemolytic fraction
dialysis against water in the cold
lyophilize

Lyophilized powder
fractionation by TEAE-cellulose column chromatography

Hemolytic fraction
dialysis against water in the cold
lyophilize

Lyophilized powder
dissolve in a small volume of 0.05 M Tris buffer (pH 8.6)
centrifuge
supernatant was dialyzed againt water in the cold
centrifuge

Precipitate (uniform shape particle)
dissolve in a small volume of the above buffer
treat as described above

Crystalline δ-hemolysin

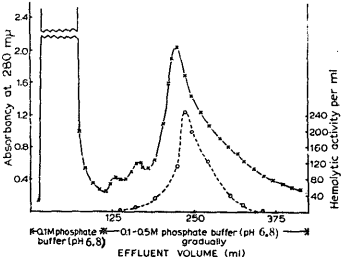

Fig. 3. Elution pattern of crude δ-hemolysin from calcium phosphate-gel column. 1.55 g of crude toxin powder (18 units/mg) was placed on a calcium phosphate column (2 × 5 cm), and eluted with buffer of increasing concentration. ×—×, absorbancy; O---O, hemolytic activity.

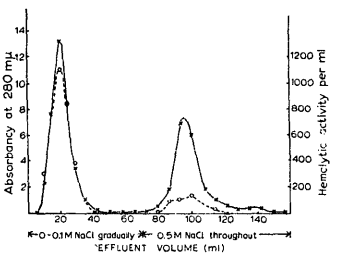

Fig. 4. Elution pattern of partially purified δ-hemolysin from TEAE-cellulose column. 185 mg of toxin powder (purified through calcium phosphate column, 130 units/mg) was placed on a TEAE-cellulose column (1 × 15 cm) buffered with 0.005 M Tris buffer (pH 8.6) and eluted with a salt gradient in the cold. ×—×, absorbancy; O---O, hemolytic activity.

The fast peak was dialyzed, lyophilized and used for crystallization. When the lyophilized powder was dissolved in the smallest amount of 0.05 M Tris buffer (pH 8.6) and the solution was dialyzed against distilled water in the cold, there developed a precipitate which had a silky sheen and uniformity of shape. Crystallization could be effected by repeating this procedure. Rod type crystals or, occasionally, lozenge type crystals were deposited (Fig. 5).

Yield and activity of the toxin at various states of the purification are shown in Table II.

δ-Hemolysin recrystallized 2–3 times was used in the following experiments.

TABLE II

RECOVERY AND ACTIVITY OF TOXIN IN THE PURIFICATION

Stage of purification	*Volume or weight*	*Activity (units)*	*Total activity (units)*	*Recovery (%)*
Culture medium, heated and centrifuged	1000 ml	40/ml	40000	100
Toxin powder, dialyzed and lyophilized	1550 mg	18/mg	28000	70
Toxin powder, purified through calcium phosphate column	435 mg	130/mg	57000	66
Toxin powder, purified through TEAE-cellulose column	68 mg	220/mg	15000	37
Crystalline toxin (2 times recryst.)*	16 mg	400/mg	6400	16

* No further increase of specific activity of the toxin was observed after recrystallization 2 times.

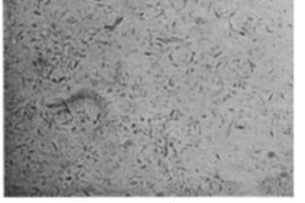

Fig. 5. Micrograph of δ-hemolysin crystals. Approx. × 300.

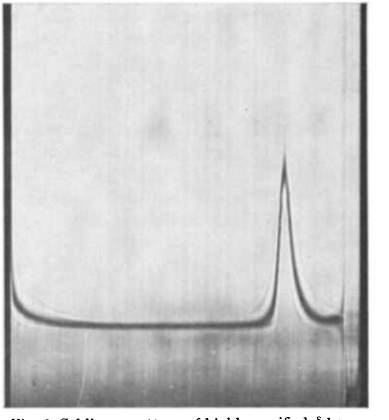

Fig. 6. Schlieren pattern of highly purified δ-hemolysin in the centrifuge at 59780 rev./min in 0.05 M phosphate buffer (pH 7.2).

Measurement of sedimentation, viscosity and molecular weight

Ultracentrifugation experiments were carried out in a Spinco Model E centrifuge at a speed of 59780 rev./min and at room temperature. The concentration of the

protein was approx. 0.4 % and the solvent employed was 0.05 M phosphate buffer at pH 7.2.

Schlieren patterns of purified toxin show single sedimentation boundaries (Fig. 6). This fact indicates a high degree of homogeneity. The sedimentation constant ($s_{20,w}$) estimated from the experimental results is 6.1 S.

Viscosity, at four different concentrations ranging from 0.2% to 0.7 % of the toxin, was measured in 0.1 M Tris buffer (pH 8.6) at 25°. Intrinsic viscosity $[\eta]$ of the toxin estimated from the experimental results is 5.5 ml/g.

From the sedimentation constant and the intrinsic viscosity, together with an assumed value for partial specific volume of 0.725 ml/g, and $\Phi^{1/3}P^{-1} = 2.6 \cdot 10^6$, the molecular weight of the toxin may be calculated from the MANDELKERN–FLORY equation[6]; a value of 74000 was obtained.

The molecular weight of the toxin was estimated also by the short column equilibrium molecular weight determination procedure[7]. Ultracentrifugation was carried out in 0.1 M Tris buffer (pH 8.6) in Spinco Model E centrifuge equipped with a light source for Rayleigh optics using a synthetic boundary cell. The molecular weight estimated from the concentration at the top of the cell is 72000, and the value obtained from the concentration at the bottom of the cell is 150000, employing an assumed partial specific volume factor of 0.725 ml/g.

Chemical composition of δ-hemolysin

The toxin was hydrolyzed completely in constant boilingpoint hydrochloric acid at 108° for 22 h in an evacuated sealed tube. The hydrolyzates were evaporated to dryness *in vacuo* over NaOH. Amino acid analysis was performed by a Spinco automatic amino acid analyzer.

Tryptophan was estimated by measuring the extinction at 294.4 and 280 mμ correcting for spurious absorption as described by GOODWIN AND MORTON[8].

The amino acid composition, assumed numbers of amino acid residues in the toxin molecule and molecular weight are presented in Table III.

Hexosamine content of the toxin was estimated by Elson–Morgan's method modified by RIMINGTON[9] after complete hydrolysis of the toxin in constant boilingpoint hydrochloric acid at 110° for 20 h. Hexosamine was not detectable in the hydrolyzates of the toxin.

Phosphorus content of the toxin estimated by Fiske–SubbaRow's method after hydrolysis is less than 0.05%.

The analysis indicates that the δ-hemolysin molecule (molecular weight 68 230 from amino acid composition) is constituted of the following 609 amino acid residues: Asp, 74; Thr, 47; Ser, 40; Glu, 42; Pro, 3; Gly, 45; Ala, 39; Val, 31; Met, 25; iLeu, 59; Leu, 45; Tyr, 7; Phe, 48; Try, 10; Lys, 88; His, 2; Arg, 4.

Tryptic digestion of δ-hemolysin

In order to distinguish whether the protein obtained actually has hemolytic capacity or whether a trace of non-proteic hemolytic substance is associated with a non-hemolytic protein, tryptic digestion was applied to the crystalline δ-hemolysin.

The tryptic hydrolysis was determined at 25°. The substrate (crystalline δ-hemolysin) was present at a concentration of 0.2 % in 0.1 M phosphate buffer (pH 7.8)

and the concentration of trypsin was 0.002 %. The course of the hydrolysis and the decrease of hemolytic activity are indicated by the curves shown in Fig. 7.

The results show that the hemolytic activity decreases with, or even more rapidly than, the hydrolysis of the protein. This fact indicates that the protein actually has the hemolytic activity, excluding the possibility that non-proteic lytic substance is a contaminant in the protein.

TABLE III

COMPOSITION AND MOLECULAR WEIGHT OF δ-HEMOLYSIN

Amino acid	*Per cent of amino acid residues**	*Assumed no. of residues/mole***	*Calculated molecular weight****
Aspartic acid	12.53	74	$679 \cdot 10^2$
Threonine§	6.98	47	$682 \cdot 10^2$
Serine§§	5.12	46	$680 \cdot 10^2$
Glutamic acid	7.09	42	$681 \cdot 10^2$
Proline	0.45	3	$659 \cdot 10^2$
Glycine	3.73	45	$689 \cdot 10^2$
Alanine	4.06	39	$683 \cdot 10^2$
Cystine/2	None detected	0	
Valine	4.55	31	$675 \cdot 10^2$
Methionine	4.80	25	$672 \cdot 10^2$
Isoleucine	9.71	59	$688 \cdot 10^2$
Leucine	7.41	45	$687 \cdot 10^2$
Tyrosine	1.68	7	$680 \cdot 10^2$
Phenylalanine	10.40	48	$679 \cdot 10^2$
Tryptophan§§§	2.61	10	$712 \cdot 10^2$
Lysine	16.55	88	$682 \cdot 10^2$
Histidine	0.41	2	$670 \cdot 10^2$
Arginine	0.94	4	$665 \cdot 10^2$
Ammonia§§§§	1.63	66	
Total	100.00	609	

* Total of individual amino acid residues is taken as 100.00.

** The nearest integral number to the calculated number of residues assuming molecular weight 68000.

*** (Molecular weight of amino acid residues ÷ 100/per cent of amino acid residues in protein) × assumed number of residues.

§ The recovery is assumed to be 90 % after hydrolysis.

§§ The recovery is assumed to be 80 % after hydrolysis.

§§§ Estimated from the extinction of protein (see text).

§§§§ Not included in the total.

Heat stability

Heat stability of crystalline δ-hemolysin was estimated in 0.02 M phosphate buffer (pH 6.8). The results are shown in Table IV. δ-Hemolysin is not very heat stable.

Solubility in chloroform–methanol

Crude toxin is completely soluble in chloroform–methanol (2:1, v/v) as described above, but crystalline δ-hemolysin is insoluble in the same solvent.

DISCUSSION

δ-Hemolysin of *S. aureus* was fractionated by column chromatographic techniques and obtained in crystalline form.

Chemical analysis indicates that the toxin is a protein, which is constituted of 609 amino acid residues and has a molecular weight of 68230. The molecular weight calculated from individual amino acid content, even the values obtained from proline which contains only three residues per molecule, from histidine which contains only two residues per molecule, or from arginine which contains only four residues per molecule, reconcile each other well (Table III). This fact indicates high homogeneity of the protein.

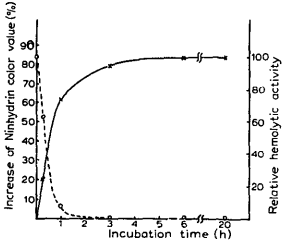

Fig. 7. The rate of hydrolysis of δ-hemolysin by trypsin at 25°. The substrate was present at a concentration of 0.2 % in 0.1 M phosphate buffer (pH 7.8) and the concentration of trypsin was 0.002 %. ×—×, ninhydrin color value; O---O, hemolytic activity.

TABLE IV

HEAT STABILITY OF δ-HEMOLYSIN

Temperature	*Time (min)*	*Per cent of inactivation*
60	30	36
60	120	71
100	30	85
100	60	92

Homogeneity of the protein was also shown by sedimentation patterns of ultracentrifugation.

The molecular weight estimated from the sedimentation constant and the intrinsic viscosity of the toxin is in good agreement with the molecular weight calculated from the amino acid composition. However, determination of the molecular weight by short column equilibrium ultracentrifugation at both the top and bottom of the ultracentrifuge cell, yielded at the top 72000 and at the bottom 150000. This probably is due to aggregation of the toxin in the solution, but may also be interpreted as due to contamination.

From the solubility of crude toxin in chloroform–methanol, the toxin was originally supposed to be lipid or lipoprotein, but by means of tryptic digestion and solubility measurement of the crystalline toxin, it has been established that the protein has the hemolytic activity. The possibility that non-proteic lytic substance contaminates the protein has been excluded.

It was reported that crude δ-hemolysin was stable to heating at 60° for 120 min (ref. 3) or at 115° for 20 min (ref. 4), but purified δ-hemolysin is relatively unstable to heating.

Very little is known about the immunological properties and pathogenicity of δ-hemolysin. Crystalline δ-toxin shows a single line precipitation reaction with antiserum obtained from rabbits immunized with the bacterial cells. The details of the immunological and biological activities of purified toxin will be reported in a separate paper.

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REFERENCES

1 R. E. O. WILLIAMS AND G. J. HARPER, *J. Pathol. Bacteriol.*, 59 (1947) 69.
2 J. MARKS AND A. C. T. VAUGHAN, *J. Pathol. Bacteriol.*, 62 (1950) 597.
3 A. W. JACKSON AND R. M. LITTLE, *Can. J. Microbiol.*, 3 (1957) 101; 4 (1958) 435, 453.
4 G. P. GLADSTONE AND W. E. VAN HEYNINGEN, *Brit. J. Exptl. Pathol.*, 38 (1957) 123.
5 A. TISELIUS, S. HJERTÉN AND O. LEVIN, *Arch. Biochem. Biophys.*, 65 (1956) 132.
6 L. MANDELKERN AND P. J. FLORY, *J. Chem. Phys.*, 20 (1952) 212.
7 E. G. RICHARDS AND H. K. SCHACHMAN, *J. Phys. Chem.*, 63 (1959) 1578.
8 T. W. GOODWIN AND R. A. MORTON, *Biochem. J.*, 40 (1940) 628.
9 C. RIMINGTON, *Biochem. J.*, 34 (1940) 931.