Purification and Preliminary Characterization of the Extra-cellular Hemolytic Toxins from *Vibrio anguillarum*

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There have been reported metalloproteases¹⁻⁴⁾, enterotoxin⁵⁾ and hemolysin^{6,7)} as the extracellular virulence factors of *Vibrio (Listonella) anguillarum*, the causative agent of vibriosis of various freshwater and marine fishes. The present communication describes the purification and preliminary characterization of novel hemolytic toxins (α and β) produced extracellularly by the organism.

An isolated strain (CS-20) from a diseased coho salmon (*Oncorhynchus kisutch*) identified as *V. anguillarum*, was used in this study. The hemolytic toxin was produced in a liquid medium containing 2% Bacto-peptone, 1% sucrose, 1% NaCl, 0.2% KH_2PO_4 , 0.025% MgSO_4 and 0.029% ZnSO_4 , pH7.2, during the stationary phase of growth at 25°C by shaking culture.

The hemolytic activity was determined by measuring the OD_{540} of liberated hemoglobin after hemolytic reactions using horse erythrocytes according to the method of Marchlewicz-Duncan⁸⁾ with minor modification. The proteolytic activity was determined by measuring the OD_{660} according to the method of Hagihara *et al.*⁹⁾. The concentration of carbohydrate was determined by measuring the OD_{625} by anthrone- H_2SO_4 reaction.

The toxin was then purified from the culture supernatant by ammonium sulfate precipitation and ion-exchange chromatography on DEAE-cellulose. Then two hemolytic fractions were obtained from the column by stepwise elution with 0.145 M NaCl containing 50 mM Tris-HCl buffer, pH7.2, and by gradient elution with 0.145-1M NaCl containing the same buffer. After ultrafiltration by Millipore immersible kit CX-10, the

Fig. 1. Elution profile of the hemolytic toxins of *V. anguillarum* for the gel filtration chromatography on Sephadex G-100. A 2-ml sample volume was applied to the column (1 × 45 cm). A: Elution profile of fraction A separated by DEAE-cellulose column chromatography. B: Elution profile of fraction B separated by the same chromatography.

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active fractions were further purified separately by gel filtration chromatography on Sephadex G-100. Two active fractions (A-1 and B-1) were obtained by the gel filtration, which were named as α - and β -hemolytic toxins (Fig. 1). Fraction A-2 showed proteolytic activity, whereas fraction B-2 showed no hemolytic or proteolytic activities.

The native toxins did not migrate by polyacrylamide gel electrophoresis. Then the molecular weights of the subunits were preliminarily determined by SDS-polyacrylamide gel slab electrophoresis. As a result, the α -hemolytic toxin was separated into three subunits, which were estimated to be approximately 79, 60 and 21 kDa. The molecular weight of the subunit of the β -hemolytic toxin was estimated to be approximately 50 kDa. The UV absorption spectrum of the α -hemolytic toxin showed a little absorbance at 280 nm. The both toxins, which were purified by the gel filtration on Sephadex G-100, were preheated at 37, 60 and 100°C for 60 min, and the activities were determined. As a result, the both toxins were stable at 37, 60 and 100°C for 60 min and the activities increased slightly with the lapse of time (Table 1). The reappearance of the thermostabilities was confirmed by further experiments. The toxins were also stable in the range of pH 5.5 to 8.5. The optimum temperature of the hemolytic activity was at 35°C, and the optimum pH of the activity was at pH 7.2.

On the contrary, the partially purified toxin, which was

obtained by ammonium sulfate fractionation with 0 to 40% saturation, was inactivated in a refrigerator for 1 or 2 weeks, however, the inactivated toxin was completely reactivated by the addition of phenol at a final concentration of 0.4%. From the results, it was assumed that the inactivation and reactivation of the toxin may be due to association and dissociation of the subunits of the toxins.

The qualitative analysis of the α -hemolytic toxin showed that the toxin contains a large amount of aldohexose and less amounts of aldopentose, aminosugar and phosphate, and protein was detected as a trace, whereas lipid were not detected. The α -hemolytic toxin was inhibited by Fe^{2+} to 35% at a concentration of 1 mM, and was also inhibited by acid phosphatase, α -D-glucosidase and α -amylase to 36, 12, 10%, respectively.

On the other hand, the α -hemolytic toxin lysed the erythrocytes of horse, rainbow trout, masu salmon, coho salmon and ayu at a concentration of 535 $\mu\text{g}/\text{ml}$ and the hemolytic activities were 1.9, 1.0, 2.0, 2.3 and 1.9 HU, respectively. The mortalities of juvenile white-spotted charr after intramuscular injections with the α -hemolytic toxin (32.8 $\mu\text{g}/\text{fish}$) and the β -hemolytic toxin (7.2 $\mu\text{g}/\text{fish}$) were 70 and 80% within 8 days, respectively. From these results, it was suggested that the both toxins are thermostable substances which may be released extracellularly from the cell surface components of the organism. Further purification are necessary to elucidate more detailed characteristics of the toxins.

Table 1. Thermostabilities of α - and β -hemolytic toxins of *V. anguillarum*

Temperature (°C)	Incubation time (min)	Hemolytic activity			
		α -hemolytic toxin		β -hemolytic toxin	
		$\Delta\text{OD}_{540}^{*1}$	HU ^{*2}	$\Delta\text{OD}_{540}^{*1}$	HU ^{*2}
4	60	0.837	1.66	0.921	1.80
	15	0.829	1.64	0.902	1.76
	30	0.830	1.64	0.905	1.77
37	60	0.832	1.65	0.904	1.77
	15	0.836	1.66	0.913	1.78
	30	0.837	1.66	0.915	1.79
60	60	0.839	1.66	0.921	1.80
	15	0.848	1.68	0.933	1.82
	30	0.865	1.71	0.942	1.84
100	60	0.886	1.75	0.952	1.86

*1 OD_{540} (total hemolysis) – OD_{540} (hemolysis, 0.85% NaCl/50 mM Tris-HCl buffer, pH 7.2).

*2 1 hemolytic unit = $\Delta\text{OD}_{540}/50\%$ hemolysis (horse red cells).

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