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The Monomeric and Oligomeric Forms of Hemolysin Purified from *Vibrio cholerae* O1, Biotype *El Tor*

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Abstract: An oligomeric form of *El Tor* hemolysin was purified from *V. cholerae* O1, biotype *El Tor,* serotype Inaba and it was examined by electron microscopy. A subunit of the oligomeric form of hemolysin had a molecular weight of 60,000 by SDS–PAGE, although this hemolysin had four or five protein bands by conventional disc electrophoresis. By Western blotting analysis, the oligomeric form of hemolysin was also recognized with anti non–O1 hemolysin antiserum, as well as with *El Tor* hemolysin. The specific activity of the oligomeric hemolysin was fourteen times lower than that of *El Tor* hemolysin.

Key words: Vibrio cholerae, Hemolysin, Oligomeric form

Vibrio cholerae non–O1 as well as *V. cholerae* O1 have been recognized as causative agents of diarrheal disease (Blake *et al.*, 1980). *V. cholerae* biotype *El Tor* was originally differentiated on the basis that these strains were hemolytic and produced hemolysin whereas classical strains did not. The majority of non–O1 *V. cholerae* strains produce heat–labile hemolysin which is identical, biologically, immunologically and physicochemically, to *El Tor* hemolysin, as previously reported by Yamamoto *et al.* (1980). These hemolysins have been shown to induce significant fluid accumulation in the intestines of animal models such as the ligated adult rabbit ileal loop test, the infant rabbit test and the suckling mice test (Ichinose *et al.*,1987). Hemolysins are considered to be diarrheic factors in addition to cholera toxin or cholera–toxin–like toxin. Little, however, is known about how hemolysins are involved in the enterotoxicity of *V. cholerae* O1 and non–O1, and the mechanism of its cytotoxicity. In this paper, we identify an oligomeric form of *El Tor* hemolysin through the purification of *El Tor* hemolysin from *V. cholerae* O1, biotype *El Tor.*

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MATERIALS AND METHODS

Cultivation: The strain used for the purification of hemolysin was *Vibrio cholerae* O1, biotype *El Tor* (N86), obtained from Toshio Miwatani, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan. A loopful of bacterial cells from a nutrient agar slant was inoculated into 5 ml of heart infusion broth (Difco Laboratories, Detroit, Michigan, U. S. A.) and incubated overnight at 37 ℃. Then 5 ml of the culture was transferred to a Roux bottle containing 150 ml of syncase broth supplemented with 3% glycerol and incubated for 18 hr at 37 ℃. Then 3 ml of the bacterial culture was inoculated into 100 ml of syncase broth supplemented with 3% glycerol in Roux bottles and incubated for 48 hr at 30 ℃ without shaking.

Purification of hemolysin: The purification of hemolysin was performed as described previously (Yamamoto *et al.*,1986). The culture (4,500 ml) was centrifuged at 25,000 x *g* for 60 min and the supernatant fluid (4,200 ml) was collected. It was mixed with 1/20 volume of 1 M Tris−hydrochloride buffer (pH 8.0) and then with 1720 g of solid ammonium sulfate (60% saturation) at 4 ℃. The mixture was centrifuged at 25,000 x *g* for 20 min and the precipitate was suspended in 30 ml of Tris−hydrochloride buffer (50 mM, pH 8.0) containing 1 mM EDTA and 3mM NaN_3 TEA buffer) and dialysed against the same buffer. The dialysate was centrifuged, and the supernatant was applied to a column (4.5 x 81 cm) of Sephadex G−100 (Pharmacia Fine Chemicals, Uppsala, Sweden). Fractions having hemolytic activity were pooled and concentrated on an Amicon PM10 membrane (Amicon Co., Lexington, Mass. U. S. A.). The concentrated sample was applied to a column of Sephadex G−100 (superfine, 2.5 x 41 cm) equilibrated with TEA buffer containing 0.25 M glucose and 5% glycerol.

Determination of hemolytic activity: Samples (1 ml) of a 2% sheep red blood cell suspension (standardized to give an optical density of 0.6 at 540 nm when lysed and diluted 10−fold with distilled water) were incubated for 30 min at 37 ℃ with samples (1 ml) of diluted hemolysin preparations. After centrifugation for 2 min at 3,000 rpm, hemoglobin released into the supernatant was measured by absorbance at 540 nm. One hemolytic unit (HU) was defined as the amount of hemolysin causing 50% hemolysis of 1 ml of 1% sheep erythrocytes solution in 30 min at 37 ℃.

Polyacrylamide gel electrophoresis: Conventional Polyacrylamide gel disc electrophoresis and sodium dodecyl sulfate−polyacrylamide slab gel electrophoresis (SDS−PAGE) were carried out as described previously by the methods of Davis (1964) and Laemmli (1970), respectively. A molecular−weight marker kit was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Western blotting: The immunochemical detection of antigen on nitrocellulose membranes was carried out by a modification of the method of Towbin *et al.* (1979). After sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protein in the gel was transferred to a nitrocellulose membrane by electrophoresis at a constant voltage of 30 V for 12 hr and then 70 V for 1 hr at 10 ℃. The electrode buffer contained 25 mM Tris,

192 mM glycine, and 20% (vol/vol) methanol (pH 8.3). The nitrocellulose membrane was incubated for 30 min with affinity-purified rabbit antibody raised against non-O1 hemolysin after being coated with 5% bovine serum albumin for 45 min. After washing three times, the membrane was incubated for 30 min with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Cappel Laboratories, Inc., Cochranville, Pa. U. S. A.) and washed three times. The nitrocellulose membrane was immersed for 10 min in PBS containing 0.02% H_2O_2 and 0.06% 1-chloro-4-naphthol, and color development was stopped by washing with distilled water. All the incubation and washing procedures were done by gently agitating the membrane at room temperature with PBS containing 0.05% Tween 20.

Estimation of molecular weight using gel filtration: Estimation of the molecular weights of the first peak fractions was done by gel filtration using high performance liquid chromatography (HPLC) on a Protein Pak 300 column (Nihon Waters Ltd. Japan) equilibrated with TEA buffer containing 0.2 M NaCl. A high molecular weight gel filtration calibration kit was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Electron microscopy: Uranyl acetate (1%) was used for negative staining of the samples. Electron microscopy was performed using a JOEL-100CX electron microscope.

Protein determination: Protein content was determined as described by Bradford (1976) with a Bio-Rad Protein Assay System (Bio-Rad Laboratories, Richmond, Cal. U. S. A.), using bovine serum albumin as a standard.

RESULTS

Purification of hemolysin: Fig. 1a shows the elution pattern of crude hemolysin on a column of Sephadex G-100 equilibrated with TEA buffer. Fractions (fraction no. 20-50) eluted with the void volume seemed to contain lipopolysaccharides in the Western blot profile, although they had hemolytic activities (data not shown). Fractions (fraction no. 80-100) having hemolytic activity, which were eluted after the bed volume, were pooled, concentrated and applied to a Sephadex G-100 superfine column, equilibrated with TEA buffer containing 0.25 M glucose and 5% glycerol (Fig. 1b). The first peak was eluted in the void volume and the second peak fraction in Fig. 1b showed hemolytic activity. They were pooled and concentrated by Amicon PM10.

Electrophoresis: Both peak fractions were examined by SDS-PAGE and conventional disc electrophoresis. As shown in Fig. 2, both fractions gave a common 60,000-molecular-weight band on SDS-PAGE. After treatments with 5 and 8 M urea, both preparations produced identical bands. Using conventional disc electrophoresis, the second peak fractions gave a single band. Therefore, the second peak fractions were identified as *El Tor* hemolysin. However, the first peak fractions gave four or five bands, which suggested several kinds of oligomeric forms (Fig. 3), and were designated as the lst peak fraction.

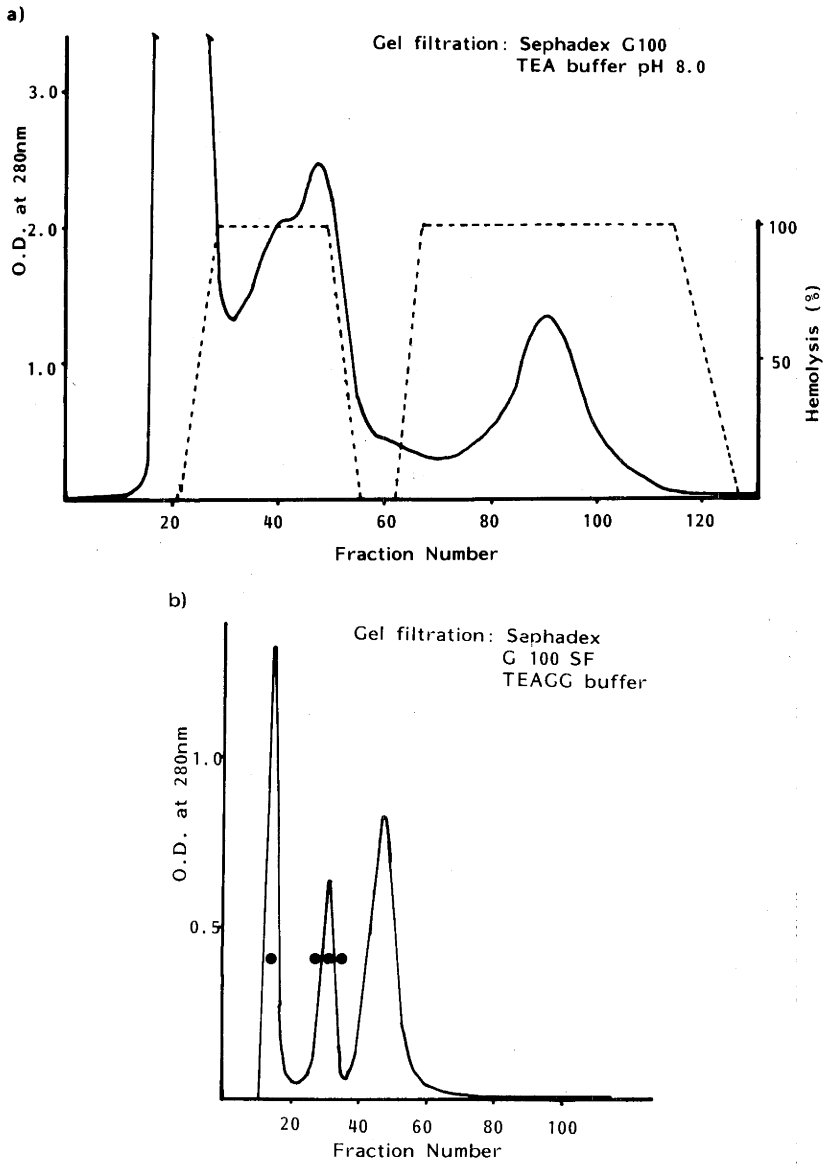

Fig.1. Gel filtration on Sephadex G−100 (1a) and G−100 superfine (1b) columns of *El Tor* hemolysin. Protein content (absorbance at 280 nm, solid line) and hemolysis (absorbance at 540 nm, dotted line in 1a and dots in 1b) were determined. Samples diluted 100−fold were assayed as described in Materials and Methods.

Western blotting of hemolysin preparations: The second peak fractions (*El Tor* hemolysin) and the lst peak fractions were examined by Western blotting analysis with anti−non−O1 Hemolysin antibody (Fig. 4). Both samples revealed a major 60,000 and a minor 32,000 molecular−weight protein band. The molecular weight of the major band coincided with that of *El Tor* hemolysin. It was considered that the lst peak fraction

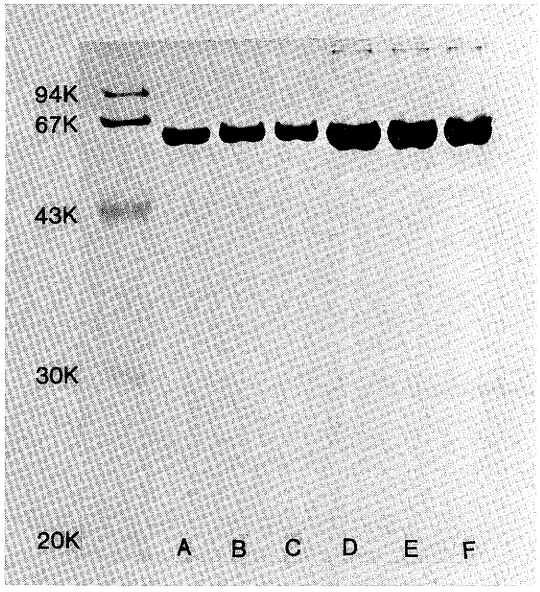

Fig.2. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of *El Tor* hemolysin. Samples of *El Tor* hemolysin (lane A, B, C) and the lst peak fraction (lane D, E, F) were applied. Samples of lane B, E and lane C, F were treated with 5 and 8 M urea 2 hr at 37 ℃, respectively. The numbers on the left show molecular weights of standard marker proteins.

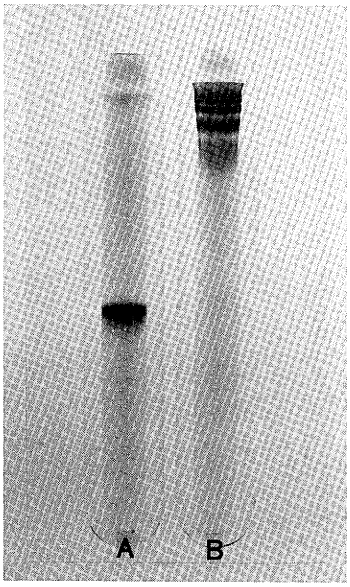

Fig.3. Polyacrylamide gel disc electrophoresis of *El Tor* hemolysin. Columns A and B contained *El Tor* hemolysin and the lst peak fraction, respectively. 5 μg samples of hemolysin were applied.

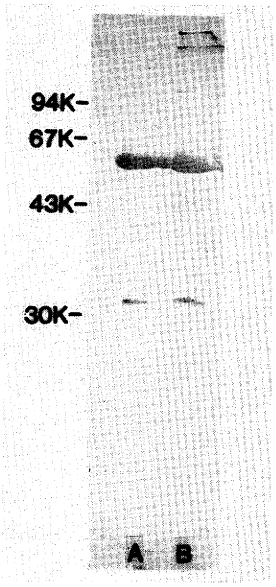

Fig.4. Western blot profile of *El Tor* hemolysin. Columns A and B contained *El Tor* hemolysin and the lst peak fraction, respectively. The numbers on the left show the molecular weights of standard protein markers.

was composed of the 60,000−molecular−weight protein recognized with non−O1 hemolysin antiserum.

Estimation of molecular weight of the lst peak fraction: HPLC was performed on a Protein Pak 300 column equilibrated with TEA buffer containing 0.2 M NaCl at a flow rate of 1 ml/min (Fig. 5a). A single, symmetrical peak was seen at a retention

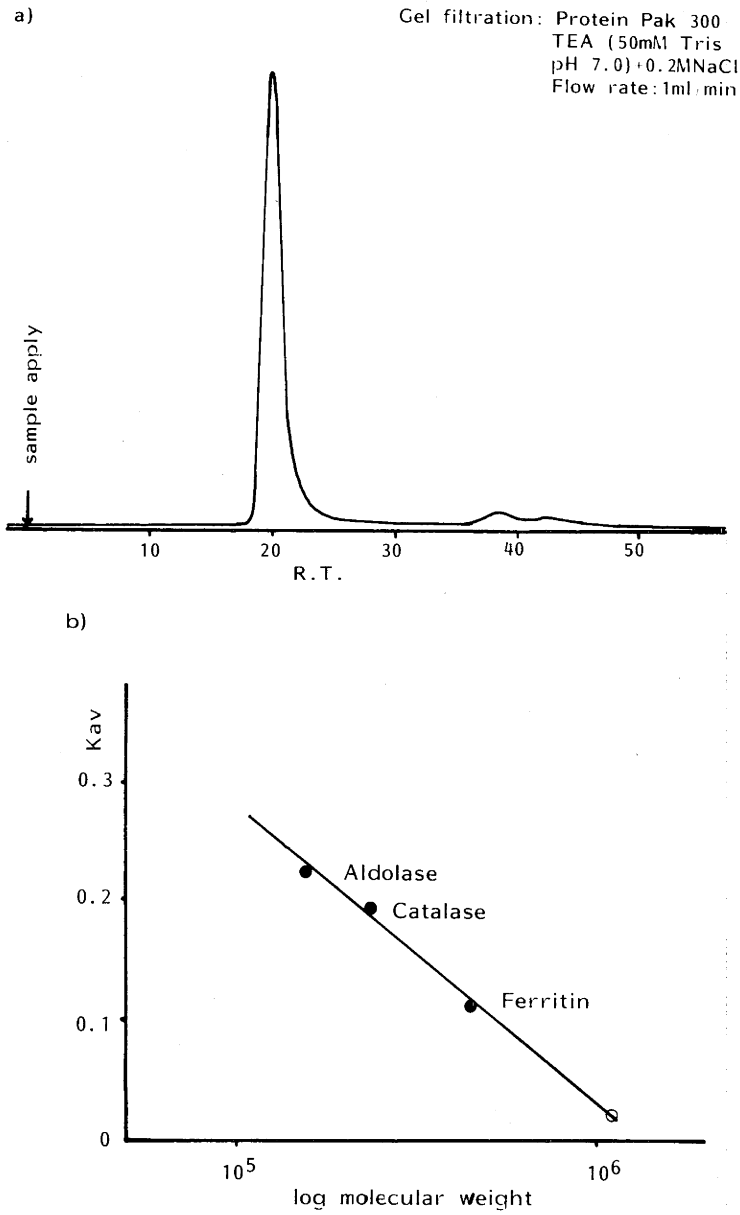

Fig.5. A chromatogram of the gel filtration performed on a Protein Pak 300 column (5a) and the calibration curve, using a high molecular weight gel filtration calibration kit (5b)

time of 20.43 min. A calibration curve using the high molecular weight gel filtration kit is shown in Fig.5b. The molecular weight of the lst peak fraction was estimated to be approximately 1,200,000.

Hemolytic activity: Hemolytic activities of *El Tor* hemolysin and the lst peak fraction were assayed as described in Materials and Methods. The specific activities of *El Tor* hemolysin and the lst peak fraction were 41.7 and 3.0 HU/μg, respectively (Fig.6). The hemolytic activity of *El Tor* hemolysin was approximately 14 times higher than that of the lst peak fraction.

Electron microscopy: The negative staining results of *El Tor* hemolysin and the lst peak fraction are shown in Fig.7a and 7b, respectively. *El Tor* hemolysin was observed to be a round particle with uranyl acetate puddled in the hollow of the particle. On the other hand, the particles of the lst peak fraction seemed to be formed of small particles, which were not necessarily uniform.

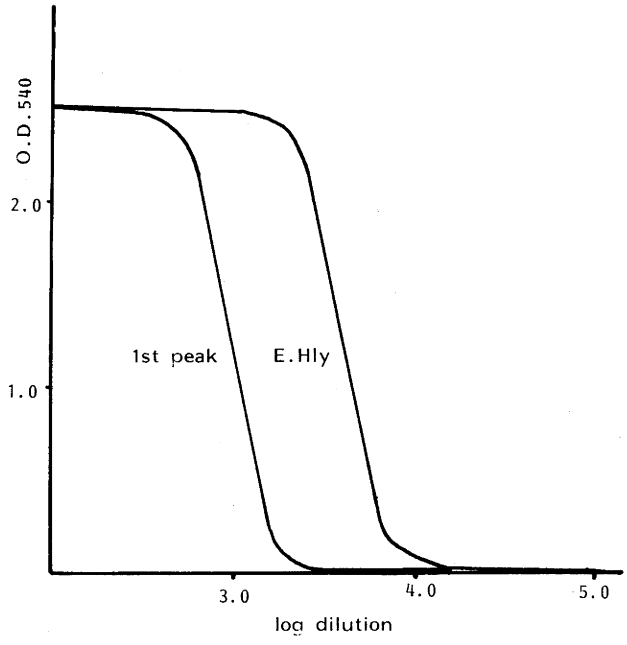

Fig.6. Dose−response curves for *El Tor* hemolysin and the lst peak fraction. Both hemolysins were assayed as described in Materials and Methods.

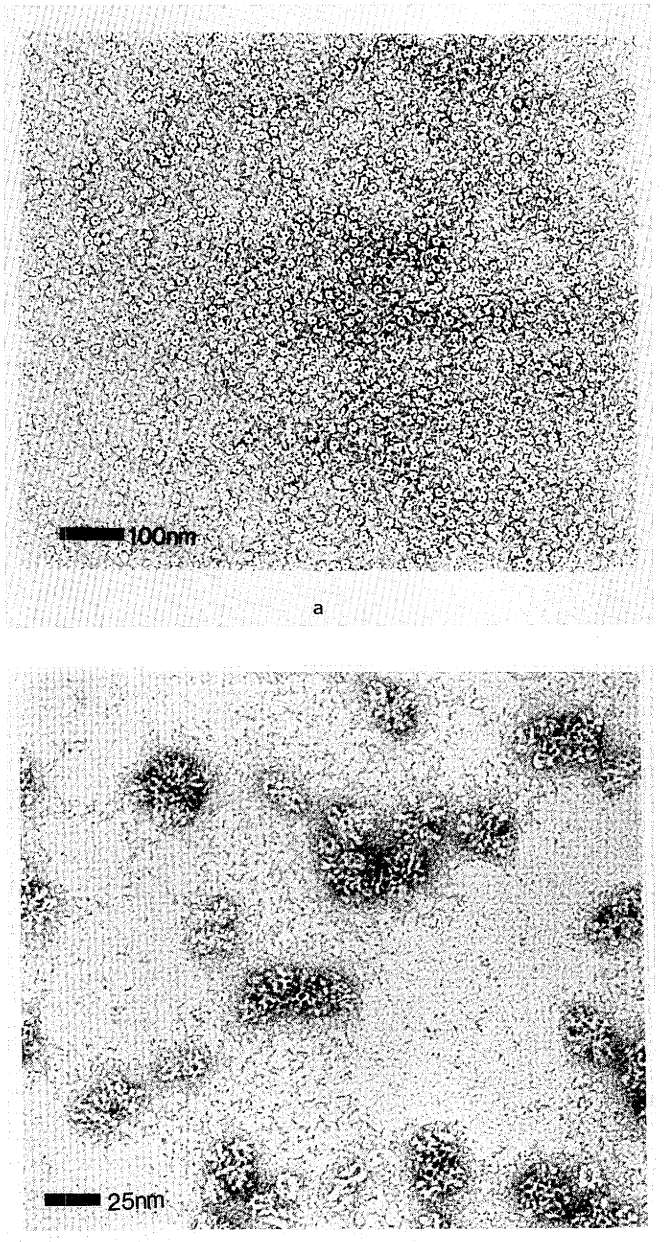

Fig.7. Electron micrograph of *El Tor* hemolysin (a) and the lst peak fraction (b). Bars indicate 100 and 25 nm, respectively.

DISCUSSION

The existence of an oligomeric form of *El Tor* hemolysin was proved by electron microscopy and it was purified from *V. cholerae* O1, biotype *El Tor,* serotype Inaba. An

estimation of the molecular weight of this hemolysin, using HPLC with a Protein Pak 300 column revealed an M. W. of approximately 1,200,000, but four or five protein bands were observed when this protein was applied to conventional disc electrophoresis. The estimated molecular weight can be considered to be an intermediate value for several kinds of oligomeric forms, because proteins with molecular weights over 400,000 can not be analyzed accurately using this column. However, the molecular weight of a subunit of this oligomeric form of hemolysin was 60,000 via SDS−PAGE. This oligomeric form of hemolysin was also recognized with anti non−O1 hemolysin antiserum as well as *El Tor* hemolysin. Minor hemolytic activity was also found in the void volume of the first column (fraction no. 20−55 in Fig. la). A major protein band in these fractions was detected with anti non−O1 hemolysin antiserum and seemed to be associated with lipopolysaccharides in the Western blot profile. The molecular weight of this major protein band ranged from 45,000 to 55,000 by SDS−PAGE (data not shown). It is, thus, considered that this minor hemolytic−active protein was coeluted with vibrio lipopolysaccharides as aggregates, as previously described by Yamamoto *et al.* (1986). However, this oligomeric form of *El Tor* hemolysin was purified from the major hemolytic−active fractions (fraction no. 80−100) in the first column. This was probably due to affinity to the Sephadex gel in which the oligomeric form of *El Tor* hemolysin coeluted with the monomeric form of hemolysin. The formation of oligomeric hemolysin was not observed during the preservation of monomeric hemolysin under the same conditions as the purification procedures, so it may be safe to assume that this oligomeric form of hemolysin was not produced by the aggregation of monomeric hemolysin in the purification process. The specific activity of the oligomeric form of hemolysin was fourteen times lower than that of monomeric hemolysin. Hemolytic activity of the oligomeric form was not the result of contamination by monomeric hemolysin, because no protein bands at the position of the monomeric form were observed by conventional disc electrophoresis. Therefore, it is fair to consider this hemolysin as an inactivated form of hemolysin, although it is unclear whether it was excreted from the vibrios together with active forms, or whether it was inactivated after excretion. However, it was probably not inactivated by exposure to oxygen, because it was not activated by treatments with reducing agents such as dithiothreithol and 2−mercaptoethanol. This inactive form of hemolysin might be applied to investigating the active site of the hemolysin molecule.

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