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Partial Characterization of Aerolysin, a Lytic Exotoxin from *Aeromonas hydrophila*

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Conditions are defined for the production, in high titers, of an extracellular hemolytic toxin of *Aeromonas hydrophila*, here termed "aerolysin." Substantial purification of the toxin was accomplished by means of salt fractionation, dialysis, and gel filtration, with a yield of 24% of the starting activity. Analysis of the product by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed a single, heavy protein band and a number of faint protein bands. The estimated molecular weight of the heavy band (50,000) was in close agreement with that (53,000) of the substance responsible for hemolytic activity as determined by gel filtration. Purified aerolysin is a labile substance, apparently protein. It is not inactivated by any of several proteases under the conditions employed nor is it inhibited by any of several lipids tested. About 0.1 μg administered to mice intravenously is lethal. The physical properties of aerolysin show considerable resemblance to those described for the exotoxin of *Pseudomonas aeruginosa*.

A substantial body of knowledge regarding the nature and properties of cytolytic toxins produced by gram-positive bacteria has accumulated. Much less information is available for comparable products of gram-negative bacteria. Among the latter, extracellular lytic agents of *Aeromonas hydrophila* (13, 14), *Acinetobacter calcoaceticus* (6, 7), and *Vibrio parahaemolyticus* (10) have recently been examined. This report describes the production, partial purification, and partial characterization of an extracellular lytic agent of *Aeromonas hydrophila*. For simplicity, and in agreement with a long-used nomenclatural convention, we refer to this agent as "aerolysin."

MATERIALS AND METHODS

Organism. *A. hydrophila* strain 38 was kindly supplied by W. Scharmann. It was maintained in nutrient broth.

Cultivation and measurement of growth. The basal culture medium consisted of 220 ml of yeast extract diffusate prepared as described earlier (3), 20 g of Casamino Acids (Difco Laboratories, Detroit, Mich.), 33 μg of thiamine, 1.2 mg of nicotinic acid, and water to make volume to 1 liter. The pH was adjusted to 7.2, the medium was divided equally between two 2-liter Erlenmeyer flasks, and sterilization was carried out for 20 min at 123 C.

Each flask was inoculated with 5 ml of a nutrient broth culture that had been incubated at 37 C until it attained an optical density (650 nm) of about 1.0. The flasks were incubated at 37 C on a rotary shaker operating at 220 cycles per min. Growth was measured as optical density in a Zeiss spectrophotometer with cuvettes of 10-mm light path and light of 650 nm. One growth unit is defined as that turbidity giving an optical density of 1.0.

Estimation of hemolytic activity. Test solutions were diluted in 0.145 M NaCl-0.01 M tris(hydroxymethyl)aminomethane (Tris; pH 7.2)-0.2% gelatin. Volumes of toxin dilutions decreasing by about 25% were delivered into tubes (12 by 75 mm), and the volume of all tubes was brought to 1 ml by addition of the same diluent. To each tube was added 1 ml of washed rabbit erythrocytes suspended in 0.145 M NaCl-0.01 M Tris, pH 7.2. The density of the erythrocyte suspension was adjusted to give an absorbance of 0.8 at 545 nm when complete lysis occurred. After mixing, the tubes were incubated at 37 C for 30 min and then briefly centrifuged. The percentage of hemolysis was estimated from the color of the hemoglobin in the supernatants as compared with that of standards. One hemolytic unit (HU) is that amount of test material needed to produce 50% hemolysis under the conditions described. A solution of aerolysin assayed with erythrocyte suspensions prepared from blood specimens from several rabbits showed the same hemolytic activity.

SDS-polyacrylamide gel electrophoresis. Molecular weight was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis according to the conditions of Weber and Osborn (12), using a 10% acrylamide system with model 12 Canalco equipment (Canalco, Rockville, Md.). The gels were stained with Coomassie brilliant blue (Colab Laboratories, Glenwood, Ill.) and destained as described by Weber and Osborn (12).

Reagents. Sodium ribonucleate (yeast) was purchased from Schwarz/Mann (Orangeburg, N.Y.),

phosphatidylcholine and cardiolipin were from Sylvana Chemical Co. (Orange, N.J.), phosphatidylethanolamine and phosphatidylserine were from Supelco, Inc. (Bellefonte, Pa.), sphyngomyelin was from General Biochemicals (Chagrin Falls, Ohio), cholesterol and Pronase were from Calbiochem (La Jolla, Calif.), trypsin and papain were from Worthington Biochemical Corp. (Freehold, N.J.), chymotrypsin and bovine plasma albumin were from Armour Pharmaceutical Co. (Chicago, Ill.), subtilisin and ovalbumin were from Sigma Chemical Co. (St. Louis, Mo.), albumin, alpha-globulin, and beta-lipoprotein fractions of human serum were from Nutritional Biochemicals Corp. (Cleveland, Ohio), and cytochrome *c* was from Boehringer and Soehne GmbH (Mannheim). The chloroform-methanol extract of brain was prepared by the method of Mueller et al. (9), and partially purified brain ganglioside was prepared by the method of Van Heyningen and Miller (11).

RESULTS

Production of aerolysin. The yeast diffusate-casein hydrolysate medium yielded heavy growth, about 10 growth units/ml, and aerolysin titers of 20 to 50 HU/ml. When one-tenth volume of a sterile 10% solution of sodium ribonucleate (yeast) was added to the medium at the time of inoculation, about twice as much growth (18 to 23 growth units) resulted, and aerolysin titers of 300 to 1,500 HU/ml were regularly obtained. Thus, sodium ribonucleate, although only doubling the growth, profoundly increased the amount of aerolysin produced. The course of growth and lysin production are illustrated in Fig. 1, which shows that the maximal amount of lysin was present shortly before the growth was maximal and that the lytic activity disappeared on prolonged incubation.

Purification. Appreciable purification of *A. hydrophila* hemolysins was reported by Wretlind et al. (14), using isoelectric focusing as a principal step. We applied this technique to the purification of material that had previously been salt-fractionated and subjected to Sephadex chromatography. The active material, however, precipitated at or near its isoelectric pH, about 4.8, and this was accompanied by disappearance of 70% of the input hemolytic activity. The specific activity of the recovered aerolysin was the same as that of the input material. Isoelectric focusing was also done in a 0 to 6 M ethylene glycol gradient in place of a 0 to 50% sucrose gradient. Very little precipitation occurred, but the recovery of activity was as low as that with sucrose and was considered unsatisfactory.

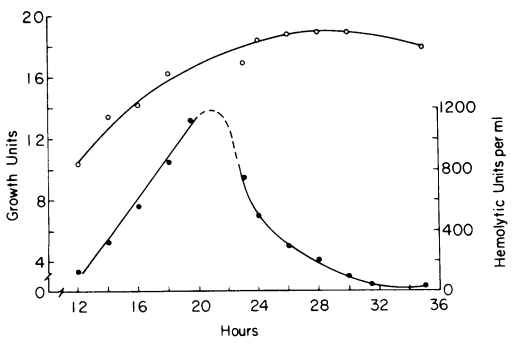

FIG. 1. *Growth and aerolysin titers as a function of time. The data are from two cultures, one of which was sampled between 12 and 23 h and one between 24 and 35 h. Samples were assayed for growth* (○) *after which they were centrifuged, and the supernatants were assayed for hemolytic activity* (●).

The following, relatively simple purification procedure was evolved. To 400 ml of culture supernatant was added, with stirring, 224 g of ammonium sulfate. After solution was complete, the mixture was allowed to stand at 4 C overnight. The precipitate was collected by centrifugation and extracted with 2.5 ml of 0.3 M sodium borate (pH 8.2) for 5 min at 20 C. The mixture was centrifuged for 7 min at 15,000 × *g*, and the supernatant fluid was discarded. Under these conditions the supernatant fluid contained extracted impurities but no aerolysin, presumably because sufficient ammonium sulfate was entrained in the precipitate to render the aerolysin insoluble in the first extraction. The sediment was extracted with a second 2.5-ml portion of the same borate buffer and recentrifuged, and the supernatant fluid was fractionated on a column (25 by 320 mm) of Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) in the presence of 0.03 M sodium borate (pH 8.2) and 0.1 M KCl at 4 C. The flow rate was 0.3 to 0.7 ml/min, and the volume of fractions collected was 4 to 5 ml. The hemolytic activity was always found in the second protein peak, but the two peaks were not always as well separated as those shown in Fig. 2. Usually, all the fractions were discarded except those comprising the center and right limb of the second peak. The latter were pooled and dialyzed for 16 h against 4 liters of cold, distilled water. The dialyzed solution was either freeze-dried or precipitated by dialysis against several hundred milliliters of 80% saturated ammonium sulfate, and stored as a suspension in a small volume of 80% saturated ammonium sulfate. The recoveries and specific activities at each step are shown in Table 1.

Nature of the purified product. The white powder obtained by the procedure just described was largely insoluble in water buffered at pH 5.0, and poorly soluble at pH 7.0. Clear

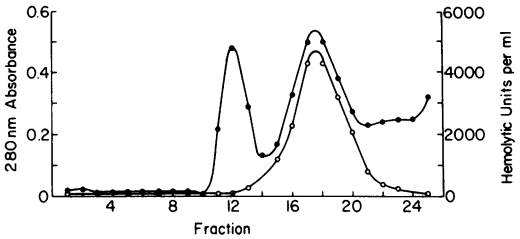

FIG. 2. *Distribution of hemolytic activity* (O) *and protein as* A_{280} (●) *among fractions from a Sephadex G-100 column. About 2.5 ml of solution containing a total of 90,000 HU was placed on the column; all of the activity was recovered in the fractions. In this experiment fractionation was done in the presence of 0.1 M sodium phosphate (pH 6.0) and 0.25 M NaCl.*

solutions at a concentration of 1 mg/ml were obtained when 0.03 M borate (pH 8.2) was used. The latter solutions had an absorbancy at 280 nm (A_{280}) of about 1.8/mg and a specific activity of 12,500 to 22,000 HU/mg, depending on the lot.

The ultraviolet light absorption of the product (Fig. 3) is typical for proteins, and the A_{280}/A_{260} of 1.59 indicates that the product is free of appreciable amounts of nucleic acids despite the large quantities of ribonucleate used in the growth medium.

Polyacrylamide gel electrophoresis in the presence of SDS yielded a pattern showing a single heavy protein band and some five faint bands (Fig. 4). The intensity of the staining suggested that the main band accounted for about 90% of the total protein.

Molecular weight. Mobility in SDS-polyacrylamide gels was calculated as the ratio of distance of migration of test material to that of cytochrome *c*. The mobilities of bovine plasma albumin, ovalbumin, and cytochrome *c* were plotted against their molecular weights on a logarithmic scale. The molecular weight of the heavy band shown in Fig. 4 was determined from its mobility on the standard curve as 50,000.

Molecular weight was also determined by gel filtration according to the principles described

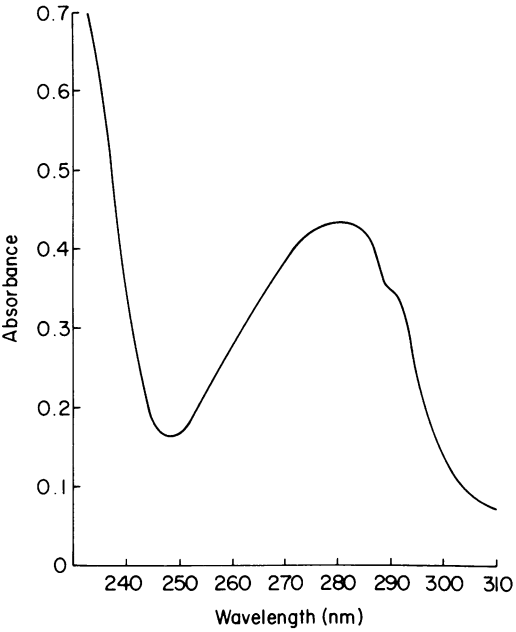

FIG. 3. *Ultraviolet absorption spectrum of a solution containing about 0.24 mg of purified aerolysin per ml of 0.03 M sodium borate, pH 8.2. Scanning was done in a Cary model 14 recording spectrophotometer using cells of 10-mm light path.*

by Andrews (1), using a Sephadex G-100 column (25 by 320 mm), 0.03 M sodium borate (pH 8.2), and 0.1 M KCl. Ovalbumin and bovine serum albumin were used as standard proteins. The elution volume of the peak of hemolytic activity corresponded to a molecular weight of 53,000.

Lability. A stock solution containing 1 mg of purified aerolysin per ml of 0.03 M sodium borate (pH 8.2) was diluted in each of a series of buffers to contain about 100 HU/ml. Portions of these solutions were allowed to stand for 1 h at 0, 20, 37, and 50 C, and they were then titrated for hemolytic activity. The activities found are expressed (Table 2) as a percentage of the hemolytic activity of the solutions kept at 0 C. It can be seen that small losses occurred at 20 C

TABLE 1. *Purification of aerolysin*

Stage	Vol (ml)	HU/ml	Total HU	Percent starting activity	A_{280}/ml	Total A_{280}	HU/A_{280}
Culture supernatant	400	1,450	580,000	100			
Ammonium sulfate precipitate	10	58,000	580,000	100	21	210	2,760
Second borate extract	2.5	165,000	413,000	71	32.5	81.3	5,100
Pooled Sephadex fractions	17.2	17,000	293,000	51	0.85	14.6	20,000
Dialyzed Sephadex pool	25	10,000	250,000	43	0.56	14.1	18,000
Freeze-dried product (7.1 mg)			140,000	24		13.0	11,000

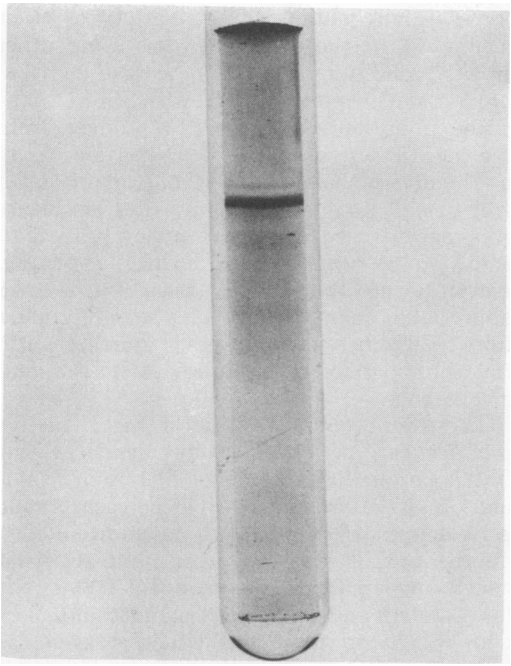

FIG. 4. *Pattern produced by SDS-polyacrylamide gel electrophoresis of 12 μg of purified aerolysin. The dark zone at the top of the gel is dye that was not removed by destaining; a similar zone was present also in control gels to which no protein had been added.*

TABLE 2. *Lability of aerolysin*

pH	Buffer	Hemolytic activity[a] at:		
		20 C	37 C	50 C
4.2	0.01 M sodium phosphate with citric acid	67	49	<5
5.2	0.01 M sodium phosphate with citric acid	89	67	<5
6.2	0.1 M sodium phosphate	83	61	<5
7.2	0.01 M Tris	83	61	<5
8.2	0.03 M sodium borate	89	<5	<5

[a] Figures indicate hemolytic activities found in solutions that had stood at indicated temperature and pH for 1 h as compared with hemoltyic activities of control solutions, and expressed as percent of the latter.

at all pH values tested. At 37 C the losses were greater, and at 50 C no residual activity was detected within the range measured.

Aerolysin that had been freeze-dried and stored dry at room temperature retained full activity for more than 7 weeks, as did aerolysin stored as a precipitate under ammonium sulfate. A solution containing 0.75 mg of aerolysin per ml of 0.01 M sodium borate (pH 8.2) retained full activity at −10 C for a 10-day period during which it had been thawed and refrozen three times.

Effect of divalent cations. A solution of purified aerolysin was titrated, by using the toxin diluent described above, when it was (i) unmodified, (ii) modified to contain 0.01 M $MgCl_2$, (iii) modified to contain 0.01 M $CaCl_2$, and (iv) modified to contain 0.01 M disodium ethylenediaminetetraacetate (EDTA). The titers obtained in the presence of the divalent cations were 10 to 15% lower than that of the control; EDTA reduced the activity by about 25%.

Effect of proteolytic enzymes. Several concentrations each of papain, trypsin, chymotrypsin, Pronase, and subtilisin were mixed with portions of purified aerolysin diluted in 0.85% NaCl buffered with 0.01 M Tris (pH 7.2) to contain about 100 HU/ml. After 1 h at 20 C the solutions were assayed for hemolytic activity (Table 3). Papain not only failed to inactivate aerolysin at any concentration tested, but at high concentration it apparently protected aerolysin against a 33% inactivation occurring in the absence of added enzyme. Similar results were obtained in experiments in which the other four proteases were used. Failure of inactivation by Pronase and trypsin was also observed when the aerolysin concentration was increased 150-fold, i.e., with a substrate concentration of about 1 mg/ml. Sephadex (G-100) gel filtration of Pronase-treated aerolysin yielded hemolytic activity only in approximately the same elution volume as that of untreated aerolysin. From this, it is deduced that no active split products were present.

Inhibition studies. The biological activities of a number of cytolytic toxins are known to be inhibited by lipids (2). For purposes of comparison, a series of lipids, or lipid-containing preparations, and normal serum and serum fractions were tested for capacity to inhibit the hemolytic activity of aerolysin. Chloroform solutions of lipids were dried as a film on the lower portion of test tubes (21 by 150 mm). Dispersions of 1 mg/ml were prepared, with the aid of a Vortex mixer, in 0.85% NaCl containing 0.01 M Tris, pH 7.2. Decreasing quantities of test substance

TABLE 3. *Effect of papain on hemolytic activity of aerolysin*

Aerolysin	Papain (μg)	HU found/ml
100 HU	100	100
100 HU	10	92
100 HU	1	67
100 HU	0	67

were allowed to interact for 10 min at 20 C with 3 HU of aerolysin in 0.85% NaCl-0.01 M Tris (pH 7.2)-0.1% gelatin. One-milliliter amounts of 0.7% washed rabbit erythrocytes were added, and after 10 min at 37 C the mixtures were centrifuged and the degree of lysis was estimated. The results (Table 4) show that none of the purified lipids inhibited, nor did a crude mixture of lipids extracted from brain. Normal sera and the alpha-globulin and beta-lipoprotein fractions, but not the albumin fraction, of normal serum showed some capacity to inhibit. It is notable that osmotically prepared rabbit erythrocyte membranes were inhibitory.

Mouse lethality. Swiss mice, strain CFW, weighing about 14 g were injected intravenously with decreasing concentrations of purified aerolysin. The volume injected was 0.2 ml, and the diluent was sterile 0.85% NaCl containing 0.1% gelatin. Mice that survived longer than 24 h were counted as survivors. The results (Table 5) indicate that the minimal lethal dose was about 0.1 μg. The survival time varied inversely with the quantity of aerolysin administered.

DISCUSSION

A significant feature of the present study is the description of a medium that consistently yields aerolysin in high titers in comparison to those usually obtained in a variety of other media, or to those obtained by using other growth conditions (13). The course of lysin production in relation to growth (Fig. 1), also differs from that seen earlier (13). Judging from the specific activity of purified aerolysin, it can be calculated that under optimal conditions of growth and toxin production a liter of culture supernatant may contain as much as 50 mg of aerolysin. Hence, if losses during purification are not too great, it should be possible to recover appreciable quantities of highly active product from relatively small volumes of starting material. This possibility is borne out by the data given above.

The results indicate that a highly active product can be obtained by means of salt fractionation, dialysis, and gel filtration. Examination of the product by SDS-polyacrylamide gel electrophoresis revealed, in addition to a number of faintly stained bands, a heavy band which appeared to make up about 90% of the total protein and which corresponded in molecular weight to that of the active agent as estimated by Sephadex gel filtration. The product, although demonstrably containing other protein contaminants, is useful for some kinds of experiments and also as material for further purification provided methods can be found that are not accompanied by unacceptably large losses in activity.

The molecular weight and lability of the active substance suggest that it is a protein. Further evidence for this belief is provided by the ultraviolet absorption spectrum of the purified product. In this context it is notable that treatment of aerolysin with none of a series of proteolytic enzymes resulted in its inactivation. It may be, however, that different results would have been obtained had other conditions been used, particularly in view of the fact that Wretlind and co-workers (14) reported aerolysin to be sensitive to trypsin and papain.

The physical behavior and biological proper-

TABLE 4. *Results of inhibition studies*

Substance	Amount required to inhibit two-thirds of the test amount[a] of aerolysin (μg)
Phosphatidyl choline	>500
Phosphatidylethanolamine	>500
Phosphatidylserine	>500
Diphosphatidylglycerol	>500
Sphingomyelin	>500
Partially purified brain ganglioside	>500
Chloroform-methanol extracted brain lipids	>500
Cholesterol	>500
Human serum	80[b]
Horse serum	100[b]
Albumin fraction of human serum	>500
Alpha-globulin fraction of human serum	200
Beta-lipoprotein fraction of human serum	500
Erythrocyte membrane suspension[c]	50[b]

[a] Three hemolytic units.
[b] Measured as microliters.
[c] Rabbit erythrocyte membranes were prepared osmotically as described by Bernheimer et al. (3).

TABLE 5. *Lethality of aerolysin for mice*

Aerolysin (μg) per mouse	No. dead/no. injected	Average survival time (min) of mice that died
4	2/2	1
2	2/2	2
1	4/4	68
0.5	4/4	90
0.25	4/4	107
0.125	4/4	180
0.062	2/4	420
Diluent only	0/2	

ties of the bacterial product studied here leave little doubt as to a close similarity between it and the *Aeromonas* cytotoxins first characterized by Wretlind and co-workers (14). The greater lethality of our material may reflect differences in the strain and weight of the mice, a true difference in specific lethal activity, or perhaps a combination of these factors. It is notable that aerolysin resembles to some extent the *Pseudomonas aeruginosa* exotoxin studied by Liu et al. (8) and by Callahan (5). The two proteins are closely similar with respect to molecular weight and charge. Both are insoluble at their isoelectric pH, both lose about 70% of their activity upon isoelectric focusing, and both show a high order of lethal potency for mice. They may prove to be members of a new class of exotoxins of gram-negative bacteria, and further delineation of their biological effects should be of interest.

ACKNOWLEDGMENTS

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