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Purification and characterisation of a hemolysin with phospholipase C activity from *Vibrio cholerae* O139

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Abstract

A hemolysin was purified from a *Vibrio cholerae* O139 strain which moved as a single protein band of 67 kDa in SDS-PAGE. The hemolysin showed high level of phospholipase C activity. The purified phospholipase C–hemolysin demonstrated enterotoxic activity in rabbit ileal loop, suckling mice and enhanced permeability of rabbit skin. The pI of the purified hemolysin was 6.4. Erythrocytes from rabbit, chicken, guinea pig, sheep and horse were sensitive to the purified hemolysin in decreasing order of intensity. Erythrocytes from human and cow were unaffected by purified hemolysin.

Keywords: Hemolysin; *Vibrio cholerae* O139; Phospholipase C

1. Introduction

Vibrio cholerae is known to produce several hemolysins. The hemolysin produced by *V. cholerae* O1 biotype Eltor (Eltor hemolysin), a product of *hlyA* gene has been shown to be enterotoxic and suggested to be a virulence factor in cholera pathogenesis [1]. Recently another hemolysin has been identified in *V. cholerae* O1 Eltor which is the product of *hlx* gene [2]. *V. cholerae* O1 of classical biotype produces a third hemolysin known as hemolysin (II) [3]. Clinical isolates of *V. cholerae* non-O1 are known to produce a thermolabile hemolysin indistinguishable from Eltor hemolysin, biologically, physicochemically and antigenically [4]. A few of the non-O1 *V. cholerae* clinical isolates also produce a thermostable hemolysin NAG-r-TDH which is similar but not identical to the thermostable direct hemolysin VpTDH [5], the principle virulence factor of diarrhoeagenic *Vibrio parahaemolyticus*.

V. cholerae O139 strains were identified for the first time from October 1992 as the causative agent of major outbreaks and epidemics of clinical cholera affecting several parts of India and its neighbouring countries almost simultaneously [6] indicating its pandemic potential. Till then the *V. cholerae* strains of O1 serovar only were thought to be capable of causing epidemics of cholera. This communication reports the purification and characterisation of a hemolysin from *V. cholerae* O139.

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2. Materials and methods

2.1. Strains and growth condition

V. cholerae O139 and *V. cholerae* O1 biotype Eltor strains isolated from hospitalised patients with acute diarrhoea were included in the study. Bacteria were grown in syncase broth [7] supplemented with 3% (v/v) glycerol at 37°C for 48 h without shaking.

2.2. Purification of hemolysin

Bacteria were grown as stated above. Solid ammonium sulphate was dissolved in the bacterial culture supernatant (390 g/l). The precipitate so formed was dissolved in 50 mM Tris-HCl containing 1 mM EDTA and 3 mM sodium azide pH 8.0 (TEA) followed by dialysis against TEA. The dialysed sample was centrifuged at $28\,000 \times g$ and the supernatant was designated as 'crude hemolysin'. The crude hemolysin was fractionated further on BioGel P-100 column, being eluted with TEA. Each fraction was assayed for hemolytic activity and protein concentration spectrophotometrically.

2.3. Quantitation of hemolytic activity

Hemolytic activity was assayed following the method as described by Tikoo et al. [8] using 2% (v/v) rabbit erythrocyte suspension in TEA which on 2-fold dilution with distilled water gave an optical density of 3 at 540 nm. Samples of hemolysin were incubated with equal volume of the standardised erythrocyte suspension for 2 h at 37°C followed by 18 h at 4°C. Hemoglobin released in the supernatant was measured spectrophotometrically at 540 nm. One hemolytic unit (HU) was defined as the amount of hemolysin causing 50% hemolysis of 1 ml of 2% standard erythrocyte suspension.

2.4. Determination of isoelectric point

Isoelectric point (pI) of the purified hemolysin was determined by chromatofocusing technique using PBE94 (Pharmacia) as polybuffer exchanger supporting gel, 0.025 M imidazole-HCl (pH 7.4) as start buffer and polybuffer74-HCl (pH 4.0) as eluent.

2.5. Rabbit ileal loop test (RIL) and passage of strain in RIL

RIL was performed essentially according to the method of De et al. [9]. Ligated ileal loops of adult rabbits were inoculated with 1 ml each of purified hemolysin and purified cholera toxin (CT, Sigma) with and without rabbit anti-CT antibody (gift from Dr. G.B. Nair, NICED). The result was expressed as volume of accumulated fluid (ml) per cm of loop (V/L ratio) on observing the animal after 18 h.

For passage of the strain in RIL, the bacteria were reisolated from the ligated loop inoculated with a live bacterial culture by draining the fluid, washing the intestinal tissue with sterile saline, incubation of the intestinal tissue in alkaline peptone water for 3–4 h at 37°C, followed by the usual procedure of isolation and confirmation of the bacteria.

2.6. Enterotoxicity test using suckling mouse

Purified hemolysin (0.1 ml) in different concentrations with 2 drops of 0.01% Evans Blue solution were administered orally to 2–3-day-old suckling mice (1.9–2.0 g). Five mice formed a group for each concentration. The degree of fluid accumulation in the intestine was determined after 18 h from the ratio of intestinal weight to the weight of the rest of the body (F/A ratio) [10]. F/A ratio of 0.09 and above was taken to indicate fluid accumulation.

2.7. Vascular permeability factor test (PF)

A 10-fold dilution (0.1 ml) of purified hemolysin in TEA was injected intradermally in the depilated back of an adult rabbit at 1 h time intervals for 4 h. The animal was injected with 2% (v/v) Evans Blue solution (1 ml/kg b. wt.) intravenously 1 h after the last injection. The diameter of bluing was measured [11].

2.8. Phospholipase C assay

Phospholipase C activity in the purified hemolysin was quantitated spectrophotometrically using *p*-nitrophenyl phosphoryl choline (NPPC) as the sub-

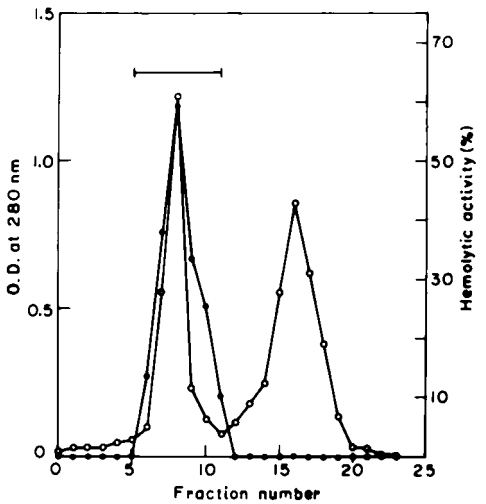

Fig. 1. Elution profile of purified hemolysin from *V. cholerae* O139 on BioGel P-100 column. Fractions indicated by the bar were pooled as purified hemolysin. ○-○, OD at 280 nm; ●-●, hemolytic activity.

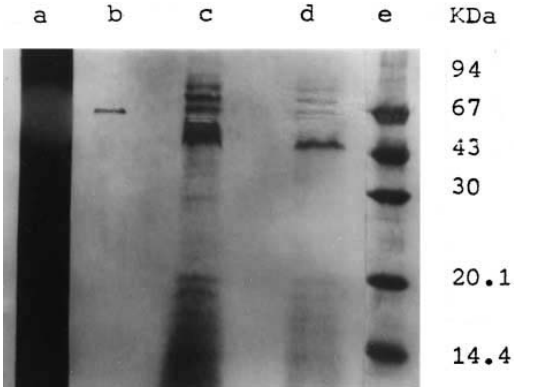

Fig. 2. SDS-PAGE of purified hemolysin, crude hemolysin and culture supernatant. Lane a: Purified hemolysin after SDS-PAGE immersed in molten blood agar plate. Lane b: Purified hemolysin. Lane c: Crude hemolysin. Lane d: Culture supernatant. Lane e: Molecular mass marker.

strate. This substrate yields phosphoryl choline and *p*-nitrophenol, a yellow chromogen which absorbs at 405 nm [12]. Phospholipase C from *Clostridium perfringens* (Sigma) was used as standard. The unit of enzyme activity was calculated by comparing the release of *p*-nitrophenol by the sample with that of the standard enzyme of known enzymatic activity.

2.9. Kinetics of thermostability

The hemolysins from *V. cholerae* O139 and *V. cholerae* O1 Eltor were heated at 60°C and 70°C in a water bath. The hemolytic activity was assayed at different time intervals.

3. Results

Out of sixteen *V. cholerae* O139 strains tested, 13 strains indicated extracellular hemolysin. On passage of four *V. cholerae* O139 strains in RIL, the hemolysin production by the strains increased considerably (data not shown). Hemolysin was purified from one of the strains of *V. cholerae* O139 which had undergone four serial passages in RIL. Table 1 records the typical data of hemolysin purification. The specific activity increased by 55-fold while recovery was 29%. The purified hemolysin constituted 1.6% of the total protein in the culture supernatant. Fig. 1 shows the elution pattern of the hemolysin in BioGel P-100 column. Eighteen nanograms of the purified hemolysin contain 1 HU. The purified hemolysin moved as a single protein band of 67 kDa molecular mass in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) as shown in Fig. 2. The single protein band in SDS-PAGE indicated hemolytic activity when the electrophoresed gel was dipped in blood agar plate (Fig. 2).

The purified hemolysin was capable of inducing fluid accumulation in RIL (Table 2) and hence was

Table 1
Steps in purification of *V. cholerae* O139 hemolysin

	Volume (ml)	Total protein (mg)	Total hemolytic activity (HU)	Recovery of hemolytic activity (%)	Specific activity (HU/μg protein)
Culture supernatant	2850	312	2.8×10^5	100	0.9
Crude hemolysin	10	70.12	1.5×10^5	53	2.1
Purified hemolysin	1	1.50	8.3×10^4	29	55.3

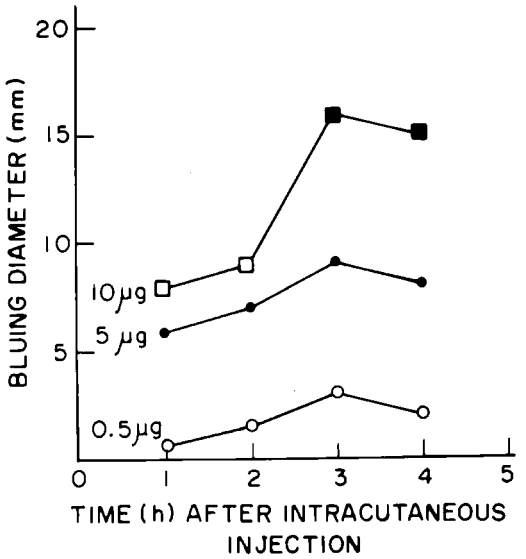

Fig. 3. Time course of the reaction in the rabbit vascular permeability factor (PF) test of purified *V. cholerae* O139 hemolysin. Amount of toxin administered: ○-○, 0.5 μg; ●-●, 5 μg; □-□, 10 μg of purified hemolysin. ■-■ indicates bluing with necrosis in the centre of injection site. Results were taken 1 h after Evans blue injection.

enterotoxic. The enterotoxicity of the purified hemolysin was not affected by presence of anti-CT antibody in the ligated loop indicating absence of CT as contaminant in the purified hemolysin. The enterotoxicity of the purified hemolysin was also indicated by fluid accumulation in infant mice intestine (Table 3) and increased vascular permeability in rabbit skin (Fig. 3) on peroral and intradermal administration of the purified hemolysin, respectively. The increased vascular permeability was best observed within 3–4 h after hemolysin injection.

The purified hemolysin of *V. cholerae* O139 demonstrated a high level of phospholipase C enzymatic activity (140 enzyme unit/mg protein).

Table 2
RIL with purified hemolysin, cholera toxin (CT) and antibody to CT (anti-CT)

Loop injected with (μg protein)	V/L[a] ratio (mean ± SD)[b]
Hemolysin (500)	1.0 ± 0.15
CT (10)	1.0 ± 0.12
CT (10)+anti-CT (10)	0.0
Hemolysin (500)+anti-CT (500)	1.0 ± 0.16

[a]Volume of fluid (ml) accumulated per unit length (cm) of rabbit gut (V/L ratio).
[b]Each result is the mean ± SD of 5 individual experiments.

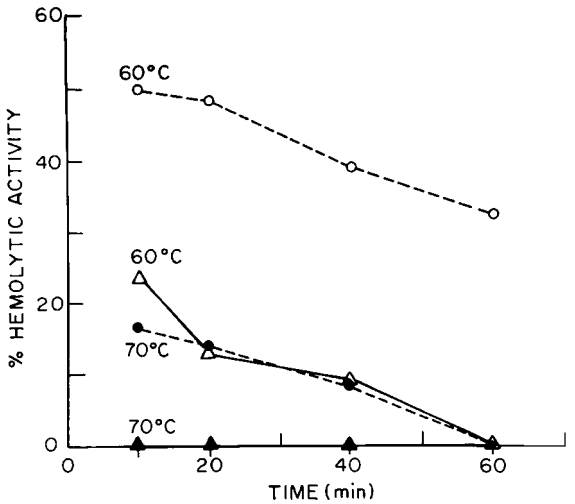

Fig. 4. Effect of temperature on hemolytic activity of *V. cholerae* O139 (○,●) and *V. cholerae* O1 Eltor (△,▲) strains. Open symbols represent 60°C and closed symbols represent 70°C.

Fig. 4 indicates that the hemolysin of *V. cholerae* O1 Eltor strain was more sensitive to heat inactivation than that of *V. cholerae* O139. The *V. cholerae* O139 hemolysin and the Eltor hemolysin lost their hemolytic activity completely by heating at 70°C for 60 and 10 min, respectively.

The pI of the hemolysin of *V. cholerae* O139 was found to be 6.4.

The purified hemolysin lysed erythrocytes from rabbit, chicken, guinea pig, sheep and horse in decreasing order of intensity. Erythrocytes from human and cow were unaffected by the purified hemolysin.

Table 3
Fluid accumulation in suckling mouse with purified hemolysin from *V. cholerae* O139

Amount of hemolysin injected orally (μg protein/mouse)	F/A ratio
125	0.10
100	0.09
75	0.08
50	0.08

$$\text{F/A ratio} = \frac{\text{Weight of intestine}}{\text{Weight of rest of the body}}$$

F/A ratio of mice fed 0.1 ml of 0.15 M phosphate-buffered saline, pH 7.4 = 0.018.

4. Discussion

This paper describes the purification of a hemolysin with phospholipase C activity from a recent epidemic strain of *V. cholerae* O139. The purified phospholipase C–hemolysin has been shown to be capable of inducing fluid secretion in rabbit as well as in mouse intestine and enhancing skin permeability in rabbit. The thermolabile Eltor hemolysin and *V. cholerae* non-O1 hemolysin have earlier been shown to be similarly enterotoxic [4] but their association with phospholipase C enzyme activity was not indicated. Although lecithinase production by *V. cholerae* O1 had been reported earlier [13], the enzyme had not been suggested to be related to either hemolytic or virulence related activity of the organism. However, it was reported earlier that on serial passage in RIL, phospholipase C activity increased concomitantly with the virulence of *V. cholerae* O1 strains [14]. Phospholipase C is known to affect host cell surface characteristics leading to altered membrane permeability [15].

The purified phospholipase C–hemolysin of *V. cholerae* O139 was unable to lyse human erythrocytes. Because in the disease cholera the organism is restricted to the lumen of the gut, intestinal epithelium remaining intact, it is of no consequence that the human erythrocytes are not hemolysed by the enterotoxic purified O139 hemolysin. Hemolysin II secreted by classical biotype of *V. cholerae* O1 and the *hlx* gene-linked hemolysin of classical and Eltor biotypes of *V. cholerae* O1 are similarly unable to lyse human erythrocytes [2]. The *hly* A gene product (Eltor hemolysin) and the antigenically identical hemolysin of clinical isolates of *V. cholerae* non-O1, non-O139, however, are known to lyse human erythocytes readily. The purified *V. cholerae* O139 hemolysin also differed from the Eltor hemolysin in pI and its thermostability kinetics. Erythrocyte susceptibility spectrum has been the basis for differentiation of multiple hemolysins excreted by a single organism such as *E. coli* [16]. The differential sensitivity of erythrocytes from various sources to the purified hemolysin from *V. cholerae* O139 in all probability is based on the general mechanism of action of cytolytic toxins, studied in details in a number of bacteria, involving rearrangement of membrane structure upon binding of the toxin to a specific receptor molecule on the membrane bilayer, followed by triggering of complex biochemical and physical events leading to destruction of the osmotic barrier [17]. This is to our knowledge the first indication that phospholipase C may have some involvement in cholera pathogenesis and further investigation is needed to confirm and clarify its role, if any, in the disease.

Acknowledgments

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