Purification and Some Properties of *Aeromonas hydrophila* Hemolysin

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A hemolysin produced by a strain of *Aeromonas hydrophila* isolated from a patient with diarrhea was purified by acid precipitation and quaternary amine-ethyl-Sephadex chromatography. The molecular weight of the hemolysin was estimated at 50,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and at 48,000 by Sephadex G-100 gel filtration. In polyacrylamide gel electrophoresis at pH 4.0 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the hemolysin migrated as a single band, whereas electrophoresis at pH 9.4 and thin-layer isoelectric focusing demonstrated multiple bands. The results may indicate charge isomers of the hemolysin. The purified hemolysin had a hemolytic activity of 134 hemolytic units per µg of protein on rabbit erythrocytes. It caused fluid accumulation in infant mouse intestines and rabbit ligated ileal loops. Purified hemolysin also elicited cytotoxicity to Vero cells and lethal toxicity to mice. All these biological activities were lost on heating for 5 min at 56°C. These findings support the notion that *A. hydrophila* hemolysin is a cytotoxic enterotoxin.

*Aeromonas hydrophila* is widely distributed in the aquatic environment and recognized as a pathogen for humans and animals, including fish (16, 24, 27). Such extracellular toxic factors as hemolysin, cytotoxin, and enterotoxin(s) produced by *A. hydrophila* have been studied in relation to its pathogenicity (1, 5, 6, 10, 15). Many cases of human acute diarrhea disease undoubtedly due to *A. hydrophila* have recently been reported in several countries (3, 5, 11, 21, 27). Many workers ascribed the enteropathogenicity of *A. hydrophila* to the production of enterotoxigenic factor(s); however, inconsistent results have been obtained on the nature of such factor(s). Ljungh et al. (19) separated a cytotoxic enterotoxin, designated according to the criteria proposed by Keusch and Donta (17), from hemolysin having no enterotoxigenic activity in the rabbit ileal loop test. Some other workers (6) indicated identity of hemolysin (cytotoxin) and enterotoxin of *A. hydrophila*. *A. hydrophila* hemolysin (cytotoxin) purified partially had an enterotoxic activity and caused fluid accumulation in infant mouse intestines and rabbit ileal loops (12, 15). Still unsettled, however, is whether the hemolytic and enterotoxigenic activities are borne by the same molecule.

To fully understand the relation between hemolysin and enterotoxin attempts were made to purify and characterize *A. hydrophila* hemolysin. We succeeded in purifying the hemolysin produced by a strain of *A. hydrophila* isolated from a patient with acute diarrhea and attempted to examine the purified hemolysin for its biological activities and particularly for its enterotoxigenic activity.

**MATERIALS AND METHODS**

**Bacterial strain.** *A. hydrophila* AH-1 was isolated in 1975 from a patient with watery diarrhea in Osaka Prefecture. Biochemical tests were performed with an API 20E System (Analytab Products, Plainview, N.Y.), and the strain was identified according to the classification of Popoff and Veron (22). The strain was stored at room temperature in the maintenance medium consisting of 0.3% yeast extract (Difco Laboratories, Detroit, Mich.), 1.0% Bacto-Casitone (Difco), 0.5% NaCl, and 0.3% agar (pH 7.0).

**Culture for hemolysin production.** The medium for hemolysin production was composed of Davis minimal broth without glucose (Difco) and 1.0% yeast extract (Difco). The stock culture was subcultured overnight at 30°C on a heart infusion agar (Difco) slant. The culture was transferred to the medium for hemolysin production (150 ml) and then incubated without shaking at 30°C. The culture was transferred to 15 liters of the medium in a 20-liter bottle, which was incubated for 9 h at 30°C with vigorous aeration.

**Assay for hemolysin.** The specimen was diluted in 0.01 M Tris-hydrochloride buffer, pH 7.2, containing 0.9% NaCl. The diluted sample was dispensed into 1-ml amounts into three tubes, to which was added 2-ml amounts of a washed rabbit erythrocyte suspension in the same buffer. After centrifugation at 1,000 × g for 5 min at 4°C, the absorbance of the supernatant was read at 540 nm. The concentration of the rabbit erythrocyte suspension was adjusted to give an absorbance of 0.5 at 540 nm when complete hemolysis occurred under the specified conditions. One hemolytic unit (HU) was defined as the minimum dose of the sample required to produce 50% hemolysis.

**Purification of hemolysin.** The culture supernatant was separated by continuous centrifugation at 15,000 × g at 4°C. An RNA (P-L, Biochemicals, Inc., Milwaukee, Wis.) solution (10 mg/ml) was added to the culture supernatant to a final concentration of 0.3 mg/ml. The mixture was adjusted to pH 4.0 with 3 N H2SO4 and stored for 2 days at 4°C to allow the precipitate to settle. The supernatant fluid was removed with an aspirator; the precipitate was packed by centrifugation for 30 min at 5,000 × g at 4°C. The precipitate was dissolved in 300 ml of 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 1 M urea. After centrifugation for 30 min at 5,000 × g at 4°C to remove insoluble materials, the supernatant was applied to a quaternary aminemethyl (QAE)-Sephadex A-50 (Pharmacia, Uppsala, Sweden) column (2 by 27 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 1 M urea. The column was washed with 80 ml of the same buffer and eluted with linear gradient of NaCl at a concentration of from 0.05 to 0.35 M in 800 ml of the buffer.

**Electrophoreses.** Electrophoreses in 6% polyacrylamide gel at pH 4.0 and 9.4 in the presence or absence of urea were
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FIG. 1. Chromatography of acid-precipitated hemolysin on a QAE-Sephadex A-50 column (2 by 27 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 1 M urea. The sample contained 243 mg of protein. Elution was with a linear gradient of NaCl in the buffer. Symbols: ●, protein contents; ○, hemolytic activity; ---, NaCl concentration.

Carried out by the methods of Reisfeld et al. (23) and Davis (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 6.0% polyacrylamide gel by the method of Weber and Osborn (28). After electrophoresis, the gel column was stained in a 0.2% Coomassie brilliant blue. The following marker proteins were used in the molecular weight estimation of hemolysin: cytochrome in monomer (molecular weight, 12,400), dimer (24,800), trimer (37,200), tetramer (49,600), and hexamer (74,400) forms (all from Oriental Yeast Kogyo Co., Osaka, Japan). Thin-layer isoelectric focusing was performed in polyacrylamide gel (1 mm thick) with a pH range of from 4 to 6 according to the method of Vesterberg (26).

Molecular sieving. The molecular weight of the purified hemolysin was estimated from the elution volume from a Sephadex G-100 (Pharmacia) column (2.5 by 97 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 1 M urea with a gel filtration calibration kit (Pharmacia).

Preparation of antiserum. Antiserum was obtained from rabbits weighing ca. 3 kg. A 1-ml portion of 20 μg of the purified hemolysin was emulsified in an equal volume of...
Freund complete adjuvant (Difco) and injected subcutaneously into a rabbit. After 4 weeks, a 1-ml portion of 20 μg of the hemolysin without adjuvant was injected subcutaneously. The same procedures were repeated twice. The animal was bled 2 weeks after the last injection.

Agar gel double-diffusion test. About 3 ml of 1.2% agarose gel in 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 1 M urea was spread over a glass plate (5 by 5 cm). After solidification, three wells were made with a stainless-steel puncher (diameter, 2 mm). Each well was filled with a 10-μl portion of the material, and the plates were incubated in a moisture chamber for 1 day at room temperature.

Protein determination. The amount of protein was determined by the method of Lowry et al. (20) with bovine serum albumin as the standard. The UV absorption of the purified hemolysin was determined with a Hitachi model 320 spectrophotometer.

Infant mouse test. A 2.0% Evans blue solution (0.01 ml) was added to 1 ml of the specimen. The sample was given intragastrically to 4- to 5-day-old mice in 0.1-ml portions with polyethylene tubing (external diameter, 1 mm) connected to a 1-ml syringe. At least five mice were used for each specimen. After being kept for appropriate periods at 22 to 25°C, the mice were sacrificed with chloroform. The ratio of the intestinal weight to the remaining body weight (FA ratio) was measured. Mice with no dye in the intestinal tract were discarded. Such a response was considered positive if the ratio was higher than 0.09 according to the criteria proposed by Dean et al. (9).

Rabbit ileal loop test. The test was performed by the method of De and Chatterje (8). The rabbits, weighing 1.6 to 1.8 kg, were fasted for 2 days before being used. A 1-ml portion of the sample was inoculated into each ligated ileal loop (ca. 7 cm in length). At least three rabbits were used for each sample. The animals were sacrificed at 6 h by intravenous injection of pentobarbital. The volume of the fluid in each loop was measured, and the volume per length was calculated.

Other biological assays. Vero cells were used to assay cytotoxicity of the hemolysin. The cells were cultured for 5
to 7 days at 37°C in Eagle minimal essential medium (Nissiu, Tokyo, Japan) supplemented with 0.8% sodium bicarbonate. The Vero cells suspended in phosphate-buffered saline were dispensed into wells on a U-titer tray (Tomy Seiko, Tokyo, Japan). Each well contained 5 × 10⁴ cells, and a 0.1-ml portion of the hemolysin was added. The tray was centrifuged briefly after incubation for 1 h at 37°C. The remaining cells were stained with a 0.2% nigrosin solution (Wako Pure Chemical, Osaka, Japan) for 20 min at 37°C. The stained cells were counted under a microscope.

Mouse lethal toxicity was assayed by intravenous injection into mice (ca. 20 g) of a 0.1-ml portion of the hemolysin, and the time to death was measured.

Neutralization tests were performed by mixing the same quantities of anti-hemolysin serum and a solution (10 μg/ml) of the purified hemolysin, incubating the mixtures for 60 min at 37°C, and assaying them for different biological activities.

RESULTS

Purification of hemolysin. Preliminary experiments were performed on the precipitation aid and the solvent to extract the hemolysin from the acid precipitate. The hemolysin was precipitated efficiently in the presence of yeast RNA at a concentration of 0.3 mg/ml. The hemolysin was extracted with 0.05 M Tris-hydrochloride buffer, pH 7.5, in the presence of 1 M urea, but 6 M urea or a higher concentration reduced the recovery.

The elution profile in QAE-Sephadex A-50 chromatography of the hemolysin is illustrated in Fig. 1. The hemolysin activity eluted as a single peak. The recovery and the specific activity at each step of purification are shown in Table 1. The hemolysin obtained at the final step of purification possessed 134 HU per μg of protein.

Characterization of hemolysin. In PAGE in pH 4.0 gel, the hemolysin migrated as a single band (Fig. 2C), but in pH 9.4 gel, it migrated in three distinct bands and occasionally an additional one or two bands (Fig. 2A). In PAGE in pH 9.4 gel containing no urea, additional bands were seen at the top of gel (Fig. 2B).

In SDS-PAGE, the hemolysin treated with 2-mercaptoethanol and that untreated migrated in a single band to the same relative position (Fig. 2D). From the migration position, the molecular weight of the hemolysin was estimated at 50,000. By gel filtration on Sephadex G-100, the molecular weight was estimated at 48,000. In thin-layer isoelectric focusing in polyacrylamide gel, the hemolysin was separated into three clear and one faint bands; the isoelectric points were calculated at 5.43, 5.41, 5.28, and 5.58, respectively (Fig. 3).

In agar gel double-diffusion analysis, the purified hemolysin and the extract from the acid precipitate each formed a single precipitin line against anti-hemolysin serum. The anti-hemolysin serum neutralized the hemolytic, the infant mouse, and the mouse lethal activities and the Vero cell toxicity.

In UV absorption spectrum, the maximum was at 280 nm, and the minimum was at 250 nm, with a shoulder at ca. 290 nm. From an A₂₈₀/A₂₆₀ of 1.81, it appeared to contain no nucleic acid.

Biological activities of hemolysin. In the infant mouse test, the hemolysin caused fluid accumulation in the intestines. Figure 4 shows the time course of the infant mouse activity of the hemolysin. The fluid accumulation appeared very rapidly. Even at 1 h after administration of hemolysin, the FA ratio (0.966 ± 0.010 standard deviation) was shown to be positive when 1 μg of hemolysin was inoculated. The ratio reached the highest level in 3 h, and the highest ratio persisted until the end of the 18-h test period. From the results, calculation of the FA ratio in the infant mouse test was made within 4 h after administration.

The dose-response curve of the hemolysin in the infant mouse test is illustrated in Fig. 5. The FA ratio was proportional to the log dose in protein in a range of 0.01 to 1 μg. The infant mouse given 10 μg of hemolysin survived, but the FA ratio (0.142 ± 0.005) was almost the same as that for the mouse given 1 μg (0.142 ± 0.004). About 30 ng of hemolysin was the minimum dose resulting in a positive reaction in the infant mouse test. After being heat treated for 5 min at 56°C, 0.1 μg of the hemolysin no longer gave a positive result.

The hemolysin also caused fluid accumulation in the rabbit ileal loop at 6 h (Table 2). The fluid accumulated was 1.5 to 2.1 ml/cm of the ligated loop at a dose of 300 μg. A 10-μg dose of hemolysin caused slight fluid accumulation in three of six rabbits (0.3 to 0.6 ml/cm). A 5-μg dose caused no reaction. After heat treatment for 5 min at 56°C, 300 μg of hemolysin per ml did not cause fluid accumulation in any of three rabbits.

The hemolysin-elicited cytotoxicity to Vero cells was determined by the failure to exclude nigrosin. The ratio of dead to live cells was proportional to the amount of the hemolysin. The minimum effective hemolysin dose was 0.3 ng. The cytotoxic effect was also inactivated by heating for 5 min at 56°C.

Intravenous injection of 4 μg of hemolysin into mice caused death within 1 min (Table 3). The minimum mouse lethal dose of the hemolysin was 0.06 μg.

DISCUSSION

Attempts were made to purify and characterize the hemolysin of a strain of A. hydrophila isolated from a patient with acute diarrhea. We established procedures for the purification of hemolysin from a culture supernatant. The procedures consisted of acid precipitation and QAE-Sephadex A-50 ion-exchange chromatography and gave a satisfactory high recovery of pure hemolysin. Acid precipitation at the initial concentration of hemolysin is simpler and more economical when handling a large volume of culture supernatant than are other methods such as ammonium sulfate precipitation or ultrafiltration. This step was very effective in removing foreign materials and markedly increased the specific activity of the hemolysin. Although mere acidification of the culture supernatant resulted in incomplete precipitation, addition of yeast RNA as an aid resulted in complete precipitation of the hemolysin, as was the case with Clostridium botulinum type C and D toxins (13).

Most efficient extraction of the hemolysin from the acid

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<th>TABLE 2. Fluid accumulation in the rabbit ileal loop caused by A. hydrophila hemolysin</th>
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<td>Hemolysin (μg) per loop</td>
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<td>300 (heated for 5 min at 56°C)</td>
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* Number of rabbits with positive results/total number of rabbits inoculated.
precipitate was attained with 0.05 M Tris-hydrochloride buffer, pH 7.5, containing urea. Bernheimer and Avigad (2) reported that freeze-dried *Aeromonas* hemolysin was insoluble in buffer at pH 7.0 or 5.0. We found that part of the purified hemolysin did not penetrate the pH 9.4 polyacrylamide gel without urea. This finding may indicate that hemolysin aggregates form large molecules. In the present study, we used a buffer containing urea for extraction of the hemolysin from the acid precipitate and for preventing formation of insoluble materials during purification.

The overall recovery of hemolysin with the present purification procedures was 65.3%, which was at least 2.5 times higher than the highest recovery ever reported by other workers (2, 4, 19).

The molecular weight of the hemolysin was calculated at 48,000 by Sephadex G-100 gel filtration or at 50,000 by SDS-PAGE. These values agree with those reported by Bernheimer and Avigad (2) and Buckley et al. (4). The UV absorption spectrum was similar to that reported by Bernheimer and Avigad (2).

Heterogeneity of the purified hemolysin was indicated by PAGE at pH 9.4 or thin-layer isoelectric focusing, whereas PAGE performed in pH 4.0 gel and SDS-PAGE gave only a single band. These results may indicate charge isomers of the purified hemolysin. Buckley et al. (4) demonstrated that the hemolysin of a strain isolated from fish was separated into two isomers that were demonstrated in the original culture filtrate. Other bacterial toxins such as staphylococcal enterotoxins are known to have charge isomers (29). The agar gel double-diffusion tests indicated immunological homogeneity of the hemolysin.

Inconsistent results of the infant mouse test have been obtained with *A. hydrophila* enterotoxin. Cumberbatch et al. (6) and Gurwitz et al. (11) obtained negative results with the tests or early death of the infant mice inoculated with the culture supernatant. The infant mouse test was found to be a simple and reliable method for detection of the enterotoxin (25). The hemolysin purified in this investigation caused fluid accumulation in the infant mouse intestines. The infant mice given 10 μg of hemolysin intragastrically survived, and the adult mice injected intravenously with ca. 0.1 μg of hemolysin died. Jiwa (14) showed heat stability of the *A. hydrophila* enterotoxin. The present study indicates the heat liability of both the hemolysin and enterotoxigenic activities; heat treatment for 5 min at 56°C inactivated both. The enterotoxigenic activity of the hemolysin did not resemble the heat-stable enterotoxin of *Escherichia coli* in the long term response of the infant mouse, the cytotoxicity to Vero cells, and the heat sensitivity, but the rapidity of the infant mouse reaction was similar to that of the toxin.

Ljungh and her co-workers (18) have demonstrated a cytotoxic enterotoxin which caused steroid secretion in Y1 adrenal cells and fluid accumulation in the rabbit ileal loop. Ljungh et al. (19) also stated that the partially purified hemolysin had no enterotoxic activity. We did not look for the cytotoxic enterotoxin; however the hemolysin induced not only fluid accumulation in the infant mouse intestines and rabbit ileal loops but also showed cytotoxicity in Vero cells. These results agree with those of Hoštacká et al. (12), who stated that the hemolytic, cytotoxic, and enterotoxic activities of *A. hydrophila* were located in the same fraction with a molecular weight of 60,000.

It would be interesting to clarify whether such biological activities are displayed by the same active site. More information on hemolysin(s) produced by different strains or under different *A. hydrophila* culture conditions may be necessary to explain the conflicting results.

**LITERATURE CITED**


