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Purification and Characterization of an Extracellular Cytolysin Produced by *Vibrio damsela*

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Large amounts of an extremely potent extracellular cytolysin produced by the halophilic bacterium *Vibrio damsela* were obtained free of detectable contamination with medium constituents and other bacterial products by sequential ammonium sulfate precipitation, gel filtration with Sephadex G-100, and hydrophobic interaction chromatography with phenyl-Sepharose CL-4B. The cytolysin is heat labile and protease sensitive and has a molecular weight (estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of ca. 69,000 and an isoelectric point of ca. 5.6. The first 10 amino-terminal amino acid residues of the cytolysin are Phe-Thr-Gln-Trp-Gly-Gly-Ser-Gly-Leu-Thr. The cytolysin was very active against erythrocytes from 4 of the 18 animal species examined (mice, rats, rabbits, damselfish) and against Chinese hamster ovary cells and was lethal for mice (ca. 1 μg/kg, intraperitoneal median lethal dose). Lysis of mouse erythrocytes by the cytolysin is a multi-hit, at least two-step process consisting of a temperature-independent, toxin-binding step followed by a temperature-dependent, membrane-perturbation step(s).

The halophilic bacterium *Vibrio damsela*, one of seven new *Vibrio* species recognized since 1976 as possible causes of human disease (26, 30), is an opportunistic pathogen that causes wound infections and fatal disease in temperate water damselfish (23), neritic sharks (11), and humans (7, 8, 23, 27). Kreger (18) has observed a correlation between the ability of 19 isolates of *V. damsela* to cause disease in mice and to produce large amounts of a cytolytic toxin in vitro. The cytolysin was physicochemically and antigenically distinct from cytolysins produced by other *Vibrio* species, and partially purified cytolysin preparations were lethal for mice and elicited grossly observable changes similar to those observed during experimental *V. damsela* infections in mice (18). This report presents a method for obtaining large amounts of highly purified cytolysin and describes some of the physicochemical and biological properties of the purified cytolysin.

MATERIALS AND METHODS

Assays. Results of preliminary experiments indicated that the stability of purified cytolysin preparations was enhanced by the addition of crystalline bovine albumin (Miles Laboratories, Inc., Naperville, Ill.) to phosphate-buffered saline (PBS; 0.067 M Na_2HPO_4–0.077 M NaCl [pH 7]) used for cytolysin dilutions. Therefore, unless prevented by the assay, and unless otherwise stated, cytolysin dilutions were prepared with PBS containing 1 mg of crystalline bovine albumin (BA) per ml.

Cytolytic activity against erythrocytes was determined by the method of Bernheimer and Schwartz (5). One hemolytic unit (HU) is defined as the amount of preparation which causes the release of 50% of the hemoglobin in a standardized (ca. 0.7% [vol/vol]) washed erythrocyte suspension. All cytolytic activity determinations, except those for the hemolytic spectrum studies, were performed with washed mouse erythrocytes.

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The purified cytolysin preparation (stage 4) was assayed for activity against Chinese hamster ovary cells as described by Kreger and Lockwood (17). The median lethal doses of the purified cytolysin were determined with mice as described for partially purified cytolysin preparations (18). Purified cytolysin (0.1 ml of stage 4 preparation containing ca. 168 μg of protein) was examined for protease activity as previously described (19, 30).

Protein was estimated by the method described by Bradford (6), with bovine gamma globulin used as the standard. The standard and the assay reagent were obtained from Bio-Rad Laboratories, Inc. (Richmond, Calif.).

Bacterium and preparation of seed culture. *V. damsela* 1421-81 was kindly supplied by F. Hickman-Brenner of the Centers for Disease Control (Atlanta, Ga.). Seed cultures were prepared as previously described (18), except the Columbia agar was inoculated with three loopsful of rapidly thawed specimens of the bacteria.

Purification of cytolysin. Unless otherwise noted, all steps were done at approximately 4°C.

Stage 1. Culture supernatant fluids. Culture supernatant fluids from six heart-infusion diffusate broth cultures were obtained as previously described (18).

Stage 2. Ammonium sulfate precipitation. Ammonium sulfate (enzyme grade; Schwarz/Mann, Cambridge, Mass.) was dissolved in the pooled culture supernatant fluids to a final concentration of ca. 60% saturation (420 g/liter). After 16 to 18 h, the precipitate was recovered by centrifugation (16,000 × *g*, 20 min) and dissolved in 8 ml of 0.1 M ammonium bicarbonate (pH 7.8). A small amount of insoluble residue was removed by centrifugation (20,000 × *g*, 20 min).

Stage 3. Sephadex G-100 gel filtration. The stage 2 preparation was applied to a column (2.6 by 95 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.1 M ammonium bicarbonate and was eluted at a flow rate of 20 ml/h (ca. 3.8 ml/cm^2 per h). Fractions (5 ml) were assayed for absorbance at 280 nm and for cytolytic activity. The cytolysin peak fractions (usually 12 to 14 fractions) were pooled.

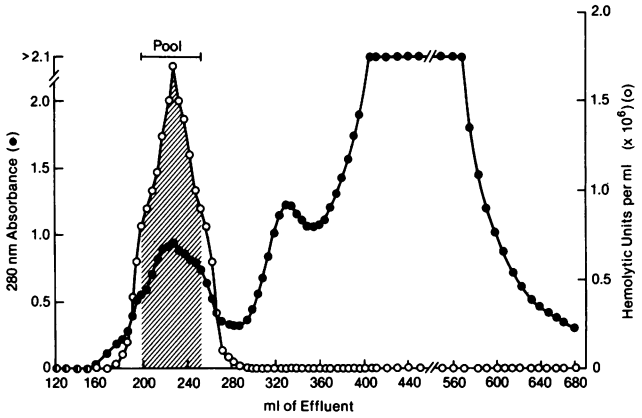

FIG. 1. Sephadex G-100 gel filtration of stage 2 cytolysin preparation. The cross-hatched area indicates the fractions that were pooled to form the stage 3 cytolysin preparation.

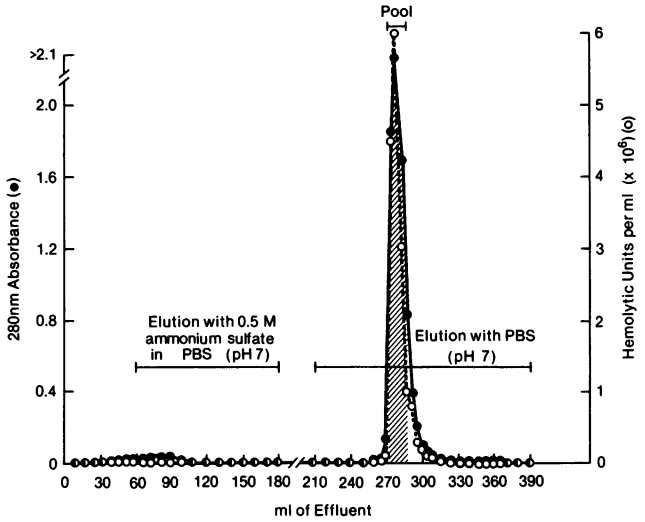

FIG. 2. Hydrophobic interaction chromatography of stage 3 cytolysin preparation. The cross-hatched area indicates the fractions that were pooled to form the stage 4 cytolysin preparation.

Stage 4. Hydrophobic interaction chromatography. Ammonium sulfate was dissolved in the stage 3 preparation to a final concentration of 0.5 M, and the preparation was applied to a column (1.6 by 30 cm) of phenyl-Sepharose CL-4B (Pharmacia) equilibrated with PBS containing 0.5 M ammonium sulfate. The column was washed (50 ml/h; 5-ml fractions were collected) with ca. three bed volumes (180 ml) of equilibrating buffer, and the cytolysin was eluted by washing the column (16 to 20 ml/h; 4- to 5-ml fractions were collected) with PBS. The fractions were assayed for absorbance at 280 nm and for cytolytic activity, and the cytolysin peak fractions (usually four fractions) were pooled.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed with an LKB 2117-401 immunoelectrophoresis kit and Tris-barbital buffer (0.02 M, pH 8.6). The general methodology described in the LKB instruction manual and application note 249 (32) and in the handbook of immunoprecipitation-in-gel techniques edited by Axelsen (2) was followed. The antiserum used in the study was raised against the stage 2 cytolysin preparation as previously described (18).

Slab SDS-PAGE. Slab sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (SDS-PAGE) was performed by a modification (10) of the method described by Laemmli (20).

Molecular weight estimation. The molecular weight of the denatured and reduced stage 4 cytolysin was estimated by the relative mobility method described by Weber et al. (33) by the slab SDS-PAGE protocol. The apparent molecular weight of the native stage 4 cytolysin was estimated by the gel filtration method described by Andrews (1) with Sephadex G-100 as previously described (18), except the equilibrating and eluting buffer was 0.1 M ammonium bicarbonate.

Analytical isoelectric focusing. Analytical thin-layer isoelectric focusing in polyacrylamide gel was performed with an LKB 2117 Multiphor electrophoresis apparatus and commercial PAG plates (pH 3.5 to 9.5) as recommended by the manufacturer. Previously described isoelectric point standards (10) were obtained from Pharmacia.

Amino acid analyses. Samples of a stage 4 cytolysin preparation were lyophilized after being concentrated and transferred into 0.1 M ammonium bicarbonate with a Centricon-30 microconcentrator (Amicon Corp., Danvers, Mass.). The amino acid composition and amino-terminal amino acid sequence of the cytolysin were determined as previously described for the *Vibrio vulnificus* cytolysin (10).

Inactivation studies. Samples of cytolysin (10,000 HU) were tested for heat sensitivity (at 25, 30, 37, 56, and 100°C for 30 min) in PBS and PBS-BA and for sensitivity to inactivation at pH 4, 6, 7, 8, and 10 (24 h at 4°C) in the absence of BA.

Sensitivity to proteases was examined by incubating the cytolysin (10,000 HU) in PBS (final volume, 1 ml) for 30 min at 30°C with soluble Pronase-CB (Calbiochem-Behring, La Jolla, Calif.) and bovine pancreatic trypsin (3× crystallized;

TABLE 1. Purification of extracellular cytolysin produced by *V. damsela* 1421-81

Purification stage	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Activity (HU/ml; $\times 10^3$)	Total activity (HU; $\times 10^6$)	Recovery (%)	Sp act (HU/mg protein; $\times 10^3$)
1. Culture supernatant fluids	1,120	0.175	196	100	112	100	571
2. Ammonium sulfate precipitation	11.6	13.125	152.3	9,400	109	97	716
3. Sephadex G-100 gel filtration	62	0.920	57	1,200	74	66	1,304
4. Hydrophobic interaction chromatography	18	1.675	30.1	3,400	61	54	2,030

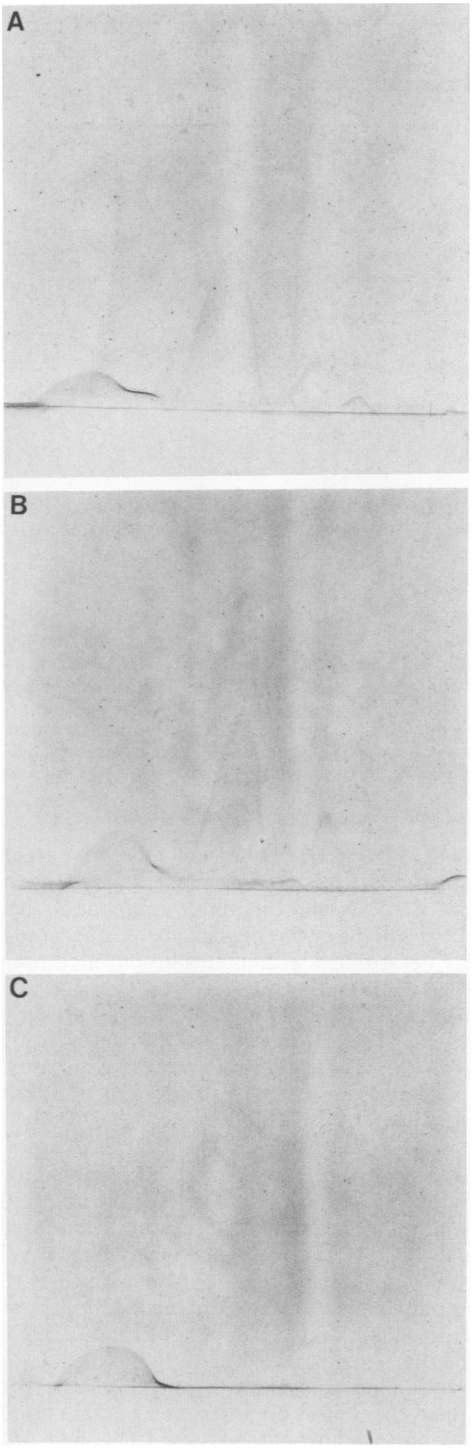

FIG. 3. Crossed immunoelectrophoresis of *V. damsela* cytolysin preparations. First dimension: Samples were placed into wells (4-mm diameter) cut in gels composed of 1.2% (wt/vol) agarose (Bio-Rad Laboratories) in Tris-barbital buffer and were electrophoresed (anode to right) at 9 V/cm for 60 min at 12°C. Second dimension: The upper part of each gel (ca. 61 cm^2) was composed of a 1.2% agarose gel (11 ml) containing 1.5 ml of anti-crude cytolysin antiserum. Electrophoresis (anode at top) was performed at 2 V/cm for 18 h at 15°C. (A) Stage 2, 100 μg; (B) stage 3, 80 μg; (C) stage 4, 40 μg. Gels were stained with Coomassie brilliant blue R-250.

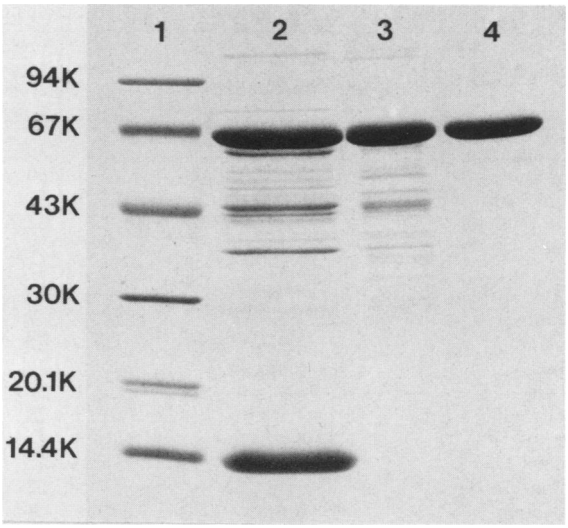

FIG. 4. SDS-PAGE of *V. damsela* cytolysin preparations. Cathode is at the top of the gel. Preparations were denatured under reducing conditions (β-mercaptoethanol in the disruption solution). Lane 1, molecular weight markers (Pharamacia; numbers are in thousands [k]); lane 2, stage 2 (100 μg); lane 3, stage 3 (40 μg); lane 4, stage 4 (30 μg). Gels were stained with Coomassie brilliant blue R-250.

Worthington Diagnostics, Freehold, N.J.), and assaying the mixtures for residual cytolytic activity.

Samples of cytolysin (10,000 HU in 0.1 ml PBS) were tested for sensitivity to inactivation by trypan blue (Eastman Kodak Co., Rochester, N.Y.), dithiothreitol (electrophoresis grade; Bio-Rad Laboratories), chelating agents (EGTA

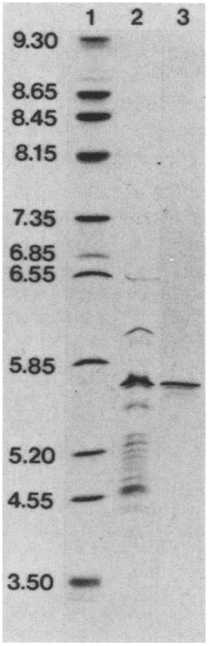

FIG. 5. Analytical thin-layer isoelectric focusing of *V. damsela* cytolysin preparations. Cathode is at the top of the gel. Lane 1, pI markers (pIs are indicated to the left of the lane); lane 2, stage 2 preparation (70 μg); lane 3, stage 4 preparation (30 μg). Gel was stained with Coomassie brilliant blue R-250

TABLE 2. Amino acid composition of *V. damsela* cytolysin

Amino acid	Concn found (mol%)	No. of residues per molecule of cytolysin[a]
Aspartic acid	16.29	98
Threonine	5.07	30
Serine	10.54	60
Glutamic acid	9.36	57
Proline	3.83	22
Glycine	5.27	28
Alanine	6.64	37
Valine	3.79	22
Methionine	1.13	7
Isoleucine	5.93	35
Leucine	6.69	40
Tyrosine	3.99	25
Phenylalanine	4.70	29
Lysine	8.40	51
Histidine	1.90	12
Arginine	3.48	22
Half-cystine	0.64	4
Tryptophan	2.34	15

[a] Based on a molecular weight of 69,000.

and disodium EDTA), glycophorin (Sigma Chemical Co., St. Louis, Mo.), and suspensions of various lipids (cholesterol, mixed bovine brain gangliosides, and cardiolipin [Sigma]; sphingomyelin and phosphatidylcholine [Supelco, Inc., Bellefonte, Pa.]; and phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine [Serdary Research Laboratories, London, Ontario, Canada]) as previously described (18), except the cytolysin-reagent mixtures (final volume, 1 ml) were incubated at 25°C for 15 min before assaying for residual activity. The organic solvents in which the lipids were dissolved were evaporated with a stream of nitrogen, and the lipids were suspended in 0.9 ml of PBS by blending with a Vortex mixer for 1 min.

The effect of various divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}) on cytolysin activity was examined by assaying the cytolysin in the presence of the reagents. The assays in the presence of Ca^{2+} and Mg^{2+} were performed with Tris-buffered saline (0.02 M Tris-hydrochloride–0.15 M NaCl [pH 7]) supplemented with 1 mg of BA per ml, instead of PBS-BA.

RESULTS AND DISCUSSION

Purification of cytolysin. The behavior of the *V. damsela* cytolysin during sequential gel filtration and hydrophobic interaction chromatography is shown in Fig. 1 and 2, respectively, and the quantitative results of the purification scheme are summarized in Table 1. The specific activity of the stage 4 cytolysin (ca. 2×10^6 HU/mg of protein) is similar to the values reported for the most potent cytolytic toxin, streptolysin S (16, 21). The stage 4 cytolysin was homogeneous by crossed immunoelectrophoresis (Fig. 3) and SDS-PAGE (Fig. 4) and did not contain detectable protease activity against azocasein. Analytical thin-layer isoelectric focusing in polyacrylamide gel (Fig. 5) showed the presence of a major, Coomassie blue-staining component and two minor, closely migrating components in the stage 4 cytolysin preparation. However, toxinogram analysis (data not shown) with an overlay of mouse erythrocytes in agarose (31) indicated that all three components possessed hemolytic activity. The microheterogeneous nature of the purified *V. damsela* cytolysin is not unique. Various other bacterial cytolysins are also known to exhibit microheterogeneity (3).

TABLE 3. Sensitivity of erythrocytes from various animal species to *V. damsela* cytolysin

Animal species[a]	Sp act (HU/mg protein; $\times 10^3$)[b]	Relative sensitivity (%)[c]
Mouse	1,600, 1,600, 1,600, 1,600	100
Rat	1,080, 1,080, 1,130	68 to 71
Damselfish	113, 113, 113	7
Rabbit	13, 13, 13, 96	0.8 to 6
Guinea pig	<0.2, <0.2, <0.2, <0.2	<0.01
Human	<0.2, <0.2, <0.2, <0.2	<0.01
Pigeon	<0.2, <0.2	<0.01
Monkey	<0.2, <0.2, <0.2. <0.2	<0.01
Cow	<0.2	<0.01
Sheep	<0.2, <0.2, <0.2, <0.2	<0.01
Pig	<0.2	<0.01
Horse	<0.2	<0.01
Chicken	<0.2	<0.01
Goat	<0.2	<0.01
Cat	<0.2	<0.01
Dog	<0.2	<0.01
Burro	<0.2	<0.01
Hamster	<0.2, <0.2	<0.01

[a] Sources of mammalian and avian bloods used to prepare the erythrocyte suspensions were as previously described (18). The damselfish blood was kindly supplied by Jo Ellen Hose (Occidental College, Los Angeles, Calif.).

[b] Multiple values were obtained with erythrocyte suspensions prepared from individual mice, rats, damselfish, rabbits, guinea pigs, humans, pigeons, monkeys, sheep, and hamsters.

[c] Compared with sensitivity of mouse erythrocytes.

Estimation of isoelectric point and molecular weight. The major cytolysin in the stage 4 preparation had an isoelectric point (pI) of ca. 5.60, and the minor components had pIs of 5.45 and 5.55. The pI of the major cytolysin is similar to the value of 5.7 determined with a partially purified preparation of the cytolysin (18).

The molecular weight of the stage 4 cytolysin was ca. 69,000 by SDS-PAGE and 52,000 by gel filtration with Sephadex G-100. The molecular weight estimated by gel filtration is similar to the molecular weight of 57,000 previously determined by gel filtration of a partially purified cytolysin preparation (18).

Amino acid analyses. The amino acid composition of the purified cytolysin is shown in Table 2. Basic, acidic, and nonpolar hydrophobic amino acid residues accounted for ca. 14, 26, and 35% of the total residues, respectively. The presence of four half-cystine residues suggests that the cytolysin could have two intrachain disulfide bonds. The minimum molecular weight calculated from all 594 residues was ca. 68,000. The similarity of the molecular weights estimated by amino acid analysis and SDS-PAGE (69,000) indicates that the cytolysin does not contain appreciable amounts of non-amino acid material. The first 10 amino-terminal amino acid residues of the stage 4 cytolysin preparation were Phe-Thr-Gln-Trp-Gly-Gly-Ser-Gly-Leu-Thr. The amino-terminal amino acid of the Kanagawa phenomenon-associated hemolysin of *Vibrio parahaemolyticus* also is phenylalanine (22).

Inactivation studies. The cytolysin lost 30, 90, 99.5, and 100% of its activity when heated for 30 min at 30, 37, 56, and 100°C, respectively. The addition of BA (1 mg/ml) to the purified cytolysin preparation increased its heat stability (loss of 0 and 70% of its activity when heated for 30 min at 37

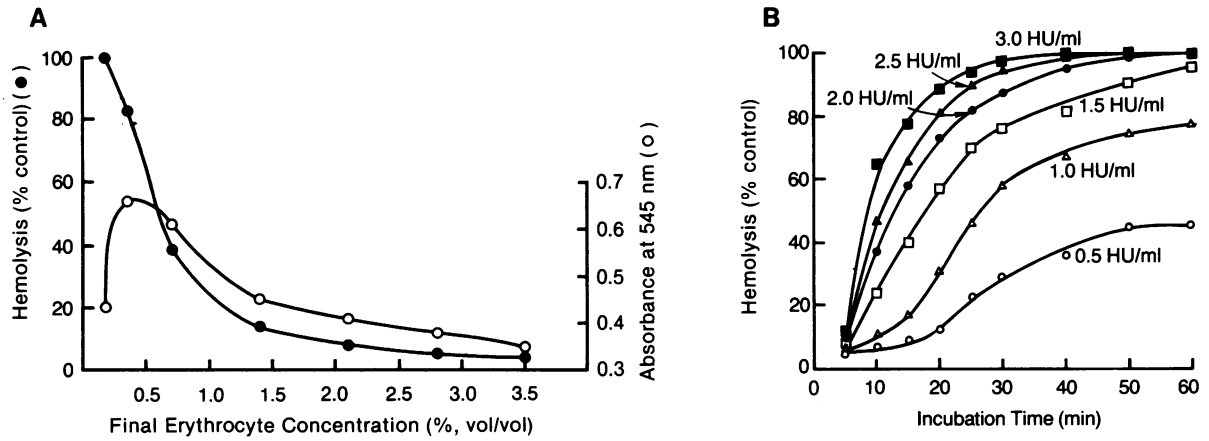

FIG. 6. Effect of erythrocyte concentration and cytolysin concentration on erythrocyte lysis by *V. damsela* cytolysin. (A) Effect of erythrocyte concentration. Samples (1 ml) of washed mouse erythrocyte suspensions were incubated with stage 4 cytolysin (2 HU in 1 ml of PBS-BA) for 30 min at 37°C, the erythrocyte-cytolysin mixtures were centrifuged (750 × *g*, 7 min) to sediment the unlysed erythrocytes, and the absorbance of the supernatant fluids was determined at 545 nm and compared with that of the control, saponin-lysed erythrocyte suspensions (0.18 to 3.5% [vol/vol]). (B) Kinetics of erythrocyte lysis as a function of cytolysin concentration. Samples (1 ml) of a washed mouse erythrocyte suspension (0.7% [vol/vol]) were incubated with 0.5 to 3 HU of stage 4 cytolysin (in 1 ml of PBS-BA) for 5 to 60 min at 37°C, and the absorbance of the supernatant fluids was compared with that of a control, saponin-lysed erythrocyte suspension.

and 56°C, respectively). The relative heat stability reported previously (18) for the partially purified cytolysin (no loss of activity when heated for 30 min at 37°C) may have been due to contaminating proteins that have a stabilizing effect similar to that of BA.

The cytolysin was unstable at pH 4, 6, and 10 (4°C for 24 h) and was inactivated by trypsin and pronase. Activity was not affected by phospholipids (phosphatidylserine, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, cardiolipin, and sphingomyelin; 100 μg), mixed gangliosides (100 μg), cholesterol (100 μg), glycophorin (100 μg), trypan blue (50 μg), dithiothreitol (5×10^{-3} M), chelating agents (EDTA and EGTA; 10^{-3} M), and divalent cations (Ca^{2+}, Mg^{2+}, and Zn^{2+}; 10^{-3} M). The previously reported inactivation of the partially purified cytolysin by EDTA, EGTA, and dithiothreitol (18) may have been due to contaminants that require the additives for inhibition of cytolysin activity.

Cytolytic activity. The cytolysin was active against erythrocytes from 4 of the 18 animal species examined (Table 3) and was most active against mouse erythrocytes. The erythrocyte spectrum differs from those of cytolysins produced by *Vibrio cholerae* biotype El Tor (12), non-O1 *V. cholerae* (34), and *V. vulnificus* (10) and from the Kanagawa phenomenon-associated hemolysin of *V. parahaemolyticus* (35). A partially purified *V. damsela* cytolysin preparation previously has been found (18) to be weakly active against 13 of the erythrocyte species refractory to the highly purified cytolysin in the current study. This observation suggests either the presence of more than one cytolysin in the partially purified preparation or that the cytolysin requires a factor (present only in the partially purified cytolysin preparation) to lyse the usually insensitive erythrocytes. The purified cytolysin also was cytolytic for Chinese hamster ovary cells in tissue culture (minimal cytotoxic dose ca. 2.5 HU [ca. 1 ng]).

Variables influencing erythrocyte lysis. Variables affecting the hemolytic process were studied by using washed mouse erythrocytes as target cells. The percentage of hemolysis decreased as the target cell concentration was increased (Fig. 6A). In addition, the absolute amount of lysis (measured by absorbance at 545 nm) increased to a peak and then decreased. These observations suggest that more than one molecule of the cytolysin (a multi-hit process) is required to lyse a single erythrocyte (13–15, 24, 28). When the dose-response data in Fig. 6B were used to construct a multi-hit survival curve as described by Inoue et al. (13) (the logarithm of the fraction of unlysed erythrocytes was plotted versus the cytolysin concentration), the ordinate intercept of the linear part of the curve indicated that hemolysis by the *V. damsela* cytolysin requires ca. two hits (data not shown).

The manner in which the rate of lysis depends on the cytolysin concentration also was revealed by analysis of the curves shown in Fig. 6B. The result of plotting the slope of the linear part of each curve as a function of the cytolysin concentration indicated that the rate of erythrocyte lysis is directly proportional to the cytolysin concentration (data not shown).

The rate of erythrocyte lysis by the cytolysin was temperature and pH dependent and was optimal at 37 to 47°C and at pH 7 to 9 (Fig. 7). Hemolysis was not observed at 4°C. Therefore, experiments were done to determine whether the cytolysin binds to mouse erythrocytes at 4°C. Cytolysin preparations (2 HU per ml of PBS-BA) were incubated with equal volumes of an erythrocyte suspension (0.7% [vol/vol]) at 4°C for up to 5 min, and samples were centrifuged at 1-min intervals. The supernatant fluids were carefully removed, and the cells were washed and suspended in PBS-BA and incubated at 37°C for 30 min. In addition, the amount of activity remaining in the supernatant fluids was determined by incubating the supernatant fluids with fresh erythrocytes at 37°C for 30 min. The amount of hemolysis that occurred was determined spectrophotometrically. We found that enough cytolysin had become associated with the erythrocytes after 1 min at 4°C to cause 80% hemolysis (data not shown). In addition, very little activity was associated with the supernatant fluids. Our results indicate that hemolysis by the *V. damsela* cytolysin is at least a two-step process consisting of a temperature-independent, cytolysin-binding step followed by a temperature-dependent, membrane-perturbation step(s) that leads to cell lysis.

The data in Fig. 7A were used to construct an Arrhenius

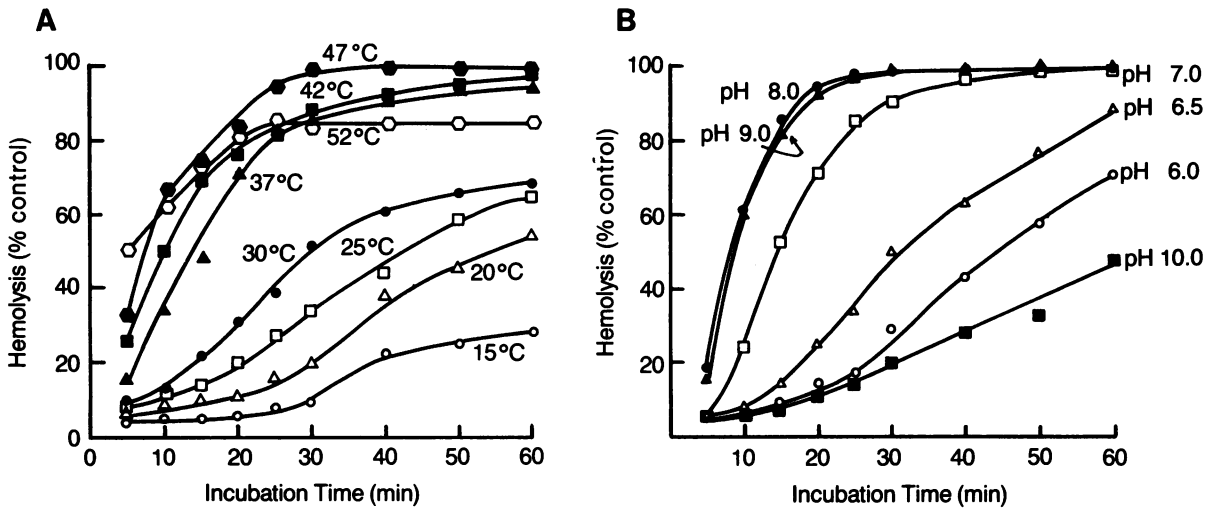

FIG. 7. Kinetics of erythrocyte lysis of *V. damsela* cytolysin as a function of temperature and pH. (A) Effect of temperature on lysis. Samples (1 ml) of a washed mouse erythrocyte suspension (0.7% [vol/vol]) were incubated with stage 4 cytolysin (2 HU in 1 ml of PBS-BA) for 5 to 60 min at 15, 20, 25, 30, 37, 42, 47, and 52°C, and the supernatant fluids of the mixtures were obtained and examined as described in the legend to Fig. 6. Hemolysis was not observed at 4°C. (B) Effect of pH on lysis. Samples (1 ml) of a washed mouse erythrocyte suspension (0.7% [vol/vol]) in PBS (adjusted to pH 6, 6.5, 7, 8, 9, and 10) were incubated with stage 4 cytolysin (2 HU in 1 ml of PBS-BA at pH 6, 6.5, 7, 8, 9, and 10) for 5 to 60 min, and the supernatant fluids of the mixtures were obtained and examined as described in the legend to Fig. 6. Hemolysis was not observed in control erythrocyte suspensions (without cytolysin) incubated for 60 min.

plot (the logarithm of the rate of lysis versus the reciprocal of the absolute temperature). The energy of activation for the cytolysin, estimated from the Arrhenius equation and the slope of the Arrhenius plot, was ca. 11,370 cal/mol (data not shown).

Lethal activity. The median lethal doses of the stage 4 cytolysin by the intraperitoneal, intravenous, and subcutaneous routes were 58 HU per mouse (ca. 1 μg/kg), 108 HU per mouse (ca. 2 μg/kg), and 1,000 HU per mouse (ca. 18 μg/kg), respectively. These values are similar to those previously determined with a partially purified cytolysin preparation (18). Mice injected subcutaneously with one median lethal dose were lethargic and had ruffled fur, encrustations around their eyelids, and severe local edema similar to that observed previously (18) in mice infected with *V. damsela* and in mice injected subcutaneously with the partially purified cytolysin preparation.

In conclusion, this paper contains additional information on the physicochemical and biological properties of a recently discovered cytolytic toxin produced by *V. damsela*. The scheme developed for obtaining large amounts of highly purified toxin should enable investigators to further examine the possible role of the toxin in the pathogenesis of *V. damsela* disease. Also, the narrow hemolytic spectrum of the cytolysin suggests that it may prove to be useful, as have other membrane-damaging toxins, as a probe in studies of membrane structure (4, 9, 25, 29).

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