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THE AMPHIPHATIC NATURE OF *PRYMNESIUM PARVUM* HEMOLYSIN

S. ULITZUR

Department of Microbiological Chemistry, Hebrew University-Hadassah Medical School, Jerusalem (Israel)

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SUMMARY

1. *Prymnesium* toxin behaves as a surface-active agent and forms aggregates above the critical concentration of 0.6 ng/ml in isotonic NaCl solution.

2. Factors affecting the critical micelle concentration of surface-active agents, such as concentration, temperature and solvents were tested for their effect on the hemolytic activity. Above concentrations of 30 μg toxin/ml in methanol, the increment of hemolytic activity is much greater than the increment in the toxin concentration and they bear no fixed relation to one another. This effect can be reversed by prolonged incubation of the titrated toxin before addition of erythrocytes. After preincubation at -10 °C, aqueous or organic solutions of the toxin show a more than 20-fold increased activity. The cold activation is also reversible and disappears when the toxin is kept at 20 °C for 10 min before titration

3. The addition of organic solvents to an aqueous solution of the toxin considerably increases the hemolytic activity.

4. The possibility that the toxin aggregates rather than the monomers are the active hemolysin is discussed.

INTRODUCTION

Toxic preparations from the phytoflagellate *Prymnesium parvum* have a broad spectrum of different biological activities[1]. In addition to the pharmacological[2–4] and ichthyotoxic activities[5–7] *Prymnesium parvum* toxin exerts lytic effects on various cell types such as erythrocytes[8], bacteria[9] and a number of nucleated cells[10–12]. In all these systems the toxins were found to exert a strong effect on biological membranes[1].

The chemical properties of the purified toxin preparation were found to be similar to those of acidic polar lipids and its chemical composition and physical properties closely resembles those of proteolipids[13]. By virtue of a non-polar lipid moiety and polar components, the *Prymnesium* toxin would be expected to show amphiphatic properties and to form aggregates or micelles in solution. Indeed, the formation of aggregates above the critical concentration and the similarity

Abbreviation: CMC, critical micelle concentration.

of the *Prymnesium* toxin to surface-active agents have been already indicated[5].

This paper describes studies of the effect of physical factors, known to influence the micellar properties of surface active agents, on the purified toxin preparation and discusses the relation between the physical state of the toxic preparation and its hemolytic activity.

MATERIALS AND METHODS

Axenic cultures of *Prymnesium parvum* Carter isolated by Reich and Kahn[14] were grown on a poor-phosphate R medium[15]. The growth conditions and the extraction and purification of toxins have been described[15]. The most purified preparation (Toxin B) containing 3000 hemolytic units per μg, which could be separated into six toxic entities by thin-layer chromatography[13] have been used throughout this study.

Tests for hemolytic activity

Standard assay

The assay of Yariv and Hestrin[8] was modified by increasing the pH to 5.5. Bovine erythrocytes were washed in the cold three times with 10 vol. of standard assay buffer (0.15 M NaCl adjusted to pH 5.5 with 0.02 M sodium citrate). Bovine blood can be stored for up to 7 days in 0.02 M sodium citrate buffer containing 0.1% glucose without a change in sensitivity of the erythrocytes toward the *Prymnesium* hemolysin[8]. The assay mixture consisting of $4 \cdot 10^6$ erythrocytes/ml buffer [340 Klett photometer units (Filter 54) when diluted 1:1 in NaCl–citrate buffer] was added to the toxin diluted serially (double dilution) in test buffer and the degree of hemolysis was determined colorimetrically in a Klett photometer (Filter 54) after incubation for 45 min at 35 °C. A unit of hemolytic activity is defined as that amount of hemolysin in 1 ml of standard assay mixture which affected 50% hemolysis[8].

RESULTS

Different concentrations of Toxin B in 0.85% NaCl solutions were dialyzed

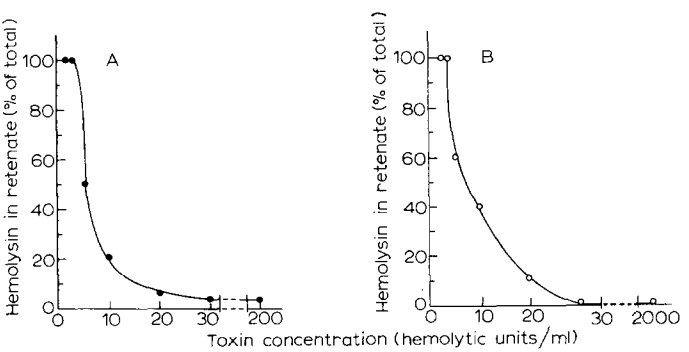

Fig. 1. Dialyzability and filterability of purified hemolysin at various concentrations. Toxin B was dialyzed (A) in cellophane bags (1 cm diameter) against 100 ml NaCl (0.15 M) at 4 °C for 25 h, and filtered (B) through 0.1 μm mean pore size membrane filter under vacuum. The hemolytic activity in the retenate and in the filtrate was determined as described in Methods.

or were filtered through a membrane filter of 0.1 μm mean pore size (Fig. 1). Only at concentrations below 2 hemolytic units/ml (0.6 ng/ml) did the toxin pass freely through the membrane or out of the cellophane bag.

This critical concentration is strongly dependent on the presence of salts in the aqueous solution. Fig. 2 shows that this critical toxin concentration in the assay buffer is 2 hemolytic units/ml, while in water it is ten times higher.

The size of hemolysin aggregates was determined by filtering a toxin solution (6000 hemolytic units/ml) through a set of membranes filters of increasing mean pore size. Fig. 3 shows that the aggregates are larger than 0.6 μm and that only 50% of the hemolysin aggregates are smaller than 1.2 μm.

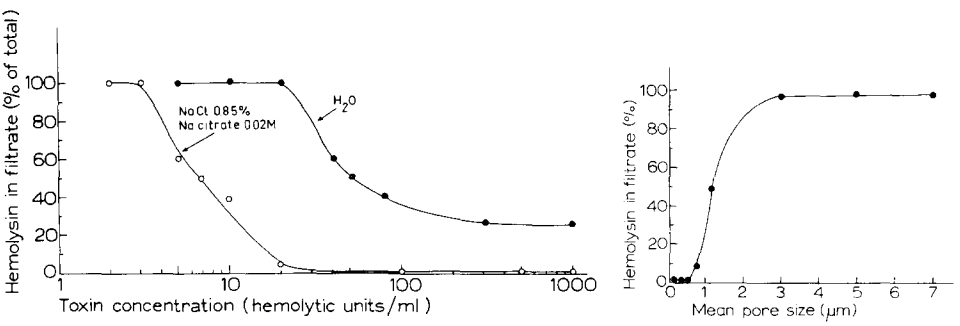

Fig. 2. The effect of salts on the hemolysin aggregate size. Solutions of Toxin B at various concentrations in distilled water or in the assay buffer were filtered through a membrane filter of 0.1 μm mean pore size. Hemolytic activity of the filtrate was assayed as described in Methods.

Fig. 3. Filterability of purified hemolysin through membrane of different mean pore size. Toxin B (6000 hemolytic units/ml) in 5 ml NaCl (0.15 M) was filtered through membrane filters of different mean pore size (0.1, 0.22, 0.45, 0.8, 1.2, 3.0, 5.0 and 7 μm). Hemolytic activity of the filtrate, given as percent of original activity, was assayed as described in Methods.

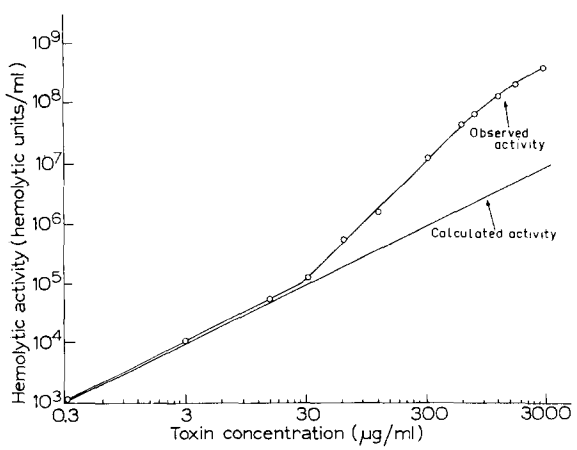

Fig. 4. Effect of toxin concentration on the expression of hemolytic activity. Methanolic solutions of Toxin B at different concentrations were tested for hemolytic activity. Erythrocyte suspensions were added within 30 s after hemolysin titration.

Factors known to affect the critical micelle concentration (CMC) of the amphiphatic agents, such as concentration, nature of solvent and temperature[16], were tested for their influence on the hemolytic activity of *Prymnesium parvum* Toxin B.

A methanolic solution of Toxin B at different concentrations was quickly titrated (30 s) in the assay buffer at 35 °C and erythrocytes were added immediately. Fig. 4 shows that under these conditions the increment of the hemolytic activity of the methanolic toxic solution containing more than 30 μg toxin/ml is much greater than the increment in the toxin concentration. An increase from 30–300 μg toxin/ml increases hemolytic activity 200-fold. As long as the methanolic solution contained less than 10^5 hemolytic units/ml the hemolytic activity increased exponentially. Maximal values, 50 times higher than the calculated values, were obtained when the methanolic solution contained $5 \cdot 10^6$ hemolytic units/ml.

This phenomenon (concentration effect) depends largely on the incubation time of the hemolysin in the assay buffer before addition of the erythrocytes. Fig. 5 shows that the hemolytic titer decreases progressively with increasing time of incubation of the hemolysin in an erythrocyte-free assay buffer. After 2 h of incubation, the hemolytic titer dropped to its calculated value. No loss in the hemolytic activity was observed after incubation of relatively low ($\leqq 10^5$ hemolytic units/ml) toxin concentrations for 2 h under the same conditions. The superactivation of the concentrated toxin depends very much on the solvents in which it is diluted. Table I shows that the maximal concentration effect is obtained only when the original Toxin B preparation is diluted in 0.85% NaCl. Dilution of the concentrated toxin in methanol, ethanol, or chloroform–methanol (2:1, v/v) or in distilled water decreased the resultant hemolytic activity 10-fold compared to dilution in 0.85% NaCl.

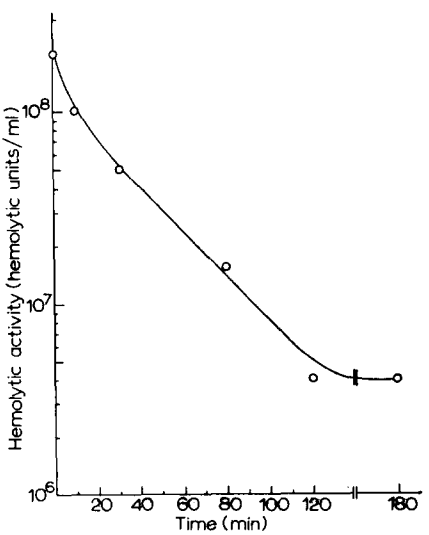

Fig. 5. Effect of incubation time in buffer solution on resultant hemolytic activity of concentrated toxin. Six assay series of concentrated methanolic solution of Toxin B ($4 \cdot 10^6$ hemolytic units/ml) were incubated at 35 °C each for different periods before the erythrocyte suspension was added. Hemolytic activity was assayed as described in Methods.

TABLE I

EFFECT OF SOLVENTS ON RESULTANT HEMOLYTIC ACTIVITY

A methanolic solution of Toxin B ($8 \cdot 10^5$ hemolytic units/ml) was diluted 100-fold in the solvents listed. Hemolytic activity was assayed immediately after titration.

Solvent	*Resultant hemolytic activity (hemolytic units/ml)*
Methanol	8000
Ethanol	8000
Chloroform–methanol (2:1, v/v)	7000
NaCl	70 000
Water	7500

TABLE II

EFFECT OF TOXIN TEMPERATURE ON RESULTANT HEMOLYTIC ACTIVITY

Toxin B ($3.5 \cdot 10^5$ hemolytic units/ml) in chloroform–methanol (2:1, v/v) or in methanolic solutions was preincubated 20 min at −10 °C. The hemolytic activity of the cooled toxin was determined immediately and after 10 min incubation at 20 °C.

Preparation	*Hemolytic activity (hemolytic units/ml)*	
	In methanol solution	*In chloroform methanol (2:1) solution*
Chilled toxin	$7 \cdot 10^6$	$1 \cdot 10^7$
Chilled toxin after incubation at 20 °C for 10 min	$4 \cdot 10^5$	$4 \cdot 10^5$

The hemolytic titers of aqueous, methanolic or chloroform–methanol (2:1, v/v) toxic solutions at concentrations above 10^5 hemolytic units/ml, pre-incubated at −10 °C for 10 min or more, were 20–30 times higher than those of controls preincubated at 20 °C (Table II). This effect (temperature effect) may be reversed by incubating the cold-treated toxin at 20 °C for 10 min before titration.

Toxin B dissolved in water shows very low hemolytic activity unless an organic solvent is added. Thus, the hemolytic activity was increased by increasing the concentration of methanol, ethanol or acetone in the aqueous solution of the toxin (Table III).

The physical configuration of the toxin assay solution affects the resultant hemolytic activity. Table IV shows that with a given volume of assay solution, hemolytic activity decreases with increasing surface area.

TABLE III

EFFECT OF TOXIN SOLVENT ON THE HEMOLYTIC ACTIVITY

A methanolic solution ($1 \cdot 10^5$ hemolytic units/ml) was added in various concentrations to distilled water. The hemolytic activity of the different aqueous solutions was tested before and after addition of different amounts of organic solvents. The organic solvents tested did not show any hemolytic activity in the concentrations tested.

Solvent added (final concn in solutions)	*Hemolytic activity (hemolytic units/ml)*	
	Before addition of solvent	*After addition of solvent*
Methanol (50%)	1	30
	10	200
Ethanol (50%)	500	1500
Acetone (50%)	500	2500
Methanol (50%)	2000	3000
Methanol (90%)	2000	5000

TABLE IV

THE EFFECT OF THE SURFACE: VOLUME RATIO ON THE HEMOLYTIC ACTIVITY

Toxin B (hemolytic units/ml) was assayed in a fixed volume (6 ml) of buffer assay system on differently-shaped containers (test tubes, beakers, petri dish) giving surface diameters of toxin solution as indicated in the table. The percent of hemolysis was determined after 45 min at 35 °C.

Diameter at the surface (cm)	*Hemolysis (%)*
0.95	50
1.3	34.5
8.2	28.3
9.5	7

DISCUSSION

An explanation for the concentration and temperature effects could involve the phenomenon known as surface ageing[17]. The molecules of surface-active agents concentrate at the surface at low concentrations below the CMC of the solution, while above the CMC the micelles remain dispersed in the bulk of the solution. The migration of the monomers to the surface is often prolonged, and equilibrium is achieved only after hours.

Most of the hemolytic aggregates are still in the bulk of the solution when erythrocytes are added shortly after the concentrated toxin is titrated. Incubation of the titrated hemolysin in the assay buffer for some time before addition of erythrocytes allowed for most of the aggregates to dissociate and for the monomers to concentrate at the surface. It may be postulated that the concentration of monomers

at the surface removes them from effective contact with the blood cells, leading to the decreased hemolytic activity observed. This idea is also supported by the fact that hemolytic activity is decreased when the surface area of the solution increases (*cf.* Table IV).

It is also suggested that the hemolytic aggregates, rather than the monomers, are responsible for the hemolytic activity of *Prymnesium* toxin. Electron microscopy of *Prymnesium* toxin in aqueous solution[1] containing $MgCl_2$ (0.05 M) at pH 9 (0.02 M Tris)[1] revealed that the toxin consists of a relatively homogeneous population of hollow spheres. The spheres have a diameter of 250–300 Å and the thickness of the enveloping leaflet is 50–55 Å. It is possible that the membrane-like leaflets of the toxin spheres penetrate the erythrocyte membrane, and the cell lyse following fusion of the toxin spheres to the cell membrane.

Armstrong[18] showed that the micelles rather than the monomers of the detergent sodium dodecyl sulfate cause damage and leakage of the yeast cell membrane.

Addition of organic solvents to aqueous solutions of the weak toxin considerably increases the hemolytic activity (*cf.* Table III). The reason for this effect is not yet clear, the organic solvents themselves in the concentrations studied did not show any hemolytic activity. It is possible that in aqueous solution the toxin forms giant micelles or aggregates[1]. The addition of organic solvents to the aqueous solution causes dissociation of these aggregates, increasing the number of the active aggregates and, consequently the hemolytic activity.

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