Studies on Pathogenic Properties of *Aeromonas liquefaciens*-II. Separation of Toxic Factors by Gel Filtration*

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In the previous paper¹⁾ it was reported that the inoculation of some motile strains of *Aeromonas* isolated from the infection of eels caused the hemorrhage and necrosis and finally the death of eels, which were usually observed in the natural infections, and that the cell free extract prepared after sonic treatment also could cause the same typical symptoms as those induced by the living organisms when it was subcutaneously or intramuscularly inoculated into animals. This crude toxic preparation was naturally supposed to contain various intracellular poisonous components besides the main toxic substance to eel. Actually it worked not only on eel but on mouse and guinea pig pathogenically, and its toxic activity was very complicated. For clearing the pathogenicity of *Aeromonas*, it would be necessary to know what kind of substance sourced from cell could cause each toxic reaction on the animals.

In the present study, fractionation of cell free preparation by gel filtration on Sephadex G-100 was undertaken as the first step for studies of identification or characterization of pathogenic substances of *Aeromonas*.

Materials and Methods

Preparation of crude toxic material. *A. liquefaciens* strain Y-62 was used. The culture media and methods were the same as described in the previous paper. Two-day cultures of the organisms on the agar plates (about 40 plates at a time) were harvested, washed twice with 0.1 M Tris-HCl buffer, pH 9.0 and resuspended in the same buffer at 400~500 mg/ml in wet weight. The cell suspension was sonicated at 10 kC by a sonic oscillator under cooling with ice water for 5 min. per 10 ml of the suspension. The sonicated material was centrifuged at 50,000×g for 60 min. The supernatant thus obtained was kept in a deep freezer at -20°C until use.

Gel filtration.²⁾ Sephadex G-100 (Pharmacia; Water regain, 12 ml/g; Particle size, 40~120 μ) was suspended in distilled water overnight, and rinsed with 0.1 M Tris-HCl buffer, pH 9.0. The columns used (3.3×35 cm or 2.3×60 cm) were made from glass tubing with a No. 2 glass filter at the bottom. The packed gel was washed with 200~400 ml of the buffer prior to the application of sample. Each 8~12 ml of the crude material containing 80~500 mg of protein was applied to the column and efflu-

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Fig. 1. Example of toxicity test on the guinea pig and eel skins.

ent was fractionated into 5ml in a tube at the room temperature from 13 to 20°C. For determining the void volume of the column, 3~5ml of 0.2% Blue Dextran 2000 (Pharmacia) was eluted under the same condition as mentioned above, and the eluant was colorimetric at 625m μ .

Determination of toxicity. Toxicities of each fraction for eel and guinea pig were determined by the skin test described in the previous paper. Inoculation example of eel and guinea pig is given in Fig. 1. Each 0.5ml of serial dilutions of toxic sample was inoculated into several points on the sides of the trunk of an eel as shown in Fig. 1. It seemed that inocula were often introduced into the subcutaneous tissues, as the common-

ly used intracutaneous injective needles were too big for the thin skin of eels. The reaction were read within 24 hours after inoculation. Characteristic lesions appeared at the site of inoculation with diluted toxic samples were local discoloration of the skin occasionally accompanied by hemorrhage. The intensity and area of discoloration decreased in parallel with increase of the dilution of the sample. When discoloration was clearly observable with the naked eye, reaction was regarded as positive.

In the case of guinea pig skin reaction, MHD (minimal hemorrhagic dose) and MND (minimal necrotic dose) were separately determined. The hemorrhagic reaction was regarded as positive when the longest diameter of hemorrhagic area was above 2mm, as the shape of the lesion was irregular. Around the necrotic lesion, nearly circular in shape, weak reddening was recognized. When the diameter of necrotic lesion, excluding the reddening area, was above 2mm, the reaction was regarded as positive.

MLD in mouse was also measured in the same way as described in the previous paper.

Determination of protein.⁹⁾ Protein concentrations of the samples were determined by the LOWRY's Folin-phenol method and bovine albumin was used as a standard.

Assay of hemolytic and proteolytic activities. (1) Hemolytic activity. Each 0.5 ml of serial dilution of the toxic sample was added to the equal amount of 2% horse erythrocyte suspension containing 0.01% marzonin in a small test tube, shaken well and incubated at 30°C for 24 hours. For determining the activity, the intensity of

red color of the supernatant was compared with that of control. The reciprocal of the highest dilution showing hemolysis was taken as the hemolytic titer.

(2) Gelatinase activity. The mixture of each 0.5 ml of 10% gelatin solution and twice serial dilutions of the sample was incubated at 30°C for 24 hours and cooled in ice water. When the mixture did not coagulate, the activity was judged to be positive. The reciprocal of the highest dilution showing liquefaction was taken as the titer of gelatinase activity.

(3) Hydrolysis of collagen and Azocoll Collagen fiber (native A. S. C.) and Azocoll (Az-8, E. p.) obtained from Dept. of Bact., N. I. H., Japan, were used. The mixture of toxic sample and collagen was incubated at 37°C for 2 hours and the destruction of the fibers was visually observed and ninhydrin positive substance produced was determined according to the method of KEMBLE *et. al.*⁴⁾

The mixture of Azocoll suspended in 0.05 M Tris-HCl buffer and the sample was incubated at 37°C for 1 hour. The red solution obtained by filtration was colorimetrically using *Clostridium welchii*'s κ -toxin as a standard.

(4) Hydrolysis of casein.⁵⁾ After toxic sample was mixed with 1% casein (Hammerstein) in 0.1 M Tris-HCl buffer and incubated for 2 hours at 30°C, 5% trichloroacetic acid was added. The mixture was then filtered and the optical density of the filtrate was read at 700 m μ . The activity was expressed as γ tyrosine released per ml sample.

Results

Toxicities of sonicated cell extract to eel, mouse and guinea pig have been demonstrated in the previous paper. Some other biological activities of the material

Table 1. Activities of two fractions separated by Sephadex gel and crude preparation.

Reaction	Fraction A	Fraction B	Crude sonic* extract
Guinea pig skin reaction			
Hemorrhage	+	—	
Necrosis	—	+	
Eel skin reaction	—	+	
Death of mouse	+	—	
Death of guinea pig	+	—	
Gelatinase	$\pm$	+	1: 320
Hemolysis for horse erythrocyte	+	$\pm$	1: 512
Hydrolysis of casein	—	+	30 γ Tyr/ml
Hydrolysis of collagen	—	—	0 mg Pro+Hyp/ml
Hydrolysis of Azocoll	—	+	1.2 U/ml
Absorption at 260 m μ	+	—	
Molish's reaction	+	$\pm$	

* Sonicated cell suspension was centrifuged at 50,000 $\times g$ for 60 min. and the supernatant fluid containing 43 mg/ml protein was used.

Fig. 2. Fractionation of sonic extract by gel filtration on Sephadex G-100 column (3.3×35 cm) with 0.1 M Tris-HCl buffer pH 9.0 as eluant.

Mark: Upper Protein concentration,
 —○— Mouse lethal toxicity,
 —●— Guinea pig hemorrhage,
 —△— Guinea pig necrosis,
 —▲— Eel necrosis.
 Lower — Absorption at 260 mμ,
 Absorption at 280 mμ,
 —○— Azocoll hydrolysis,
 — — Gelatinase.

Blue Dextran 2000 and the other with toxic peak for eel, being dispersed in Sephadex gel. Symptoms caused by these 2 fraction groups were apparently different from each other: one was visually noticed to be hemorrhage and the other mainly necrosis.

Fractions which killed mouse by intraperitoneal inoculation emerged in the same position as that of the first peak toxic to guinea pig. Inoculation of 1 ml of the original solution containing about 2 mg of protein also killed guinea pigs. Fractions No. 14~17 causing hemorrhage to the guinea pig skin and death of mice and guinea pigs and fractions No. 21~26 causing hemorrhage and necrosis to the eel skin and necrosis to the guinea pig skin were pooled and designated as Fractions A and B, respectively. On the lethality of these 2 fractions for eel, no definite results were obtained as shown in Table 2.

The result of subcutaneous injection of Fraction B into eels is shown in Plate I-1. The visual discoloration at the inoculation site of the skin after 12~18 hours developed to necrosis within 24 hours. A large amount of inoculation (0.4 ml/100 g

are given in Table 1. Ten ml of this preparation containing approximately 400 mg of protein was fractionated on Sephadex G-100 column. Each fraction obtained was initially examined for its toxicities to the animals and the titer of toxic fractions found was obtained by inoculating into animals with 2 or 3.2-fold serial dilutions. Hemolytic and proteolytic activities were also examined. The elution pattern of these activities are shown in Fig. 2.

In the eel skin test, a single elution peak was observed in fractions No. 23~25. Inoculation of the original solution of fractions No. 11~20 gave some slight change on the eel skin but it was negligible as compared with the clear reaction occurred by inoculation of fractions No. 21~27.

In the case of guinea pig skin test, 2 reaction positive peaks emerged in fractions No. 14~16 and fractions No. 23~24. The first peak was found to be associated with the void volume of the column which was measured by using

Table 2. Mortality of eels caused by effluents from Sephadex G-100 gel filtration.

Inoculum (mg of protein)	No. of eels inoculated	Mortality				
		within 24 hr	24-48 hr	3 days	after 3 days	survived
Fraction A						
0.6~1.0	6	0/6	2/6	1/6	0/6	3/6
1.1~5.0	3	0/3	1/3	0/3	1/3	1/3
Fraction B						
0.1~0.5	5	0/5	0/5	1/5	0/5	4/5
0.6~1.0	8	3/8	2/8	0/8	0/8	3/8
1.1~5.0	6	3/6	1/6	0/6	0/6	2/6
5.0~5.6	1	0/1	0/1	0/1	0/1	1/1

Body weight of eels: 70~120 g, Water temperature: 18~23°C.

body weight) caused edematous and swollen lesion containing infiltrated matters. Hemorrhage and congestion were also observed in and around the lesions. No definite distinction was recognized between the symptoms and those caused by inoculation of the crude sonic extract. The histological sections of inoculation site are shown in Plate I-2 and 3. Destruction of the muscle tissue was observed in the sections which were taken from the fish within 24 hours after inoculation. Simple hemorrhage and congestion were also noticed in the dermal and muscle tissues near the inoculation site. Toxicity of Fraction B to eel was estimated at 640 MND/ml.

Inoculation of Fraction A at the original concentration did not show any visible change at the inoculation site of eel skin, but some of eels died within the observation period of a week. Inoculation of more concentrated solution of the fraction or some attempts to elevate the toxicity are left for further study. These results show that the formation of regional symptoms of eels is due to a toxic factor contained in Fraction B.

In guinea pigs inoculated intracutaneously with Fraction A, only dermal hemorrhage and congestion appeared, and the lesion was formed irregularly along the capillary vessels at the inoculation site of skin 12~24 hours after inoculation. These hemorrhagic areas did not develop to edematous lesion containing purulent matter, but was recovered in a week forming a scab. The hemorrhagic activity of Fraction A to guinea pig was estimated at 160 MHD/ml.

On the other hand, inoculation of Fraction B caused discoloration of the inoculation site in 12~24 hours, which was round in shape and developed to necrotic lesion containing purulent matter under the epidermal tissue in 48 hours. The necrotic area was surrounded by weak reddening but the tone of the color and shape was different from that of hemorrhage caused by inoculation of Fraction A. The necrotic activity of Fraction B, estimated at 1,024 MND/ml, was considerably intensive, as compared with the hemorrhagic one. The large amount of inoculation (4~5 ml in total) of this fraction did not cause the death of guinea pig but only heavy necrosis

at the inoculation site. The lesions caused by these 2 fractions are shown in Plate II.

The death of mouse was caused by Fraction A. The animals died within 3 days after inoculation showing similar symptoms to those in the case of inoculation of crude toxic preparation. The visual changes of animals before their death were decrease of the activity and general prostration. This fact with the results of mortality of inoculated guinea pig shows that lethal factor to animals is contained in Fraction A.

Some other properties of Fractions A and B are summarized in Table 1.

A fairly large amount of the protein contained in sonic extract was eluted in association with Fraction A as shown in Fig. 2. A broad peak of protein was noticed at the position of Fraction B. The optical density at $260\text{m}\mu$ was higher than that at $280\text{m}\mu$ at the position of Fraction A, while the reverse was the case at the position of Fraction B. In addition, Fraction A showed fluorescence. These results suggest the existence of nucleic acid or its related substance in Fraction A⁶⁾. Result of MOLISCH's reaction shows that Fraction B hardly contained carbohydrate.

About the biological activities concerned, Fraction A contained hemolysin for horse erythrocyte and the trace of gelatinase, but no activity of hydrolyzing Azocoll and casein. While in Fraction B, hydrolyzing activity of gelatin, casein and Azocoll and slight hemolysin were detected. Neither of the fractions contained collagenase activity.

Gel filtration of the sonic extract on a longer Sephadex G-100 column ($2.3 \times 60\text{ cm}$) also gave the similar elution pattern.

Toxic fractions (No. 23 and 24) to eel were combined and concentrated by salting out with $(\text{NH}_4)_2\text{SO}_4$ at 50% saturation. The

precipitates (approximately 20 mg in total protein) dissolved in Tris-HCl buffer were re-gel filtered on the same column and followed by the longer one. The elution pattern is shown in Fig. 3. Peaks of necrotic toxicity to eel and guinea pig were not separable and a broad peak of protein was obtained at the same position.

Fig. 3. Re-gel filtration of necrotic fractions on Sephadex G-100 ($3.3 \times 35\text{ cm}$) with 0.1M Tris-HCl buffer pH 9.0 as eluant.

Mark: — · — Protein concentration,
—○— Guinea pig necrosis,
—●— Eel necrosis.

Discussion

The fraction eluted at the void volume of Sephadex G-100 column caused the death of mouse and guinea pig and hemorrhage to the guinea pig skin. Al-

though it should be necessary to examine whether these different toxic activities are due to the same substance or not, the toxic factor(s) responsible to all these symptoms was shown to have high molecular weight more than 10^6 .⁷⁾ On the other hand, necrotic factors for eel and guinea pig were eluted in the same fractions, dispersing in the gel. Rechromatography of the fractions on a longer column could not separate these toxic activities. From these results, it may be considered that the necroses of the both animals were caused by a single toxic component of smaller molecular size. For confirming this point, a further attempt to separate these activities is now being made.

Pathogenicity of Fraction A for eel has not been cleared: the inoculation with a larger amount of it into eel could not induced the definite mortality of the animal, nor the damage in the skin, while the symptoms in guinea pig and mouse were obvious, even when they received a smaller amount. It seemed that some other factor(s) in another fraction might participate in the death of eel and that, in the case of the eel, some invisible symptoms such as extension of capillary or increase in palpitation might have occurred as HOSHINA⁸⁾ indicated.

Both of Fractions A and B were found to contain some in vitro activities which were possibly thought to play some role in the destruction of the animal tissues. Concerning the hemolytic activity, LIU *et al.*⁹⁾ studied on pathogenesis of *Pseudomonas aeruginosa*, closely related to *Aeromonas* in the etiological position and indicated that extracellular hemolysin from the bacterium could cause the severe destruction of the rabbit skin, but very low mortality of mouse. The high lethality of mouse, on the other hand, was induced by another cellular fraction which was not reactive to the rabbit skin. In the present study, the relation between the hemolysin and the toxic factors for the animals seems to be reverse to that in the case of *Pseudomonas* and it would be impossible to consider that hemolysin in *Aeromonas* plays the similar pathogenic roles to that in *Pseudomonas*. However, it might be necessary to identify the toxic substances for the animals in relation with these in vitro activities.

Though there remain several problems to be solved, it is apparent from the present study that at least 2 different substances are involved in the pathogenesis of *Aeromonas* to eel, mouse and guinea pig, and that the toxic substance dispersed in the gel had great significance on symptoms of eels.

Summary

The 50,000×g centrifugal supernatant of sonicated cell suspension of *A. liquefaciens* strain Y-62 was gel filtered on Sephadex G-100 column. Factors causing the death of mouse and guinea pig, hemorrhage to the guinea pig skin passed through Sephadex gel without dispersing, while hemorrhage and necrosis in the eel skin and necrosis in the guinea pig skin were dispersed in Sephadex gel and these activities were not separable. These indicate that the sonic extract of the organisms contained

at least 2 different toxic substances.

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Explanation of plates I.

Reaction of eels 24 hours after intracutaneous injection of fractions No. 21-24 of gel filtration on Sephadex G-100.

1. Lesion of eels inoculated with fractions (Average protein concentration, about 1.5 mg/ml; Dilution, $\times 4 \dots \times 320$; Dose, 0.1 ml/point; Average body weight of eels, 80 g).

2. Section of the skin of an eel inoculated with 0.1 ml of fraction No. 24 (Protein concentration, about 2.5 mg/ml). $\times 250$

3. Section of the muscle of the same eel as that in Photo. 2. $\times 250$

Explanation of plates II.

Reactions of guinea pigs 24 hours after intracutaneous inoculation of 0.1 ml of serial dilutions of the effluents from gel filtration on Sephadex G-100.

1. Hemorrhagic reactions caused by the inoculation of fractions No. 14-16 (Average protein concentration, 2 mg/ml; Dilution, $\times 4 \dots \times 64$).

2. Necrotic reactions caused by the inoculation of fractions No. 21-24 (Protein concentration, 1.5 mg/ml; Dilution, $\times 4 \dots \times 64$).