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Research Article

EVALUATION OF HEMOLYSIN ACTIVITY AMONG THE ENVIRONMENTAL ISOLATES OF *AEROMONAS HYDROPHILA*

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ABSTRACT
Nineteen environmental strains of *Aeromonas hydrophila* were evaluated for the production of hemolysin. The 47.36 percent of them showed positive hemolysin activity. Three of the isolates showed the significant hemolysin activities in the culture supernatants over a broad range of temperatures (30 - 50°C). At 37 ^{o}C, the hemolysin activity in the culture supernatant was stable when incubated at pHs between 5 to 9 for 5 h retaining significant activity. A hemolysin produced by a strain PB27 was purified by ammonium sulfate precipitation and phenyl sepharose CL-4B chromatography. The molecular weight of the hemolysin was estimated at 47.7 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified hemolysin had a hemolytic activity of 3.03 hemolytic units per microgram of protein on human erythrocytes at 37°C. Although, the hemolysin activity in the culture supernatants from the isolates at a temperature above 50 ^{o}C was abolished, purified hemolysin from the strain PB27 showed better stability, retaining its significant activity after incubation at 60 °C for 1 h.

***Keywords*:** *Aeromonas Hydrophila, Hemolysin, Enteropathogen, Environmental Isolates, Enterotoxic*

INTRODUCTION
Aeromonas hydrophila are ubiquitous microorganisms found in aquatic environments, food samples and the intestines of apparently healthy humans and humans with diarrhea (Hanninen and Siitonen, 1995). This species comprise mesophilic motile and psychrophilic non-motile gram-negative organisms. Bacteria of *Aeromonas* genus belong to Vibrionaceae family. The growth potential of *A. hydrophila* in drinking water, also with low concentration of organic compounds, is well established (Van der Kooij and Hijnen, 1988). *A. hydrophila* is recognized as a pathogen for humans and animals, including fish (Janda and Abbott, 1998). Among bacterial etiological agents of diarrhea, *A. hydrophila* is increasingly recognized as an enteric pathogen (Janda and Abbott, 1998). However, the pathogenic mechanisms by which this enteropathogen cause diarrhea are not yet well established. Extracellular toxic factors such as hemolysin, cytotoxin, and enterotoxin(s) produced by *A. hydrophila* have been studied in relation to its pathogenicity (Asao *et al*., 1984). Determination of the etiologic agents of diarrhoea is an important step for therapy and in implementing control measures. It is equally even more important also to determine the source of the infection. Most of all the enteropathogens are water borne. Hence, it is rational to characterize the environmental isolates. In the present study, we have evaluated the hemolysin activity among some environmental isolates of *A. hydrophila*, and purified and partially characterized the hemolysin from an environmental isolate of *A. hydrophila*.

MATERIALS AND METHODS
Strains
Nineteen environmental strains of *Aeromonas hydrophila* were included in the present study. These strains were isolated from the samples of natural surface water collected from the different sampling sites located in diarrhea endemic zones in Kolkata, India. These strains were selected from our laboratory collection. The strains were stored in 15 % glycerol stock at 27 ^{0}C.

Preparation of Cell-Free Culture Supernatants
Brain Heart Infusion Broth (BHIB, Hi-media) supplemented with 1% NaCl was used for assessing production of hemolysin by the strains. The test strains were grown in the above media at 37°C with

shaking (200 rpm) for 18 h. After centrifugation (15,000×g for 20 min at 4°C), the culture supernatant was used for hemolysin assays.

Assay of Hemolysin Activity

Hemolytic activities of the strains with erythrocytes from human were determined as described previously (Rammamurthy *et al.*, 1993). The amount of released hemoglobin in the supernatant was measured spectrophotometrically (Hitachi, U-3210; Japan) at 540 nm. An optical density of ≥0.45 was considered as positive for hemolysin.

Finally, results were expressed as the percentage of lysis by comparing these optical density values with that of an identical erythrocytes suspension lysed (100%) with an equal volume of TritonX-100 solution. One hemolytic unit (HU) was defined as the minimum dose of the sample required to produce 50% hemolysis.

The Effect of Temperature and pH on Activity and Stability

Thermostability was analyzed by measuring the residual activity after incubating the culture supernatant or purified hemolysin (10mM Tris-HCl, 1 mM EDTA, pH 7.5) at 30°C, 40°C, 50°C, and 60°C for 1 h in a sealed tube.

After each steps of incubation, the samples were centrifuged and hemolysin activity was determined as described above. To determine the effect of pH on enzyme stability, the culture supernatants were incubated in various buffers (pH 4.0 to 10.0) at 37^{o}C for 5 h. The buffers (10 mM) used were sodium acetate (pH 3.0–5.0), potassium phosphate (pH 6.0–7.0), Tris–HCl (pH 8.0–9.0), and sodium bicarbonate (pH 10.0-11.0).

Purification of Hemolysin

An overnight growth of *A. hydrophila* strain PB27 was inoculated into 100 ml Brain Heart Infusion Broth (BHIB, Hi-media) supplemented with 1% NaCl contained in 500 ml conical flask. The flask was incubated at 37°C with shaking (150 rpm) for 16 h.

After centrifugation (10,000 × g for 20 min at 4°C), the cell-free culture supernatant was assayed for hemolysin activity as described above. Hemolysin was purified from total one litre of the cell-free culture supernatant.

The cell-free culture supernatant was concentrated by ammonium sulfate precipitation (50%, w/v) at 4°C. The precipitate was dissolved in 10 mM Tris-HCl, pH 7.5 supplemented with 1mM EDTA and 1 mM phenylmethane sulphonyl fluoride (PMSF) (SRL). The sample was dialyzed against the same buffer. Then, 5 ml of this concentrated sample was loaded (in two batches, 2.5 ml per batch) onto a phenyl sepharose CL-4B column (4 × 1.5 cm i.d.), equilibrated with Tris-HCl buffer (10mM, pH 7.5). The column was washed with two bed volume of Tris-HCl buffer (10mM, pH 7.5) and the bound protein was eluted with ethylene glycol (30%, v/v). Fractions of 1 ml contain hemolysin activity were pooled, dialysed against 10mM Tris-HCl, 1 mM EDTA, pH 7.5 containing PMSF. The dialyzed protein samples were tested for purity on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as described by Laemmli (1970) using Bio-Rad electrophoresis apparatus. Protein bands were stained with coomassie blue. The molecular mass of hemolysin was determined graphically by interpolation from a logarithmic graph of molecular mass versus relative migrations using standard proteins (Fermentas).

Protein Concentration

Protein concentrations were determined by using a Bradford protein assay kit (Fermentas) following the manufacturer's instructions.

RESULTS AND DISCUSSION

The 47.36 percent of the environmental strains of *A. hydrophila* showed positive hemolysin activity (Table 1).

Three of the isolates (PB 27, 40 and 42) showed the significant hemolysin activities in the culture supernatants over a broad range of temperatures (30 - 50°C) (Table 2). At 37 ^{o}C, the hemolysin activity in the culture supernatant was stable when incubated at pHs between 5 to 9 for 5 h retaining significant activity (Table 3).

Table 1: Hemolysin Activity of the *A. hydrophila* Strains

Strains of *A. hydrophila*	Hemolysin Activity (HU)*
PB 22	0
PB 23	1.60
PB 24	0
PB 25	0
PB 26	1.63
PB 27	1.46
PB 28	1.50
PB 30	0
PB 31	1.07
PB 32	1.34
PB 33	1.53
PB 35	0
PB 36	0
PB 37	0
PB 38	0
PB 39	0
PB 40	1.46
PB 41	0
PB 42	1.65

* One hemolytic unit (HU) was defined as the minimum dose of the sample causing 50% hemolysis .

Table 2: Effect of Temperature on Hemolysin Activity

Temperature	Hemolysin Activity (HU) *								
	Strains								
	PB23	PB26	PB27	PB28	PB31	PB32	PB33	PB40	PB42
30℃	1.00	2.01	1.89	1.86	ND	1.59	1.91	1.73	1.74
40℃	1.28	2.22	1.94	2.06	1.98	1.76	1.76	1.85	1.52
50℃	0	0	2.21	0	0	0	0	1.77	1.43

* One hemolytic unit (HU) was defined as the minimum dose of the sample causing 50% hemolysis.

Table 3: Effect of pH on Hemolysin Activity

pH	Hemolysin Activity (HU) *								
	Strains								
	PB23	PB26	PB27	PB28	PB31	PB32	PB33	PB40	PB42
4	0	0	0	0	0	0	0	0	0
5	0	1.38	1.24	0	1.57	0	1.41	1.33	0
6	1.02	1.45	1.51	1.45	1.48	1.6	1.45	1.48	1.44
7	1.15	1.45	1.45	1.5	1.45	1.5	1.44	1.48	1.6
8	1.86	1.38	1.66	1.66	1.16	1.35	1.48	1.19	0
9	1.67	0	0	0	0	0	1.46	0	0
10	0	0	0	0	0	0	0	0	0

* One hemolytic unit (HU) was defined as the minimum dose of the sample causing 50% hemolysis.

A hemolysin produced by a strain (strain PB27) of the *A. hydrophila* isolates included here was purified by ammonium sulfate precipitation and phenyl sepharose CL-4B chromatography. This hemolysin was purified 2.7-fold. A summary of the purification data is presented in Table 4. The active fractions after different purification steps were electrophoresed on 10% SDS-PAGE using protein standards. A single

band was observed with the active fraction after gel filtration (FPLC) indicating the complete purification of the enzyme (Figure 1). SDS-PAGE analysis of purified hemolysin from *A. hydrophila* strain PB27 yielded molecular mass of 47.7 kDa (Figure 1).

Table 4: Purification of Hemolysin from *Aeromonas hydrophila* Strain PB 27

Purification Methods	Volume	Total Protein (mg)	Total Activity (HU)	Specific Activity (HU/mg)	Purification (fold)	Yield (%)
Culture supernatant	1000	980	1080	1.1	1	100
Concentrated supernatant[a]	2	3.26	5.36	1.64	1.49	0.49
Phenyl Sepharose CL-4B chromatography	1.5	1.17	3.55	3.03	2.7	0.32

*One hemolytic unit (HU) was defined as the minimum dose of the sample causing 50% hemolysis.
[a]The cell-free culture supernatant was concentrated by ammonium sulfate precipitation (50%, w/v) at 4°C.

The hemolysin activity in the culture supernatants from the isolates at a temperature above 50 °C was abolished. However, purified hemolysin from the strain PB27 showed better stability, retaining its significant activity after incubation at 60 °C for 1 h (Figure 2).

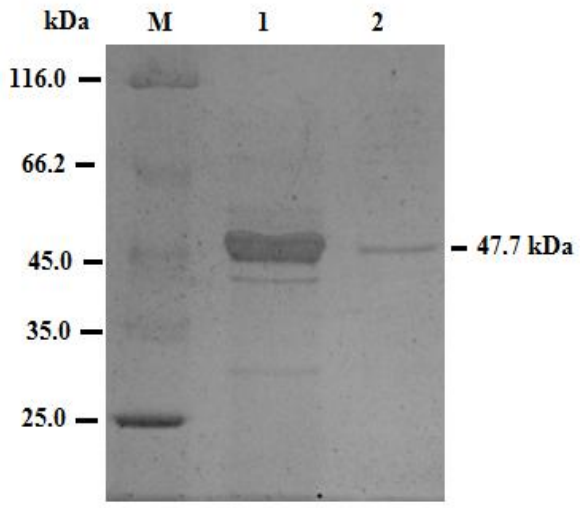

Figure 1: SDS-PAGE Analysis of Purified Hemolysin Protein from *A. hydrophila* Strain PB 27; Protein Marker (Lane 1), Crude Dialysed (Lane 2) and Pooled Phenyl-sepharose (Lane 3) Fractions Containing Hemolysin Protein were Analyzed by SDS-PAGE and Coomassie Blue staining

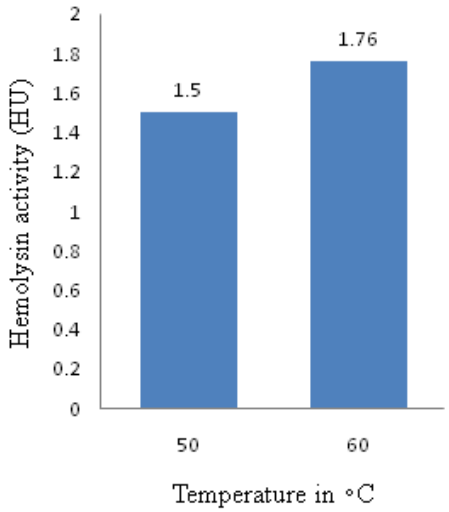

Figure 2: Thermostability of Hemolysin Purified from *A. hydrophila* Strain PB27

Although, the pathogenesis of *Aeromonas* infections remains poorly understood, mesophilic *Aeromonas spp.* can express a range of virulence factors (Gosling, 1996), including attachment mechanisms and production of a number of toxins. The primary toxins produced are hemolysins, of which the most significant is aerolysin, expressed by many strains of *A. hydrophila* and *A. sobria* (Howard *et al*., 1996; Janda, 1991). This is a heat-labile β-hemolysin, which exhibits phospholipase A and C activity. It is a pore-forming cytolysin able to insert into the cell membrane bilayer causing leakage of cytoplasmic contents. Hemolytic enterotoxins have been reported by some authors (Gosling, 1996). The role of

hemolysin in pathogenesis has been the subject of considerable dispute. One human volunteer study indirectly indicated that hemolysin does not have a role in the pathogenesis of *V. cholerae* (Levine *et al.*, 1988).

On the other hand, there is ample evidence for cytotoxic (Zitzer *et al.*, 1997; McCardell *et al.*, 1985) and enterotoxic (Ichinose *et al.*, 1987; McCardell *et al.*, 1985) activities of hemolysin. A 30-fold increase in the 50% lethal dose in infant mice has been reported from an El Tor *hlyA* mutant (Williams *et al.*, 1993). Expression of virulence factors, including hemolysins and proteases, by aeromonads has been shown to be influenced by environmental temperature (Eley *et al.*, 1993; Mateos *et al.*, 1993). There is abundant evidence to suggest associations between mesophilic aeromonads and diarrhoea, and production of enterotoxin has been demonstrated. In two comprehensive studies, Burke *et al.*, (1984) and Kirov *et al.*, (1993) compared *Aeromonas* strains isolated from drinking waters, rivers, water reservoirs and humans. These investigators found that both clinical and environmental strains were capable of producing enterotoxins. Hemolysin production and cytotoxin production are properties that are often associated with gastrointestinal infections caused by aeromonads (Singh and Sanyal, 1992). Further work is needed to clarify the pathogenic mechanisms of *Aeromonas spp*. and substantiate the causative role of these organisms in gastroenteritis. Although, it is still indistinct which virulence factor/s of *A. hydrophila* is responsible for causing diarrhea in human, the environmental isolates possessing several virulence factors including hemolysin in water may be potential enteric pathogen.

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