Studies on Pathogenic Properties of *Aeromonas liquefaciens*-III. Some Chemical and Antigenic Properties of Toxic Factors*

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As previously reported¹⁾, gel filtration of the cell free preparation from 2-day cultures of *A. liquefaciens* gave 2 toxic fractions: one caused hemorrhage to guinea pig and death to mouse and the other necrosis both to eel and guinea pig. In the present study, some chemical and antigenic properties of these toxic factors were compared with each other to know whether the different toxic activities found in the same fraction are ascribable to a single component or not.

Materials

Seeds for cultures were taken from several storages of *A. liquefaciens* strain Y-62. Sonic extracts were prepared from 2-day-old plate cultures. The extracts were filtered through Sephadex G-100. The effluents of the gel filtrations and the crude sonic extracts were used as toxic samples.

The antisera used for neutralization of toxic activity were prepared by immunizing rabbits, weighing about 3 kg, with sonic extracts detoxified by addition of 0.5% formalin and incubation at 30°C for more than 10 days. Immunization was made by the following schedule: a mixture of each 0.1 ml of the antigen and saturated Al_2O_3 solution (7 mg/ml) was inoculated subcutaneously in the neck of the animal 3 times at intervals of 2 days. These inoculations were followed by the second series of inoculations with the antigen of 0.5 ml at one time and by the third ones with 1.0 ml, without adjuvant. These groups of inoculations were given at intervals of 20 days. Two weeks after the final injection, the rabbits were bled to death. The sera were added with 0.01% of marzonin as a preservative.

Experimental and Results

Detailed procedures for determination of toxicity to each animal and examination of biological properties such as proteolytic and hemolytic activities were already described^{1,2)}.

Dialysis and salting out of toxic factors. Sonic extracts were packed in cellulose tubes and dialyzed against 0.02 M Tris-HCl buffer, pH 9.0 or 0.02 M phosphate buffer, pH 7.0 at 4°C overnight. Toxicities of the dialysates were tested by using eel, guinea pig and mouse. Results shown in Table 1 indicate that the toxic factors are undialyzable.

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Table 1. Dialysis of toxic factors*

Factor	Toxicity**	
	before dial.	after dial.
Guinea pig necrosis (MND)	16,000	15,360
Eel necrosis (MND)	10,240	8,960
Mouse lethality (MLD)	800	1,120

* The sample for guinea pig and eel skin test and that for mouse lethality test were dialyzed against 0.1 M Tris-HCl buffer, pH 9.0 and 0.1 M phosphate buffer, pH 7.0, respectively.

** Toxicity per ml sample was multiplied by the total volume (ml) of solution in a cellulose tube.

Fractionation of sonic extracts with $(\text{NH}_4)_2\text{SO}_4$ was made as follows. The extracts dissolved in 0.1 M Tris-HCl buffer, pH 9.0 were added with saturated $(\text{NH}_4)_2\text{SO}_4$ solution, allowed to stand for 3–24 hours at 4°C and centrifuged at 10,000 rpm for 15 min. The supernatants were again added with saturated $(\text{NH}_4)_2\text{SO}_4$ solution and the precipitates formed were centrifuged off similarly. Thus, the precipitates at 0–25%, 25–40%, 40–50% and 0–50% saturations were obtained and dissolved in the buffer for determination of toxicities. Results obtained are summarized in Table 2. The precipitates formed at 40–50% saturation exhibited the highest necrotic activities for both eel and guinea pig. At 50% saturation, almost all of the original toxicities including the hemorrhagic activity for guinea pig and the lethality for mouse were recovered in the precipitate. Therefore, salting out at 50% saturation and dialysis were mainly used for concentration of test samples.

Table 2. Salting out of the toxic factors at different saturations.

Saturation (%)	Recovery in precipitate (%)*			
	protein	guinea pig necrosis	eel necrosis	mouse lethality
0–25	0.9	0–10		
25–40	45	20–45		
40–50	50	60–82	60–85	
0–50	94	100 (100**)	80–100	100

* The range of the recovery was obtained from 3–5 times trials.

** Guinea pig hemorrhage.

Some factors influencing the stability of toxins. (1) Heating, freezing and lyophilization. Small test tubes each containing 1 ml of the sonic extract which had been kept at room temperature (22°C) for about 30 min. were put into water baths adjusted to 40, 50, 60, 80 and 100°C, respectively. Immediately after being heated for 5, 15, 30 and 60 min., the samples were cooled in ice water for 1–2 hours and examined for their toxicities. The results are given in Table 3. The necrotic activities for guinea pig and eel and gelatinase activity disappeared almost completely by heating at 50°C for 15 min. On the other hand,

Table 3. Stability of the toxic factors in sonic extracts.

Treatment	Toxicity								Gelatinase	
	mouse		guinea pig				eel		titer %	
	MLD/ml	%	MHD/ml	%	MND/ml	%	MND/ml	%		%
Heating:										
Control (not heated)	512	100	1,600	100	5,120	100	1,280	100	320	100
40°C 15 min.	512	100	1,600	100	680	12			32	10
50 15	128	25	500	31	0	0	0	0	10	1
60 5	64	13	320	20					0	0
15	32	6	160	10	0	0	0	0	0	0
30	32	6	160	10					0	0
60	32	6	160	10					0	0
80 15	16	3	50	3	0	0			0	0
100 15	32	6	50	3	0	0	0	0	0	0
Freezing:										
Control	512	100	5,000	100	50	100	120	100		
-70°C	512	100	5,000	100	50	100	120	100		
Lyophilization:										
Control	640	100			80	100				
Lyophilized	0	0			0	0				

neither the lethal activity for mouse nor the hemorrhagic one for guinea pig was influenced by heating at 40°C for 15 min. They were reduced to 25–30% of the control by heating at 50°C, and remained to some extent even after heating at 100°C. These results indicate that the necrotic factors are more heat-labile than the others. However, distinct differences were not observed in heat-stability between the necrotic factors for guinea pig and eel, or between the lethal factor for mouse and the hemorrhagic one for guinea pig.

The effect of freezing on the stability was investigated. The samples of sonic extracts and fractions from gel filtration were rapidly frozen at -70°C with dry ice in acetone and stored at -20°C in a deep freezer for more than 5 days. All the toxic activities for eel, guinea pig and mouse remained unchanged after the treatment, and the initial toxicities were maintained for 2 months, when the samples had been kept frozen without melting. However, a considerable decrease of toxicity occurred, when freezing and melting were repeated several times during storage. For example, toxicity of the sonic extract estimated to be 16,000 MND/ml in the guinea pig skin test, decreased to 2,560–5,120 MND/ml after 2-month storage at -20°C, during which melting of the frozen sample at room temperature was repeated 4 times.

The effect of lyophilization was examined as follows. The supernatant from centrifugation at 100,000×g for 90 min. of the sonicated cell suspension was dialyzed against distilled water at 5°C overnight. The dialysate which retained 80 MND/ml of toxicity in the guinea pig skin test was rapidly frozen at -70°C and lyophilized. The resulting powder dissolved in 0.02 M Tris-HCl buffer, pH 9.0 showed no toxicity. The eel skin test of the

sample lyophilized in the same way as the above also indicated that the treatment caused complete destruction of the toxic factor.

(2) pH. Five ml of the $50,000 \times g$ centrifugal supernatant packed in a cellulose tube was dialyzed against each 250 ml of buffer solutions adjusted to pH 6.0, 7.0, 8.0 and 9.0, respectively. The dialysis was carried out at 4°C for 3 days during which the buffer solutions were renewed 4 times. Decreases in toxicities of the dialysates were varied with pH as shown in Table 4. The hemorrhagic factor for guinea pig and the lethal one for mouse were more stable in acid side than in alkaline side. On the other hand, decrease of necrotic toxicities for guinea pig and eel was less in alkaline condition.

Table 4. Effect of pH on the stability of toxic factors.

pH	Toxicity**				
	guinea pig			eel	mouse
	MHD or MND /ml	n.r.§	h.r.§	MND/ml	MLD/ml
Control*	5,120	+	+	2,560	160
6.0	640	—	+		80
7.0	80	trace	+	80	80
8.0	80	+	trace	640	
9.0	1,280	+	—	640	0

* Supernatant from centrifugation of sonicated cell suspension at $50,000 \times g$ for 60 min.

** Each 5 ml of the above sonic extracts was dialyzed against the following buffer solution at 4°C for 3 days: pH 6.0 and 7.0, 0.1 M phosphate buffer; pH 8.0 and 9.0, 0.1 M Tris-HCl buffer. The buffers were renewed 4 times during the period.

§ n.r.: necrotic reaction, h.r.: hemorrhagic reaction.

(3) Proteolytic enzymes. Destruction of both hemorrhagic and necrotic factors by proteolytic enzymes such as trypsin and chymotrypsin was examined. Two toxic fractions obtained by gel filtration were concentrated by salting out with $(\text{NH}_4)_2\text{SO}_4$ at 50% saturation.

Table 5. Effect of trypsin and chymotrypsin on the toxicity.

Enzyme	Guinea pig skin reaction	
	necrosis MND/ml	hemorrhage MHD/ml
Control		
Untreated	640	32
After incubation	160	20
Trypsin	20	20
Chymotrypsin	10	20

Toxin: Supernatant from centrifugation at $50,000 \times g$ for 60 min. was gel-filtrated through Sephadex G-100 and the fractions No. 14-16 and No. 22-24 were used as samples¹⁾. Enzyme: $100 \mu\text{g/ml}$. Buffer: 0.02M phosphate, pH 7.0. Digestion: at 37°C for 60 min.

The precipitates dialyzed against 0.02 M phosphate buffer, pH 7.0 were used as toxic samples. Each 0.9 ml of toxic samples was mixed with 0.1 ml of trypsin or chymotrypsin solution prepared by dissolving the enzyme in the above buffer at the concentration of 1 mg/ml. The mixtures were incubated at 37°C for 1 hour. As a control, the sample solution was incubated without addition of the enzymes. Toxicities of the incubated samples were measured by the guinea pig skin test. As shown in Table 5, the necrotic activity was lowered to 1/8 of the control after trypsin treatment and to 1/16 by chymotrypsin. While in the case of hemorrhagic activity, no influence of these enzymes was observed. The inoculations of these enzyme solutions at this concentration hardly affected the guinea pig skin.

Antigenicity of toxic factors. In the guinea pig skin test, the toxicity of the sonic extract (protein concentration: approximately 56 mg/ml) added with 0.5% formalin gradually decreased from 10^4 to 10^2 MND/ml during the incubation period of 10 days at 30°C, as shown in Fig. 1. However, the reaction appeared on the skin 10 days after injection

Fig. 1. Formation of sonic extract toxoid of *Aeromonas* by addition of 0.5% formalin. The supernatant of centrifugation at 10,000 rpm for 15 min. (protein concentration: approximately 56 mg/ml) was used and incubation was carried out at 30°C. Toxicity was determined by guinea pig skin test.

of the extract was largely hemorrhagic, and necrosis was hardly observed. Neutralization test was done using the antisera prepared as described above. Each 0.5 ml of diluted antisera (2-, 4- and 8-fold dilutions) was mixed in a small test tube with the equal amount of 2-fold serial dilutions of the sonic extracts and allowed to stand at room temperature for 30 min. The mixtures were inoculated into guinea pigs and eels in parallel with the serial dilutions of the original sonic extracts. The neutralization of activities with the antisera

Table 6. Neutralization of toxic activities with antisera prepared by using sonic extract as an antigen.

Reaction	Titer of toxic activity*				Toxicity of original sample	Neutralization**
	dilution of antiserum					
	1:2	1:4	1:8	no serum		
Guinea pig skin reaction						
Hemorrhage	64	64	64	64	320 (MHD/ml)	0 (MHD)
Necrosis	16	32	64	640	3,200 (MND/ml)	200 (MND)
Eel skin reaction	8	16	32	64	640 (MND/ml)	80 (MND)
Gelatin liquefaction	4	8	16	128	128	16
Hemolysis of horse erythrocyte	512	128	128	128	128	0

* Reciprocals of the highest dilution which caused reaction when animal was injected with toxin-antiserum mixture. Protein concentration of the original toxic sample was about 50 mg/ml.

** Toxicity or titer neutralized with 1 ml of antiserum.

was measured by comparing toxic titers of the mixtures with those of the original toxic samples. The results are given in Table 6. The hemorrhagic activity for guinea pig was unaltered by addition of the antiserum, while the necrotic activity for eel and guinea pig decreased by the treatment. The gelatinase activity was also inhibited by the antiserum. The hemolytic titer in the table showed no influence of the antiserum. However, as the antiserum itself seemed to have some lytic effect on erythrocytes, there remains a possibility that an antihemolytic substance is slightly produced in the serum. From these results it is evident that the toxoid of the factor causing the guinea pig necrosis was formed by addition of 0.5% formalin and the necrotic toxicity for both eel and guinea pig was neutralized with the antiserum. As compared with these antigenicity shown in the necrotic factors, no remarkable antigenic property of hemorrhagic factor for guinea pig was observed.

Discussion

No remarkable differences in the chemical and antigenic characteristics were recognized between the eel necrotic and the guinea pig necrotic factors and between the guinea pig hemorrhagic and the mouse lethal factors. While contrasts of characteristics were observed between the 2 pairs of factors. These chemical and antigenic characterization confirmed the qualitative differences between the toxic factors eluted separately in gel filtration, but could not clarify yet whether toxic activities found in the same fractions were expressed by different cellular substances of similar molecular size or by a single component.

Generally, bacterial toxins are classified into 2 types by their chemical, physical and antigenical properties: one of them is protein in nature and the other a complex composed of polysaccharide, protein and lipid. According to PANOS and AJL³⁾ or PILLEMER and ROBBINS⁴⁾, the former type of toxin is generally heat-labile, undialyzable, destroyed by the exposure to proteolytic enzymes, detoxified by addition of formalin and neutralized with

the antisera prepared from the toxin or toxoid. While the latter type has the almost contrary characteristics. From these criteria, it may be considered that the necrotic factors for guinea pig and eel in the present study are protein and the hemorrhagic factor for guinea pig and the lethal factor for mouse are complex compound rather than simple protein. In addition, these toxic factors are probably included in the group of endotoxin, because their toxicities are not so intensive as those of exotoxin and they are obtained from somatic preparation and because other gram negative families related to *Aeromonas* are known as producers of endotoxins^{5,6)}.

Summary

The examination of some chemical and antigenic properties of the toxic factors of *A. liquefaciens* indicated that there were no remarkable differences in their characteristics between the necrotic factors for guinea pig and eel, and between the hemorrhagic factor for guinea pig and the lethal factor of mouse. The necrosis may be caused by bacterial protein, while the hemorrhage and the death may result from more complicated cellular substance.

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References

- 1) T. SHIMIZU: This Bull., **35**, 55-66 (1969).
- 2) T. SHIMIZU: *Ibid.*, **35**, 163-172 (1969).
- 3) C. PANOS and S. J. AJL: *Ann. Rev. Microbiol.*, **17**, 297-328 (1964).
- 4) L. PILLEMER and K. C. ROBBINS: *Ann. Rev. Microbiol.*, **3**, 265-288 (1949).
- 5) C. E. LANKFORD: *Ann. N. Y. Acad. Sci.*, **88**, 1203-1212 (1960).
- 6) R. S. BERK: *J. Bacteriol.*, **88**, 559-565 (1964).