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Purification and Characterization of Cytolytic Toxins Produced by *Aeromonas hydrophila* and *Aeromonas veronii* Biotype Sobria Strains

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Abstract

Cytolytic toxins produced by *Aeromonas hydrophila* and *Aeromonas veronii* biotype sobria strains were partially purified from culture filtrates by two steps of purification: ammonium sulfate precipitation and hydrophobic chromatography using Phenyl-Sepharose CL-4B. Hemolytic activity was detected in one or two peaks in elution profile. Purified toxins were also cytotoxic to Vero and CHO cells. Moreover, these toxins revealed cytotonic activity to CHO cells.

Key words: *Aeromonas* spp., cytolytic toxins, cytotoxic activity

Introduction

The genus *Aeromonas* represents a group of gram-negative, facultatively anaerobic organisms, widely spread in water habitats (Holmes *et al*., 1996). The genus is taxonomically complex and includes at least 20 species that can be differentiated on the basis of DNA-DNA hybridization (Altwegg, 1999; Pidiyar *et al*., 2002; Harf-Monteil *et al*., 2004). Species belonging to hybridization group 1 (HG 1, *A. hydrophila*), HG 4 (*A. caviae*) and HG 8/10 (*A. veronii* biotype sobria) are the most prevalently isolated from the stool of humans with gastroenteritis, which mainly affects young children, the elderly and immunocompromised patients. Human diarrheal diseases range from a mild self-limiting, acute diarrhea to a cholera-like dysenteric illness or a more persistent diarrhea (Altwegg, 1999).

Various virulence factors appear to be involved in *Aeromonas* sp.-associated gastroenteritis: enterotoxins, cytotoxins, hemolysins and extracellular enzymes. However, it is still unclear which toxin is responsible for the diarrhea symptoms (Laohachai *et al*., 2003).

Strains of *A. hydrophila* and *A. veronii* biotype sobria produce β-hemolysin which is called aerolysin, cytotoxic enterotoxin or cytolytic enterotoxin cross-reactive with cholera toxin (Thornley *et al*., 1997). Recent studies indicated the production of four hemolytic enterotoxins by different *Aeromonas* spp. isolates (Chopra and Houston, 1999; Albert *et al*., 2000, Trower *et al*., 2000). *Aeromonas* spp. can produce at least one non-enterotoxigenic β-hemolysin and possibly several β-hemolytic enterotoxins. Albert *et al*. (2000) identified three distinct genes encoding enterotoxins in *Aeromonas* spp. One of them encodes cytotoxic enterotoxin (Act) and two encode cytotonic enterotoxin. One cytotonic enterotoxin is heat stable at 56°C (Ast) and the other one is heat labile at 56°C (Alt). Site-directed mutagenesis on Act indicated possibly different loci on a single chain which may be associated with various biological activities. Chopra and Houston (1999) reported that Act is an aerolysin with hemolytic, cytotoxic and enterotoxic activity.

Much work on the mechanistic level of aerolysin action has concentrated on erythrocytes and there is still little known about its interaction with epitelial cells (Thornley *et al*., 1997). Our study was undertaken to examine hemolytic, cytotoxic and cytotonic activity of partially purified cytolytic toxins produced by *A. hydrophila* and *A. veronii* biotype sobria strains isolated from stool of humans with gastroenteritis.

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Experimental

Material and Methods

Bacterial strains and culture methods. Toxins were isolated from strains of *A. hydrophila* (HG 1) RK 70363, RK 217215, RK 226254 and *A. veronii* biotype sobria (HG 8/10) RK 43939 and RK 66113. The strains were isolated from fecal specimens of patients with diarrhea symptoms and were obtained from dr R. Kong (Hong Kong City University). The identification of examined isolates was performed on the basis of their phenotypic properties, confirmed by DNA-DNA hybridization (Szczuka and Kaznowski, 2004) and by 16S rDNA RLFP analysis as described previously (Figueras *et al*., 2000). The isolates were maintained at – 75°C in brain heart infusion broth (BHI, Difco) containing 50% (v/v) glycerol. *Aeromonas* spp. strains were grown for 24 hours on tryptic soy broth (TSB, Difco) supplemented with 0.6% yeast extract (TSB-YE) at 37°C. After overnight incubation, 250 ml of TSB-YE was inoculated with 0.5 ml of culture supernatant and incubated at 37°C for 24 hours in water bath with shaking at 120 rpm. The culture was centrifuged then at 10 000 g for 50 minutes and the pellet was discarded. Culture supernatant was sterilized through 0.22 μm-pore-size low protein binding Millex GV filters (Millipore). Two protease inhibitors, soybean tripsin inhibitor (1 mg liter^{-1}) and phenylmethylsulfonyl fluoride (1 mM) were added to the culture filtrates in order to prevent proteolytic degradation (Rose *et al*., 1989).

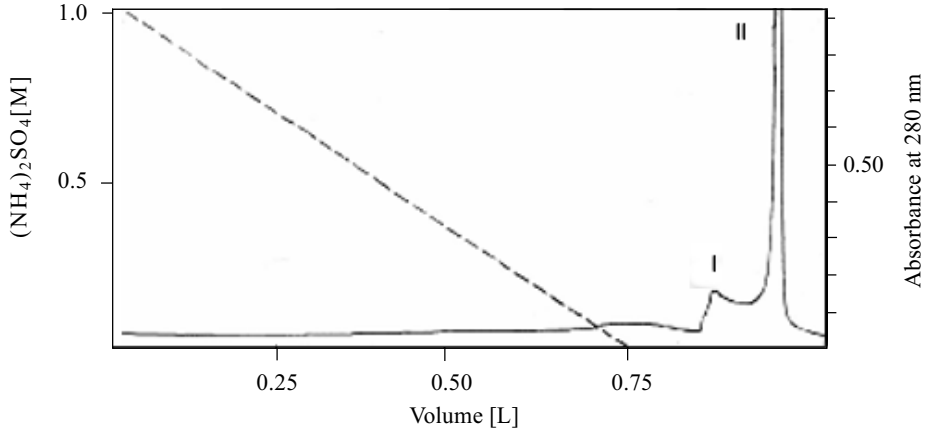
Partial toxin purification. Ammonium sulfate $(NH_4)_2SO_4$ (Difco) was added to the culture supernatants to give 60% saturation. The pH was adjusted to 6.0 and the supernatants were stored overnight at 4°C. The precipitate was centrifuged at 10 000 g for 50 minutes at 4°C and the pellet was dissolved in 1 M ammonium sulfate and adjusted to pH 7.0. For hydrophobic interaction chromatography (HIC) the redissolved pellet was dialyzed against 1 M ammonium sulfate, applied to a column of Phenyl-Sepharose CL-4B (1×180 cm) and washed with 1 M ammonium sulfate (pH 7.0) until absorbance at 280 nm reached zero. Bound protein was eluted with a decreasing linear gradient from 1 M to 0 M ammonium sulfate. Fractions of 3 ml volume were collected (GradiFrac, Pharmacia). The protein concentration in culture supernatant filtrates, ammonium sulfate precipitates and fractions of partially purified toxins was determined by the Bradford protein assay (Bradford, 1976).

Hemolysin activity. Hemolysin assay was performed by using a suspension of 1% human erythrocytes according to Nowotny (1979). Serial dilutions (from 1:2 to 1:512) of each sample in phosphate-buffered saline (PBS) were incubated at 37°C for 1 hour with equal volume of erythrocyte suspension. After incubation at 4°C for 1 hour, the samples were centrifuged at 800 g for 10 minutes to remove unlysed cells. Absorbance was measured on a microplate reader SUMAL PE 2 at 545 nm. Hemolytic activity was expressed in hemolytic units per miligram of protein (HU mg). HU was defined as the reciprocal of the highest dilution of toxin in which 50% hemolysis was observed.

Cytotoxic and cytotonic activity. Cytotoxic activity was measured on African monkey kidney cells (Vero) and Chinese hamster ovary cells (CHO), whereas cytotonic activity was examined on CHO cells as described previously (Krzymińska *et al*., 2003). Cells were cultivated on Minimum Essential Medium Eagle (MEM, Sigma) supplemented with 5% fetal bovine serum (FBS, Sigma), 2 mM glutamine, 80 IU ml^{-1} of penicillin, 80 μg ml^{-1} of streptomycin and 1mg ml^{-1} of nystatin. Cells were seeded in microtitre plates at a density of 1×10^4 cells ml^{-1}. Serial twofold dilutions of culture supernatant was added to the monolayer and the plate was incubated for 24 hours at 37°C in atmosphere containing 5% CO_2 and assessed under an inverted microscope. Cytotoxic activity was identified as rounding and detachment of 50% cells whereas cytotonic activity was recognized as elongation of CHO cells. Cytotoxic and cytotonic activities were expressed in total units per miligram of protein. Cytotoxic and cytotonic units were expressed as the reciprocal of the highest dilution yelding a positive result. Heat stability of culture supernatant fluids and HIC fractions with purified toxins was detected after heating at 56°C for 20 minutes.

Results

Partial purification of cytolytic enterotoxin. Cytolytic toxins produced by *A. hydrophila* and *A. veronii* biotype sobria were purified from culture supernatant in two steps which included ammonium sulfate precipitation and hydrophobic column chromatography. The elution patterns from this column (Fig. 1 and 2)



The dialyzed protein solution was applied to a column of Phenyl Sepharose CL-4B and was washed with 1 M ammonium sulfate until the absorbance at 280 nm returned to zero. Bound protein was eluted with linear gradient of 75 ml of 1 M ammonium sulfate and 75 ml of water. Flow rate 1ml min^{-1} (GradiFrac System, Pharmacia)

Fig. 1. Elution profile of toxins isolated from *A. hydrophila* RK 217215.

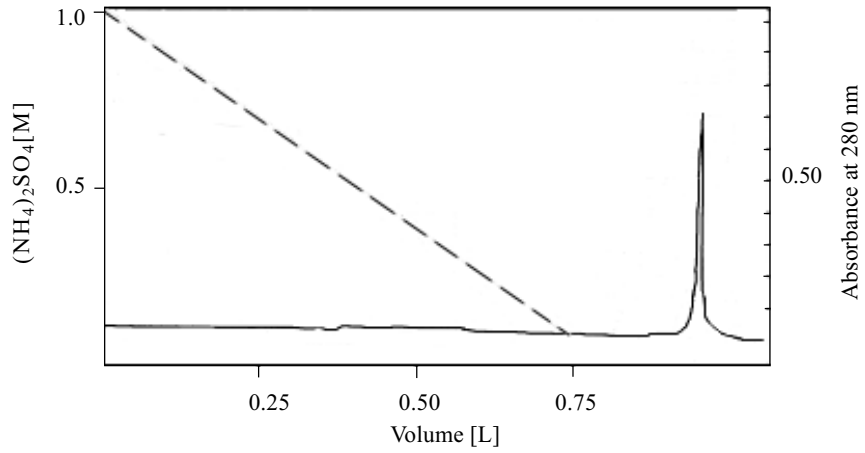


Fig 2. Elution profile of toxin isolated from *A. hydrophila* RK 226254.

showed that the toxin was eluted as a single peak (*A. hydrophila* RK 70363 and 226254) or as a double peak (*A. hydrophila* RK 217215, and *A. veronii* biotype sobria RK 43939, 66113). Table I summarizes the data on the purification of cytolytic toxins. Specific hemolytic activity of these toxins gradually increased with the decline in the amount of protein, suggesting true purification. Ammonium sulfate precipitation followed by dialysis resulted in an increase in specific activity (1.2-fold to 27.5-fold). Next the proteins were applied to Phenyl-Sepharose CL-4B. After chromatography, the specific activity increased about 32-fold for *A. hydrophila* RK 226254. Low grade of the purification may suggest that culture supernatant and pooled HIC fractions should be concentrated.

Cytotoxic and cytotonic activity. Cytotoxic activities to Vero and CHO cells of culture supernatant, ammonium sulfate precipitates and fractions of partially purified cytolytic toxins are shown in Table II.

Table I
Partial purification of cytolytic enterotoxins produced by *Aeromona*s spp. strains

Strain No		Fraction	Total vol. (ml)	Total protein (mg)	Total hemolytic activity[a] (HU)	Specific activity[b] (HU mg^{-1})	Purification fold	Recovery (%)
A. hydrophila RK 70363		supernatant	200	23.6	3.1×10^5	1.3×10^4	1.0	100
		ammonium sulfate	2	0.3	2.6×10^4	8.7×10^4	6.7	8.4
		HIC-I[c]	3	0.2	1.9×10^4	9.5×10^4	7.3	6.1
	RK 217215	supernatant	200	19.2	1.9×10^6	1.0×10^5	1.0	100
		ammonium sulfate	2,0	0.3	3.6×10^4	1.2×10^5	1.2	1.9
		HIC-I	3.0	0.2	2.6×10^4	1.3×10^5	1.3	1.4
		HIC-II[d]	3.0	0.3	8.4×10^4	2.8×10^5	2.8	4.4
	RK 226254	supernatant	200	13.6	9.4×10^3	6.9×10^2	1.0	100
		ammonium sulfate	2.0	0.2	3.8×10^3	1.9×10^4	27.5	40.4
		HIC-I	3.0	0.2	3.0×10^3	1.5×10^4	31.9	21.7
A.veronii biotype sobria RK 43939		supernatant	200	9.5	6.5×10^3	6.9×10^2	1.0	100
		ammonium sulfate	2.0	0.6	1.4×10^3	2.3×10^3	3.3	21.5
		HIC-I	3.0	0.1	0	0	0	0
		HIC-II	3.0	0.2	7.2×10^2	3.6×10^3	5.2	11.1
	RK 66113	supernatant	200	4.2	9.2×10^3	2.2×10^3	1.0	100
		ammonium sulfate	2.0	1.2	8.8×10^3	7.2×10^3	3.3	95.6
		HIC-I	3.0	0.04	0	0	0	0
		HIC-II	3.0	0.08	9.6×10^2	1.2×10^4	5.4	10.4

a – reciprocal of the highest dilution of toxin showing 50% hemolysis of 1% erythrocytes (HU × volume)
b – total HU per mg of protein
c – first peak on Phenyl-Sepharose CL-4B column
d – second peak on Phenyl-Sepharose CL-4B column

Table II
Cytotoxic activity of partially purified toxins and their fractions to Vero and CHO cells

Strain No		Purification step	Specific cytotoxic activity[a] (total units mg^{-1})			
			Vero		CHO	
			unheated sample	preheated sample[b]	unheated sample	preheated sample
A. hydrophila	RK 70363	supernatant	1.4×10^{3}	8.1×10^{1}	2.7×10^{3}	8.4×10^{1}
		ammonium sulfate	1.6×10^{5}	ND [c]	1.3×10^{3}	ND
		HIC-I	3.0×10^{2}	1.3×10^{1}	6.0×10^{2}	1.3×10^{1}
	RK 217215	supernatant	1.7×10^{3}	2.1×10^{2}	1.3×10^{4}	2.1×10^{2}
		ammonium sulfate	5.3×10^{3}	ND	4.3×10^{3}	ND
		HIC-I	1.5×10^{2}	1.0×10^{2}	4.8×10^{3}	1.5×10^{2}
		HIC-II	8.0×10^{2}	1.0×10^{2}	1.2×10^{4}	1.0×10^{2}
	RK 226254	supernatant	2.3×10^{3}	1.4×10^{2}	2.3×10^{3}	0
		ammonium sulfate	1.6×10^{5}	ND	1.6×10^{5}	ND
		HIC-I	1.2×10^{3}	1.5×10^{2}	6.0×10^{2}	3.0×10^{1}
A. veronii biotype sobria	RK 43939	supernatant	6.7×10^{3}	4.2×10^{2}	2.5×10^{4}	8.4×10^{2}
		ammonium sulfate	3.3×10^{5}	ND	5.3×10^{2}	ND
		HIC-I	2.0×10^{2}	1.0×10^{2}	6.0×10^{2}	1.0×10^{2}
		HIC-II	2.4×10^{4}	3.2×10^{2}	4.8×10^{3}	3.0×10^{2}
	RK 66113	supernatant	3.1×10^{4}	9.5×10^{2}	3.0×10^{4}	9.5×10^{2}
		ammonium sulfate	1.3×10^{5}	ND	5.3×10^{2}	ND
		HIC-I	1.5×10^{4}	0	7.2×10^{2}	0
		HIC-II	3.0×10^{3}	3.7×10^{2}	6.0×10^{3}	7.5×10^{2}

a – total cytotoxic units (reciprocal of the highest dilution showing rounding and detachment of 50% Vero or CHO cells) per mg of protein
b – sample preheated at 56°C for 20 minutes
c – not determined

Table III
Cytotonic activity of partially purified toxins and their fractions to CHO cells

Strain No		Purification step	Specific cytotonic activity[a] (total units mg^{-1})	
			unheated sample	preheated sample[b]
A.hydrophila	RK 70363	Supernatant	0	1.7×10^{2}
		ammonium sulfate	0	ND[c]
		HIC-I	0	1.5×10^{2}
	RK 217215	Supernatant	0	2.1×10^{2}
		ammonium sulfate	0	ND
		HIC-I	0	1.5×10^{2}
		HIC-II	0	2.0×10^{2}
	RK 226254	Supernatant	0	5.9×10^{2}
		ammonium sulfate	0	ND
		HIC-I	0	3.0×10^{2}
A.veronii biotype sobria	RK 43939	Supernatant	0	8.4×10^{2}
		ammonium sulfate	0	ND
		HIC-I	0	0
		HIC-II	0	6.0×10^{2}
	RK 66113	Supernatant	0	9.5×10^{2}
		ammonium sulfate	0	ND
		HIC-I	0	0
		HIC-II	0	7.5×10^{2}

a – total cytotonic units (the reciprocal of the highest dilution showing elongation CHO cells × volume) per mg of protein
b – sample preheated at 56°C for 20 min
c – not determined

Cytotoxic activity was present only in the first peak eluted from *A. hydrophila* (RK 70363, RK 226254) or in both peaks (RK 217215), whereas toxins eluted from *A. veronii* biotype sobria revealed cytotoxic activity in the first and the second peaks. Specific cytotoxic activity of culture supernatants of examined strains to Vero cells, ranging from 1.4×10^3 to 3.1×10^4 units per mg of protein was progressively decreasing during purification. Preheating of culture supernatant and fractions of purified toxins at 56°C for 20 minutes caused decline of specific cytotoxic activity and revealed low cytotonic activity to CHO cells, which decreased during purification. Cytotonic activity to CHO cells of partially purified toxins and their fractions are shown in Table III. Specific cytotonic activity of preheated toxins ranged from 1.5×10^2 to 7.5×10^2 units per mg of protein.

Discussion

A variety of extracellular proteins produced by *Aeromonas* spp. isolates have been implicated in the pathogenesis of gastroenteritis. It is important to determine activity of separate toxins to establish their role in gastrointestinal disease. Therefore, cytolytic toxins produced by *A. hydrophila* and *A. veronii* biotype sobria were partially purified from culture filtrates by using two-step purification.

Cytolytic toxins present in culture filtrates were salt-precipitable at 60% saturation of ammonium sulfate. Hemolytic activity was detected in one or two peaks in elution profile obtained during elution of dialyzed ammonium precipitate on Phenyl-Sepharose CL-4B column. Toxins produced by *A. veronii* biotype sobria eluted in hydrophobic chromatography in the second peak revealed hemolytic activity, whereas these from the first peak were non-hemolytic (Table I). Cytolytic toxins isolated from *A. hydrophila* culture supernatant showed hemolytic activity in the first peak or in both. The highest specific hemolytic activity (2.8×10^5 HU per mg) was observed for the toxin eluted in the second peak and isolated from *A. hydrophila* RK 217215. Rose *et al*. (1989) purified cytolytic toxin produced by *A. hydrophila* isolated from a human specimen and calculated specific activity of the toxin to 4×10^6 HU per mg of protein.

It appeared, that the main targets of cytolytic toxins produced by *Aeromonas* strains causing gastroenteritis are epithelial cells of the intestine. However little is known about interaction of this toxin with mammalian cells. Our results indicated that cytolytic toxins produced by *Aeromonas* spp. strains were also cytotoxic to Vero and CHO cells (Table II). The results are in agreement with observations of Rose *et al*. (1989) who stated that cytolytic toxin from *A. hydrophila* strain revealed cytotoxic and enterotoxic activity as well as mice lethality. Our results showed a decrease of specific cytotoxic activity to Vero and CHO cells during purification which could have been due to removal of other cytotoxic enterotoxins from culture supernatants of examined strains. Evidence for the existence of more than one cytolytic toxin has been reported previously (Asao *et al*., 1984; Chopra and Houston 1999; Fujii *et al*., 1998).

Interestingly, partially purified cytolytic toxins in our study demonstrated also low cytotonic activity revealed to CHO cells only after preheating (56°C for 20 min) of culture supernatant and fractions of purified toxins. Preheating of these samples caused inactivation of heat-labile toxins which destroyed CHO monolayer. Cytotonic activity of these toxins could be associated with an increase of cAMP concentration. Fujii *et al*. (2003) demonstrated that hemolysin produced by *A. sobria* strains increased intracellular cyclic AMP concentration in cultured colonic epithelial cells.

Our results showed that cytolytic toxins produced by isolates of *A. hydrophila* and *A. veronii* biotype sobria revealed hemolytic, cytotoxic and cytotonic activities. These observations suggested that cytolytic toxins play an essential role in *Aeromonas* sp.-associated gastroenteritis.

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