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Purification and Characterization of Two Hemolysins Produced by *Bacillus cereus*

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Abstract

A strain, UI-10, was isolated from soil and identified as a strain of *Bacillus cereus*. The strain was shown to produce two hemolysins. The two hemolysins were purified by fractionation with ammonium sulfate and various column chromatographies. Apparent molecular weights of the two hemolysins were 49,000 for H1 and 30,000 for H2. Both the hemolysins exhibited the Arrhenius effect, but their effective temperatures on the effect were different each other. At a low concentration, cholesterol completely inhibited the hemolytic activity of H1 but not H2. The hemolytic activities of H1 and H2 were enhanced by SH compounds such as cysteine (0.1%), dithiothreitol (1 mM) and 2-mercaptoethanol (0.1%). When rabbit erythrocyte ghosts were treated with H1, ring and arc-shaped structures appeared on the membranes.

Introduction

B. cereus is widely distributed in natural environments and is one of the commonest aerobe spore-bearer in soil. The organism is commonly found in dehydrated cereals and cause the spoilage of milk and eggs. As described previously (7), cornflour sauce, milk puddings and rice disks are infectious vehicles of the organism for human. Among the metabolites of *B. cereus*, a hemolytic agent, cereolysin, has been well characterized (1, 2, 4, 19). Several studies (6, 8, 13, 16, 17) proposed that the hemolytic, phospholipolytic and lethal activities of the organism were associated with three distinct extracellular products,

which correspond to hemolysin, phospholipase C and lethal toxin, respectively. However, the relationship between the three activities of the organism remains unclear.

During the course of the investigations on the biological and biochemical activities of bacteria isolated from soil, we found an isolate, a strain of *B. cereus*, which secretes two hemolysins in the culture supernatant. Several studies concerning the Bacillus strains which produce two hemolysins have been reported (3, 6, 9, 14, 19). However, the properties of the hemolysins have not been well characterized.

In the present study, we isolated the two hemolysins produced by *B. cereus* UI-10 and examined the interaction between the hemolysins and rabbit erythrocyte membrane ghosts using electron microscopy.

Materials and Methods

Isolation of a strain of *B. cereus* from soil

For the isolation of a strain of *B. cereus*, a clod in the garden of our campus was suspended in autoclaved water at a concentration of about 1g (wet weight) per 3 ml and blended. After standing for 5 min, the upper portion was centrifuged at 10,000 × g for 10 min, and the resulted sediments were smeared on a selection medium for *B. cereus*, NGKG agar base plate (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 20% egg-yolk and incubated at 30℃ for 18 to 24 h. The colonies suspected to be *B. cereus* were isolated according to the working manual and identified as a strain of *B. cereus* according to the method of Sinagawa (15). According to the ability to produce the hemolysin, one strain designated UI-10 was selected and used in this work. In comparison with the bacteriological and biochemical properties of an authentic strain of *B. cereus*, 4433/73 (19), the strain UI-10 was shown to be a typical strain of *B. cereus*. The strain was maintained on a slant of Heart Infusion (HI) agar (Nissui) at room temperature and was transferred routinely every two months. Working cultures were maintained on HI blood agar plates.

Bacterial growth and hemolysin production

Bacterial growth and hemolysin production were performed according to the method of Coolbaugh and Williams (3). One loopful culture of a working blood agar plate was inoculated into 5 ml of HI broth supplemented with 0.5% glucose (HIBG) in a 100-ml Erlenmeyer flask. After incubation at 37℃ for 16 to 18 h in a shaking water bath, the culture was centrifuged at 15,000 × g for 20 min, and the cell pellets were resuspended in 250 ml of the same broth in a 500-ml Sakaguchi flask. The second culture was incubated at 37℃ in a water bath with shaking. At an indicated time, the growth of the bacteria was followed by measuring the absorbance of the culture at 660 nm and a 0.5-ml aliquot of the culture was withdrawn to assay its hemolytic activity.

Assay of hemolytic activity

Hemolytic activity was determined as follows: 0.2 ml of test samples in a series of two-fold dilution in 20 mM Tris-HCl buffer, pH 7.2, containing 0.9% NaCl (TS buffer) was mixed with an equal volume of 0.5% rabbit erythrocytes (RBC) in a 96-well microtiter U-plate (Becton Dickinson, NJ, USA) and allowed to stand at 37℃ for 30 min. One unit of hemolysin was defined as the reciprocal of the highest dilution of a sample that gave rise to complete hemolysis. In an alternative assay, 0.2 ml of the samples was mixed with 1 ml of 20 mM Tris buffer (pH 7.2) containing 0.9% NaCl and 5% glycerol in a test tube and 0.2 ml of 5% RBC was added to the mixture. After incubation at 37℃ for 30 min, the mixture centrifuged briefly and the amounts of hemoglobin released in the supernatant fluids was assayed by measuring the absorbances of the hemoglobin solutions at 545 nm.

Effect of temperature and cholesterol on hemolytic activity

In the experiments as to the effect of temperature on hemolytic activity, 0.2 ml of purified hemolysin was incubated at various temperatures for 5 min. After incubation, the samples were cooled in an ice water bath and the hemolytic activities were assayed as described above. In the experiments with cholesterol, varying concentrations of cholesterol were mixed with three units of a hemolysin in a total volume of 0.2 ml. After a 10-min incubation at room temperature, 0.4 ml of 0.5% RBC suspension was added to the mixtures. The mixtures were further incubated at 37℃ for 30 min and centrifuged briefly to obtain clear supernatant fluids. The hemolytic activities of the supernatants were assayed as described above.

Effect of thiol compounds on hemolytic activity

The activation of the hemolysins by cysteine was examined according to the method of Bernheimer and Grushoff (1) with a slight modification: 0.2 ml of purified hemolysins, ranging from 1/4 to 8 units/ml, was mixed with an equal volume of 0.1 M phosphate buffer (pH 7.4) containing 0.1% cysteine and the mixture was allowed to stand for 10 min at room temperature, after which the hemolytic activity of the mixture was measured as described above. One mM of dithiothreitol (DTT) or 0.1% 2-mercaptoethanol (2-ME) was tested with respect to its effect on hemolytic activity in the same manner as described in the experiments with cysteine.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (10) using a 12% separation gel as described previously (18). Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as a standard.

Preparation of erythrocyte ghosts and electron microscopy

Erythrocyte ghosts were prepared by osmotic lysis according to the method of Duncan

and Shlegel (5): 50% rabbit erythrocyte suspensions were added to a 1-ml drop of water and the ghosts were taken up on 400 mesh grids covered with carbon-coated formvar. The grids were floated on a drop of purified hemolysin (50 units/ml) in TS buffer, kept at 37℃ for 30 min and the excess fluid was removed with filter paper before negative staining.

The grids prepared as described above were fixed with 2.5% glutaraldehyde and negatively stained with 2% ammonium molybdate (18). The specimens were examined in a Hitachi HU-11 electron microscope operating at 75 KV as described previously (18).

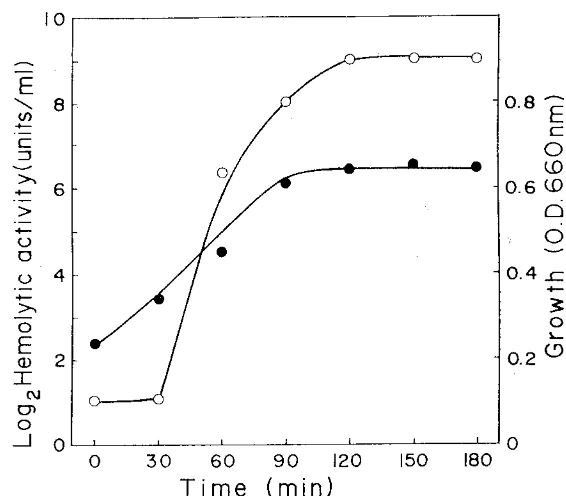

Fig. 1. Growth of an isolate UI-10 and hemolysin production. Organisms obtained from 5 ml of the first culture were grown in 250 ml of HIBG in 500 ml Sakaguchi flasks with shaking at 37℃ as described under Materials and Methods. ○, hemolytic activity in the culture supernatant; ●, absorbance at 660 nm.

Results

A strain of *B. cereus*, HI-10, was isolated and identified as described under Materials and Methods. The properties of the isolate were similar to those of *B. cereus* 4433/73 (Table 1). The culture supernatant of UI-10 showed a highly hemolytic activity (1 :

Table 1. Morphological and biochemical properties of an isolate IU-10 and strain 4433/73

	Strain	
Property[a]	UI-10	4433/73
Rod-shape	+	+
Gram-positivity	+	+
Motility	+	+
Central spore	+	+
Growth on Simmon's citrate medium	+	+
Catalase activity	+	+
Reduction of nitrate to nitrite	+	+
Starch hydrolysis	+	+
Lecithinase activity	+	+
Gelatin liquefaction	+	+
Indole production fermentation of sugar	−	−
Glucose	+	+
Arabinose	−	−
Mannitol	−	−
Xylose	−	−

[a] Each biochemical test including sugar fermentation was carried out according to the method described by MacFaddin (12).

512), when it was grown at 37℃ with vigorous shaking in a HI broth containing 0.5% glucose. The growth of the isolate and the production of hemolysin in a preparative second culture of the isolate were illustrated in Fig. 1. Hemolytic activity in the culture fluid was detected 30 min after culture and reached a maximum in about 90 min. The high levels of the hemolytic activity were maintained for at least 3 h without any reduction. The culture fluid obtained from a 2-h culture was used to isolate the hemolysin(s).

Purification of the hemolysins

Hemolysins in a 2-h culture fluid were fractionated by ammonium sulfate and the precipitate formed between 30 and 80% saturation was collected by centrifugation. The precipitate was dissolved in a small volume of 50 mM Tris-HCl buffer (pH 8.0), allowed to stand for 1 h with stirring and centrifuged at 40,000×g for 30 min. The supernatant obtained was applied on a Phenyl-Sepharose CL-4B column (1.8×30 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 M ammonium sulfate. The hemolytic activities were found to be in the fraction obtained by washing with the same buffer (H1) and in the fraction obtained by elution with a linear gradient of ethylene glycol (0 to 80%) as shown in Fig. 2. Each fraction was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) overnight. The dialysate was concentrated to a small volume by ultrafiltration. Finally, the hemolysins were separately purified using a Thiopropyl-Sepharose 6B column (1.0×15 cm) equilibrated with 100 mM sodium acetate buffer (pH 4.5) and the hemolysins were eluted with a

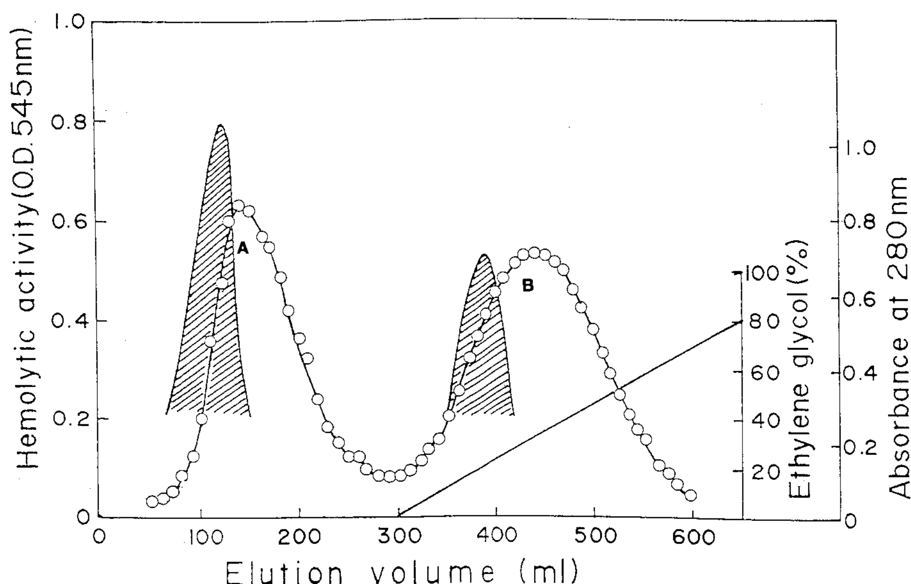

Fig. 2. Phenyl-Sepharose CL-4B chromatography of the hemolysin. The column chromatography was carried out as described under Materials and Methods.
(A) Washed fraction with 50 mM Tris-HCl (pH 8.0);
(B) Eluted fraction with a gradient from 0% to 80% ethylene glycol;
○, absorbance at 280 nm. Hatched area represents the hemolytic activity.

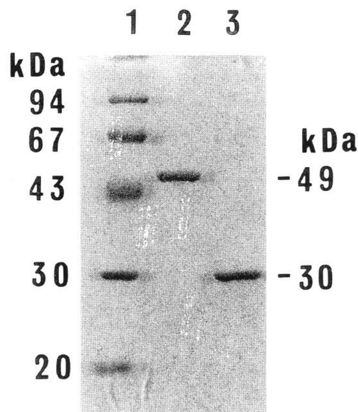

Fig. 3 SDS-PAGE of purified H1 and H2.
Lane 1, Molecular mass standards; 2, H1; 3, H2.
Two μg of the protein was applied on the gels.

linear gradient of pH 4 to 7.5 in 0.1 M phosphate buffer. H1 was eluted at pH 5, and H2 at pH 6.5. The two purified hemolysins were shown to be homogeneous by SDS-PAGE (Fig. 3). The molecular weights of H1 and H2 were estimated to be 49,000 and 30,000, respectively, by SDS-PAGE.

Effect of cholesterol and sulfhydryl compounds on the hemolytic activities of H1 and H2

Cholesterol affected the hemolytic activities of the two hemolysins in a different manner from another (Fig. 4). The cholesterol showed a noticeable suppression on the hemolytic activity of H1 at μM levels and completely inactivated H1 at a concentration of 1 mM. However, no effect was observed on the hemolytic activity of H2 under the same conditions (Fig. 4). As shown in Fig. 5, cysteine enhanced the hemolytic activities of H1 and H2 and the hemolytic activity of H2 was stimulated more strongly than that of H1. The experiments with DTT and 2-ME showed similar results to those described above.

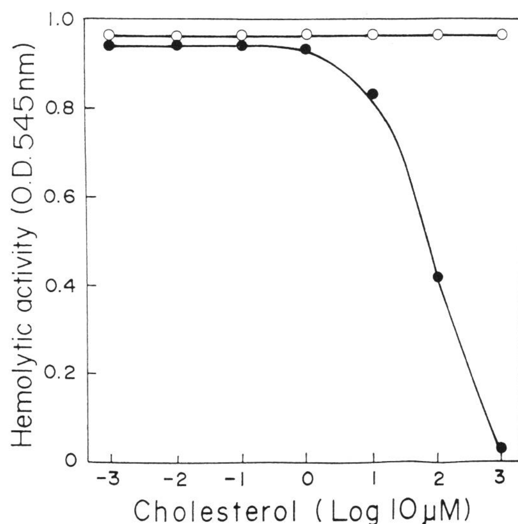

Fig. 4. Effect of cholesterol on the hemolytic activities of H1 and H2. Reaction mixture consisted of 0.2 ml of hemolysin (35 unit/ml) and 0.2 ml of 5% RBC in 1ml of TS buffer containing 5% glycerol and the hemolytic activity was determined by measuring an absorbance at 545 nm.
●, H1; ○, H2.

Effect of temperature on the hemolytic activities of H1 and H2

The effect of temperature on the hemolytic activities of the hemolysins, that is, the Arrhenius effect (19), was examined. As shown in Fig. 6, at 50℃ or higher temperatures, H1 lost its original activity. On the other hand, H2 was inactivated at 70℃, but gradually recovered at higher temperatures and at 100℃, showed about 100% of the original activity (Fig. 6B). However, in the presence of glycerol, H1 showed a minimal hemolytic activity between 70 and 80℃ and the activity gradually recovered over 80℃, indicating that H1 shows the Arrhenius effect in the presence of glycerol. On the other hand, H2 was stable in the presence of glycerol in the same range of temperature as described above.

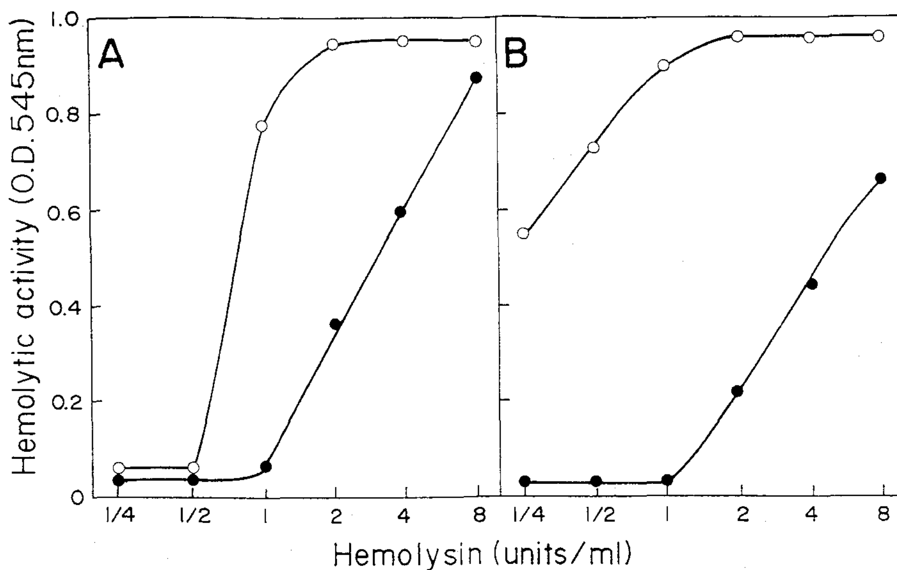

Fig. 5. Effect of cysteine on the hemolytic activities of H1 (A) and H2 (B). Reaction mixtures were consisted of 0.25 ml of hemolysin, ranging from 1/4 to 8 with an equal volume of 0.1% cysteine. The mixture was incubated at room temperature for 10 min, after which 0.5 ml RBC was added to the mixture.
○, in the presence of cysteine; ●, in the absence of cysteine.

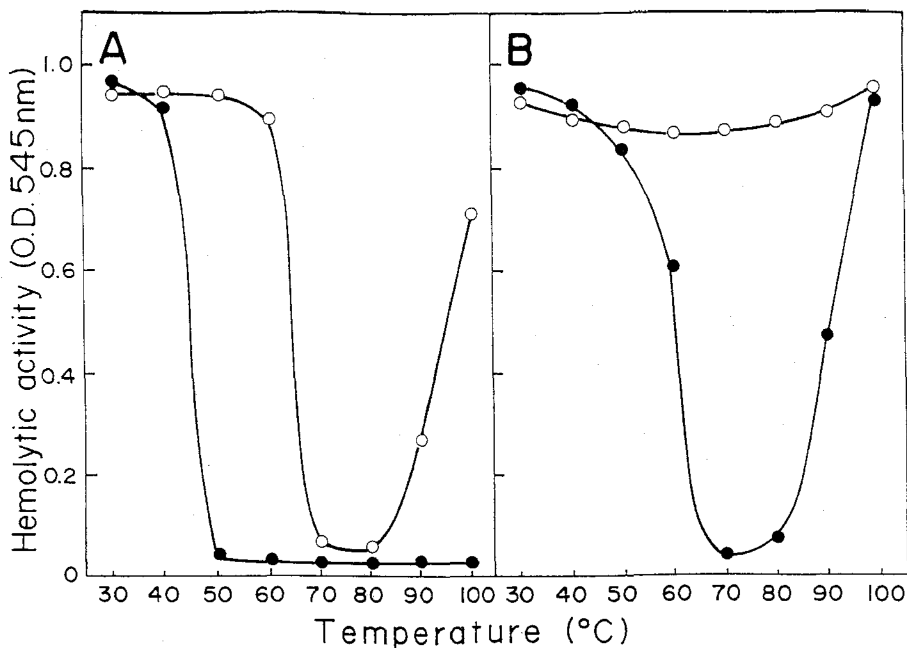

Fig. 6. Effect of temperature on the hemolytic activities of H1 (A) and H2 (B). The hemolysin was incubated at various temperatures for 5 min and its hemolytic activity was assayed as described under Materials and Methods.
○, in the presence of glycerol; ●, in the absence of glycerol.

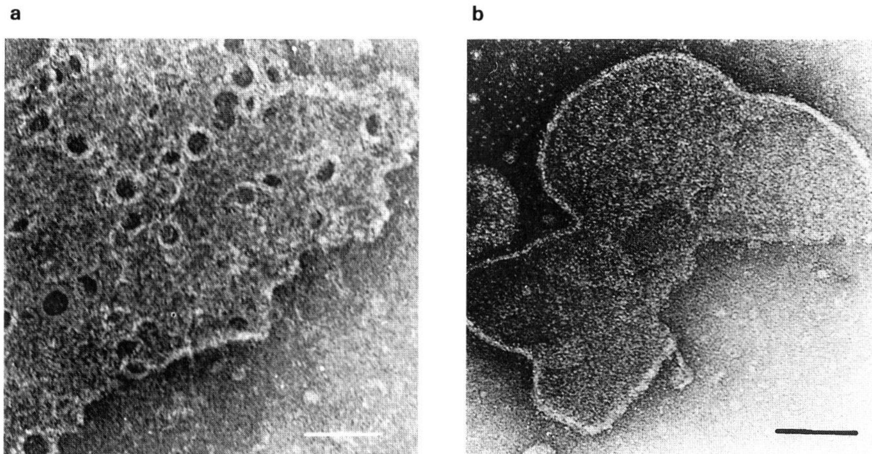

Fig. 7. Electron micrographs of rabbit erythrocyte ghosts treated (a) and untreated (b) with purified H1. The ghosts were treated with H1 in a concentration of 5,000 units/ml and incubated at 37℃ for 30 min. Bars represent a length of 100 nm.

Electron microscopic observation

Effect of the hemolysins on the morphology of rabbit erythrocyte membranes was examined using electron microscopy (Fig. 7). The membranes from control cells, which had been osmotically lysed on a water droplet, showed an intact structure (Fig. 7a). When erythrocyte membranes were lysed by H1, ring or arc-shaped structures were shown to come out on the surface of the membranes (Fig. 7b). However, H2 could induce no significant changes in the structures of the H2-treated membranes (data not shown).

Discussion

B. cereus is a bacterial species which is well known as potential food-poisoning organism and widely distributed in the environment. The strain used in this work, designated UI-10 was isolated from soil in the garden of our university and identified as a strain of *B. cereus* by the morphological studies with gram and spore staining and some biological studies on VP reaction, gelatin liquefaction, starch hydrolysis and the fermentation of glucose, mannitol, arabinose and xylose (Table 1). The isolate UI-10 produced highly active hemolysin(s) in the supernatants of the cultures.

Two kinds of hemolysin, H1 and H2, were separated by Phenyl-Sepharose CL-4B chromatography (Fig. 2). The fact that *B. cereus* produces two kinds of hemolysins is not surprised, because the production of some hemolysins in the organism have been observed by several investigators (3, 6, 17). The homogeneous hemolysin proteins were obtained by Thiopropyl-Sepharose 6B chromatography.

The two purified hemolysins showed no turbidity in diluted egg-yolk, indicating that they are free from phospholipase C activity. Johnson and Boventre (8) reported that the hemolytic, phospholipolytic and lethal activities of *B. cereus* showed different behavior dur-

ing the growth of the organism and that the three activities are not those exhibited by a single protein. Our present result is compatible with that described by Johnson and Boventre (8). The molecular masses of H1 and H2 were estimated to be 49 and 30 kDa, respectively, by SDS-PAGE and correspond to those of H-I hemolysin (cereolysin) and H-II hemolysin, which were reported by Turnbull (19).

In order to compare the hemolysins obtained by this work with H-I and H-II from *B. cereus* reported by Coolbaugh and Williams (3), we examined the effects of cholesterol, thiol compounds and temperature on the hemolytic activities of our hemolysins. Cholesterol inhibited the hemolytic activity of H1, but H2 was insensitive to the inhibitor, indicating that H1 and H2 may act on a receptor or substrate on the rabbit erythrocyte membranes in a different manner. This finding is consistent with that observed by Coolbaugh and Williams (3). In the absence of glycerol, H1 did not show any Arrhenius effect, but a typical Arrhenius effect was shown with respect to H2. However, in the presence of glycerol, H1 was observed to exhibit an Arrhenius effect and H2 was stable within the temperatures tested. Similar phenomena were observed with respect to H-I and H-II by Coolbaugh and Williams (3), although they reported that H-II exhibited a slight Arrhenius effect. Furthermore, thiol compounds such as cysteine, 2-ME and DTT stimulated both of the hemolysins. Interestingly, when rabbit erythrocyte membranes were treated with H1, the hemolysin induced ring and arc-shaped structures on the membranes, but H2 showed no effect on the membrane structure.

The above observations indicate that H1 and H2 are identical to H-I (cereolysin) and H-II, respectively, which were reported by Coolbaugh and Williams (3) and Turnbull (19). Further detailed investigations on the identity of our hemolysins with H-I and H-II and the mechanism of their hemolysis should be required. However, the present study provides useful information in understanding the hemolytic action of the hemolysins.

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