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Extracellular Phospholipase A_2 and Lysophospholipase Produced by *Vibrio vulnificus*

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Phospholipase A_2 and lysophospholipase activities were detected in the culture supernatant fluids of a virulent strain of *Vibrio vulnificus*. The phospholipase A_2 was inactivated by heating at 56°C for 30 min, had an apparent molecular weight of ≥80,000 (estimated by gel filtration with Sephadex G-75), and a pI of ca. 5.0. Phospholipid hydrolysis was unaffected by Ca^{2+} or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and was optimal at pH 5.0 to 5.5. The lysophospholipase was not affected by heating at 56°C for 30 min but was inactivated at 100°C and had an apparent molecular weight of ≥80,000 and a pI of ca. 4.0. The enzymes were detected coincidentally with a previously described extracellular cytolysin of *V. vulnificus*; however, they were physically separable from the toxin (which did not possess phospholipase A, C, or D activity) by gel filtration with Sephadex G-75.

Vibrio vulnificus, previously called "lactose-positive *Vibrio*" and *Beneckea vulnifica* (2–4, 7, 13, 17–19, 21, 28, 29), is a halophilic bacterium recently recognized as an etiological agent of severe wound infections, septicemia, meningitis, pneumonia, and keratitis in humans (5, 7, 8, 10, 12, 13, 16–22, 28, 29, 32, 34). The bacterium produces an extracellular toxin(s) possessing cytolytic activity against mammalian erythrocytes and Chinese hamster ovary cells, vascular permeability factor activity in guinea pig skin, and lethal activity for mice (23). We recently found that a crude preparation of the cytolysin caused cell lysis and degradation of membrane phospholipids of Madin-Darby canine kidney cells, and our observation prompted us to investigate the possibility that the cytolysin is a phospholipase. In this paper, we report that *V. vulnificus* E4125 produces an extracellular phospholipase A_2 and a lysophospholipase, but that the enzymes are distinct from the major cytolysin produced by the bacterium.

MATERIALS AND METHODS

Bacterium. *V. vulnificus* E4125 was kindly supplied by R. E. Weaver and D. G. Hollis of the Centers for Disease Control, Atlanta, Ga. The bacterium was isolated from the blood of a septicemic human and had a median lethal dose for 6-week-old (25- to 28-g) Dub:(ICR) strain randomly bred albino mice of ca. 5×10^6 CFU injected subcutaneously (23).

Preparation of CFC and partially purified cytolysin. The bacteria were grown for ca. 7 h in heart infusion broth (Difco Laboratories, Detroit, Mich.), and the culture filtrate was obtained as previously described (23). The culture filtrate (ca. 300 ml) was dialyzed for 48 h at 4°C against 4 liters of 0.01 M Na_2HPO_4-HCl (pH 7) buffer (buffer was changed three times during dialysis), and after lyophilization the culture filtrate concentrate (CFC) was stored at −10°C. The heart infusion broth concentrate which was used as a control was obtained by dialyzing and lyophilizing the uninoculated broth as described above.

A partially purified cytolysin preparation was obtained by slowly dissolving ammonium sulfate (enzyme grade; Schwarz/Mann, Inc., Spring Valley, N.Y.) in the culture filtrate, with gentle stirring at 4°C, to a final concentration of ca. 50% saturation (350 g/liter). After 18 h at 4°C, the precipitate was recovered by centrifugation (16,000 × *g*, 15 min, 4°C) and dissolved in 1 volume of cold phosphate-buffered saline (PBS; 0.02 M Na_2HPO_4-HCl, 0.15 M NaCl, pH 7.4), which was 1/75th the volume of the original culture filtrate. A small amount of insoluble residue was removed by centrifugation, and the preparation was stored at −70°C.

Preparation of radiolabeled lipid substrates. Mixtures of phospholipids double labeled with ^3H and ^{14}C (used to assay for phospholipase activity and to obtain radiolabeled diglycerides for lipase assay) or single labeled with ^{14}C (used to obtain radiolabeled 1-acyl lysophospholipids) were prepared from Madin-Darby canine kidney cells (Flow Laboratories, Inc., McLean, Va.) grown in medium supplemented with [5,6,8,9,11,12,14,15-^3H]arachidonic acid and [1-^{14}C]palmitic acid (Amersham Corp., Arlington Heights, Ill.) or [1-^{14}C]palmitic acid (14). The preparations were depleted of neutral lipids by silicic acid chromatography (15) and stored in an atmosphere of nitrogen at −10°C. The single-labeled phospholipids obtained in this manner were labeled in the sn-1 position with ^{14}C. The double-labeled phospholipids were labeled in the sn-1 position with ^{14}C and in the sn-2 position with both ^{14}C and ^3H, because snake venom phospholipase A_2 hydrolysis (method described below) was found to remove 92% of the ^3H label and 60% of the ^{14}C label.

Purified ^{32}P-^3H-labeled phosphatidylcholine (PC) used to determine the specificity of the *V. vulnificus* phospholipase A was obtained from Madin-Darby canine kidney cells cultured in the presence of ^{32}P (1 mCi/ml) and [^3H]arachidonic acid (10 μCi/ml) for 16 h at 37°C. The double-labeled phospholipids were extracted and depleted of neutral lipids, and the individual phospholipid classes were separated by high-pressure liquid chromatography (Dupont 850 Liquid Chromatography System equipped with a heated column compartment; Du Pont Co., Wilmington, Del.) with a Zorbaxsil column (4.6 mm by 25 cm; Du Pont). The phospholipids were eluted at 50°C and a flow rate of 2 ml/min with a solvent gradient containing 1.5 to 9% water in a mixture of 2-

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propanol and hexane (10:9 [vol/vol]). Purified ^{32}P-^{3}H-labeled PC was dried under nitrogen, dissolved in chloroform, and stored under nitrogen at −10°C. The purified PC was predominantly labeled in the sn-2 position with ^{3}H because ca. 88% of the ^{3}H label could be removed from the purified PC by hydrolysis with snake venom phospholipase A_2 (see Table 3).

Mixtures of radiolabeled 1-acyl lysophospholipids used to assay for lysophospholipase activity were prepared as follows. Mixtures of phospholipids single-labeled in the sn-1 position with [^{14}C]palmitate (1.5×10^6 ^{14}C dpm) and 250 μl of a solution of hexadecyltrimethylammonium chloride (0.02 M, in chloroform; Eastman Kodak, Rochester, N.Y.) contained in a 15-ml, round-bottom screw-capped tube were dried under nitrogen. The dried film was dissolved in 1 ml of ethyl ether, and 1 mg of a preparation of snake venom (*Crotalus adamanteus*) phospholipase A_2 (Sigma Chemical Co., St. Louis, Mo.) contained in 1 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4) supplemented with 0.01 M $CaCl_2$ was added. The tube was flushed with nitrogen, capped tightly, and shaken for 2 h at room temperature. The ether layer was evaporated under nitrogen, the lipids were extracted and depleted of neutral lipids, and the preparation was stored under nitrogen at −10°C. Hydrolysis of the substrate was 85 to 90% complete.

Radiolabeled diglycerides used to assay for lipase activity were prepared by hydrolyzing ^{3}H-^{14}C-labeled phospholipids (ca. 10^6 ^{3}H dpm in 2 ml of ethyl ether) with 2 mg of a *Bacillus cereus* phospholipase C preparation (Sigma) contained in 2 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4). The mixture was incubated under nitrogen for 3 h at room temperature with shaking, the generated diglycerides were extracted by a modification of the Bligh and Dyer method (9), and the preparation was stored under nitrogen at −10°C.

Phospholipase and lysophospholipase assays. Lipid substrates containing at least 50,000 ^{3}H dpm (0.25 Ci of ^{3}H per mol of phosphorus; phosphorus was determined as described by Rouser et al. [33]), 15,000 ^{14}C dpm, or 75,000 ^{32}P cpm in 25 μl of absolute ethanol were incubated for 1 or 2 h with shaking at 37°C with 1 ml of the preparations to be assayed. The incubation time, the concentration of the preparations assayed and the buffer used varied according to the purpose of the experiment. The reactions were terminated by adding methanol (2 ml), the lipids were extracted by a modification of the Bligh and Dyer method (9), and the lipid extracts were dried under nitrogen and dissolved in 50 to 100 μl of chloroform. Lipid standards (Serdary Research Laboratories, London, Ontario, Canada) and samples of the lipid extracts of the incubation mixtures were spotted onto Silica Gel 60 plates (American Scientific Products, McGaw Park, Ill.), and the plates were developed in two different solvent systems. Neutral lipids were separated in a system of hexane-ethyl ether-formic acid (90:60:6 [vol/vol/vol]), and phospholipids were separated in a system of chloroform-methanol-acetic acid-water (75:48:12.5:4.5 [vol/vol/vol/vol]). Lipid spots were visualized with iodine vapors and scraped into vials (17 by 54 mm) containing 6 ml of Budget-Solve scintillation fluid (Research Products International Corp., Mount Prospect, Ill.), and the radioactivity was determined in a liquid scintillation counter.

The peak fraction of phospholipase A_2 activity obtained by isoelectric focusing (method described below) was assayed for lipase activity against the labeled diglycerides. The assay conditions were as described above, and the reaction products were separated in the solvent system used for neutral lipids.

Specimens (200 μg) of the CFC and of the peak fraction of phospholipase A_2 activity obtained by isoelectric focusing (1 ml of a 1:10 dilution) were assayed for phospholipase C activity against *p*-nitrophenylphosphorylcholine (Calbiochem-Behring, La Jolla, Calif.) as described by Kurioka and Matsuda (24), except the assay mixtures were incubated for 90 min instead of 60 min.

Cytolysin assays. Two methods were used to estimate cytolytic activity against mammalian erythrocytes.

(i) Microtiter plate assay. Adjacent wells of 96-well microtiter plates having wells with conical bottoms (American Scientific Products) contained 100 μl of a serial doubling dilution of the preparation to be assayed and 50 μl of a standardized (0.7% [vol/vol]) sheep erythrocyte suspension in PBS (0.067 M Na_2HPO_4, 0.077 M NaCl [pH 7]). The plates were covered with plastic wrap and incubated at 37°C for 30 min. The cytolysin titer was determined after storing the plates for an additional 10 min at 4°C to allow unlysed erythrocytes to sediment. The cytolysin titer is defined as the reciprocal of the highest dilution causing complete hemolysis.

(ii) Tube assay. Cytolytic activity against mouse erythrocytes was determined by the tube method of Bernheimer and Schwartz (6). One hemolytic unit is defined as the amount of preparation which causes the release of 50% of the hemoglobin in the standardized (0.7% [vol/vol]) erythrocyte suspension.

Gel filtration. Gel filtration of a partially purified cytolysin preparation (10 ml containing ca. 500,000 hemolytic units) was performed as previously described (23), except Sephadex G-75 was used instead of Sephadex G-100. Fractions (5 ml) were assayed for cytolytic activity against mouse erythrocytes and for absorbance at 280 nm. Selected fractions also were assayed for phospholipase and lysophospholipase activities.

Glycerol density gradient isoelectric focusing. A sample of the CFC (45 mg in 5 ml of deionized water; included in the light gradient solution) was fractionated by high-speed isoelectric focusing (25, 36) at 4°C in a pH 3.5 to 5.0 glycerol density gradient containing glycine (1% [wt/vol]) and formed at 15 W for 17 h with an LKB 8100-1 column (LKB Instruments, Inc., Gaithersburg, Md.). The pH of each fraction (4 ml) was determined at 4°C, and the fractions were assayed for cytolysin, phospholipase, and lysophospholipase activities.

RESULTS

Extracellular phospholipase and lysophospholipase activity. The CFC hydrolyzed both acyl ester bonds of all classes of phospholipids, except sphingomyelin (Table 1). Free fatty acids were the only labeled neutral lipids generated by and detected after incubation of the ^{3}H-^{14}C-labeled phospholipids with the CFC, and the CFC did not possess detectable phospholipase C activity against *p*-nitrophenylphosphorylcholine (data not shown).

Phospholipase activity was not affected by incubating the CFC for 1 h at 37°C before the addition of the substrate (data not shown) but was abolished by heating at 56°C for 30 min (Table 1). Phospholipid hydrolysis by the CFC was detected from pH 4 to 9 but was optimal at pH 5.0 to 5.5, and neither the addition of Ca^{2+} (1 to 12.5 mM, final concentration in 0.1 M Tris-hydrochloride, 0.15 M NaCl [pH 7.5]) nor ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (2 mM, final concentration) to the assay mixtures altered the hydrolytic activity (data not shown).

The CFC also deacylated 1-acyl lysophospholipids (Table

TABLE 1. Hydrolysis of ^{3}H-^{14}C-labeled phospholipid substrate preparation by *V. vulnificus* CFC

Prepn tested	% Radiolabel recovered in:[a]									
	Sph		PC		PS-PI		PE		FFA[b]	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
CFC[c]	0.5	9.2	1.2	5.4	4.4	5.3	7.4	8.1	86.1	67.3
CFC-56°C[d]	0.9	8.1	15.3	53.1	13.3	10.5	65.1	20.9	4.5	6.1
Heart infusion broth concentrate	0.6	9.4	15.4	52.3	14.3	11.5	64.9	21.6	3.6	4.5
PBS[e]	0.8	8.3	15.2	52.3	14.2	11.2	61.4	22.1	7.6	6.3

[a] Abbreviations: Sph, sphingomyelin; PC, phosphatidylcholine; PS-PI, phosphatidylserine-phosphatidylinositol; PE, phosphatidylethanolamine; FFA, free fatty acids.
[b] Free fatty acids were the only labeled neutral lipids generated by and detected after incubation of the substrate preparation with the CFC.
[c] CFC (2 mg in 1 ml of PBS) was incubated for 1 h at 37°C with the phospholipid substrate.
[d] CFC (2 mg in 1 ml of PBS) was heated at 56°C for 30 min before incubation with the substrate.
[e] Composition, 0.067 M Na_2HPO_4-0.077 M NaCl (pH 7).

TABLE 2. Lysophospholipase activity in *V. vulnificus* CFC

Prepn tested	% Hydrolysis of 1-acyl lysophospholipids[a]
CFC[b]	57.6
CFC-56°C[c]	53.9
CFC-100°C[d]	13.4
PBS[e]	2.1

[a] Free fatty acids were the only labeled neutral lipids generated by and detected after incubation of the substrate with the CFC.
[b] CFC (2 mg in 1 ml of PBS) was incubated for 1 h at 37°C with the substrate preparation.
[c] CFC (2 mg in 1 ml of PBS) was heated at 56°C for 30 min before incubation with the substrate.
[d] CFC (2 mg in 1 ml of PBS) was heated at 100°C for 30 min before incubation with the substrate.
[e] Composition, 0.067 M Na_2HPO_4–0.077 M NaCl (pH 7).

2); however, the lysophospholipase activity was not affected by heating at 56°C for 30 min. Heating at 100°C for 30 min eliminated most of the activity.

The phospholipase purified by isoelectric focusing did not possess detectable phospholipase C activity against *p*-nitrophenylphosphorylcholine or lipase activity against labeled diglycerides (data not shown). The reaction products generated by hydrolysis of ^{32}P-^{3}H-labeled PC by the purified phospholipase (^{3}H-labeled fatty acids and ^{32}P-labeled lysophospholipids) are consistent with the conclusion that the enzyme is a phospholipase A_2 (Table 3).

Physicochemical differentiation of the phospholipase A_2, lysophospholipase, and cytolysin produced by *V. vulnificus*. Although the kinetics of appearance of extracellular phospholipase and cytolysin activities were similar (Fig. 1) and culture filtrates obtained from 7-h cultures contained phospholipase, lysophospholipase, and cytolysin activities (Tables 1 and 2, Fig. 1), the two enzymes were easily separated from the major cytolysin of the bacterium by gel filtration with Sephadex G-75 (Fig. 2 and Table 4). Phospholipase A_2 and lysophospholipase activities were eluted at the column void volume and therefore were associated with enzymes with apparent molecular weights of ≥80,000. However, the major extracellular cytolysin, which has been reported to be inactivated by heating at 56°C for 30 min and to have an apparent molecular weight of ca. 38,500 (23), was retarded by the Sephadex G-75 gel. In addition, the major peak of cytolysin activity did not release free fatty acids (Table 4), diglycerides, or phosphatidic acid (data not shown) from the phospholipid substrate, thus confirming the toxin's lack of phospholipase A, C, and D activity, respectively.

The results obtained by isoelectric focusing of the CFC further differentiated the extracellular enzymes from one another and the cytolysin from the phospholipase (Fig. 3). The lysophospholipase and cytolysin had pI values of ca. 4.0 and 3.8, respectively, whereas the phospholipase A_2 had a pI of ca. 5.0. A small amount of apparent lysophospholipase activity associated with the peak of phospholipase A_2 activity (Fig. 3, fraction 18) was abolished by heating at 56°C for 30 min, a treatment which had been found to not affect lysophospholipase activity (Table 2). Therefore, the lysophospholipase activity in the fraction apparently was an artifact caused by the action of the phospholipase A_2 on undegraded phospholipid contaminants in the lysophospholipid substrate preparation or by a low activity of phospholipase against lysophospholipids.

DISCUSSION

The results of the studies described in this paper indicate that *V. vulnificus* produces an extracellular phospholipase

TABLE 3. Hydrolysis of ^{32}P-^{3}H-labeled phosphatidylcholine by partially purified *V. vulnificus* phospholipase A_2 and snake venom phospholipase A_2

Treatment of substrate	% Radiolabel recovered in:[a]							
	PC		LPC		PA		FFA	
	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P
V. vulnificus phospholipase A_2[b]	50.2	78.0	4.4	21.1	0.4	0.3	44.5	0.3
Snake venom phospholipase A_2[c]	5.0	18.2	3.7	72.2	3.3	7.1	87.7	1.5
No enzyme	91.0	97.0	1.4	2.5	2.1	0.3	4.2	0.1

[a] Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PA, phosphatidic acid; FFA, free fatty acids.
[b] A sample (0.1 ml) of the phospholipase peak fraction obtained by isoelectric focusing was diluted with 0.9 ml of PBS and was incubated with the substrate for 2 h at 37°C.
[c] Hydrolysis was performed as described in the text.

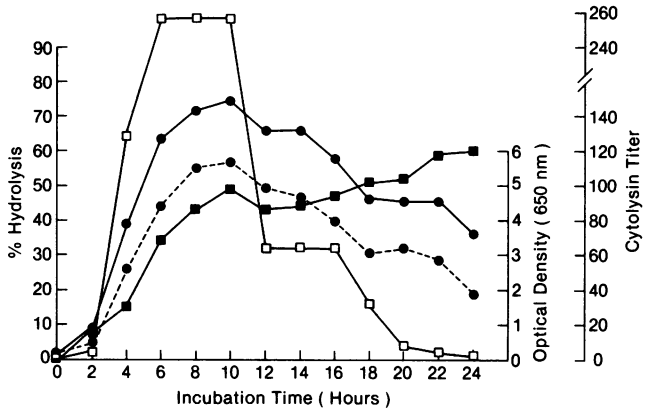

FIG. 1. Relationship between growth of *V. vulnificus* E4125 and the amount of extracellular phospholipase and cytolysin activities. The bacteria were grown as previously described (23) in a 2-liter flask containing 200 ml of heart infusion broth. Growth was followed turbidimetrically at 650 nm in cuvettes (1-cm light path) in a model DB-GT spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) (■). Culture filtrates were assayed for cytolytic activity against sheep erythrocytes by the microtiter plate method (□), and samples (0.1 ml) of the culture filtrates were diluted with 0.9 ml of PBS and assayed for phospholipase activity (●) against a mixture of ^{3}H-^{14}C-labeled phospholipids (1 h at 37°C), as described in the text (solid line indicates release of ^{3}H-labeled fatty acids and the dashed line indicates release of ^{14}C-labeled fatty acids.)

A_2 and lysophospholipase which are distinct from a previously recognized cytolysin (23) of the bacterium. A related bacterium, *Vibrio parahaemolyticus*, also has been found to produce an extracellular phospholipase A and a lysophospholipase (30, 37). In addition, various other members of the family *Vibrionaceae*, such as *Aeromonas hydrophila* and *Aeromonas salmonicida*, have been reported to produce an extracellular glycerophospholipid:cholesterol acyltransferase which also possesses lysophospholipase activity and, in the absence of cholesterol, phospholipase A activity (11, 26, 27).

Fatty acids esterified at both the sn-1 and sn-2 positions of double-labeled phosphoglycerides (i.e., phospholipids with a glycerol backbone) were released during incubation of the phospholipids with the CFC of the bacterium, and labeled mono- or diglyceride reaction products were not detected in the incubation mixtures. Evidence that hydrolysis of both acyl ester bonds was not caused by the sequential action of a phospholipase C and a nonspecific lipase, or by the sole action of a lipase, was obtained by the observation that

TABLE 4. Phospholipase, lysophospholipase, and cytolytic activities in selected fractions obtained by Sephadex G-75 gel filtration of partially purified *V. vulnificus* cytolysin

Fraction no.	Cytolytic activity (hemolytic units per ml)[b]	% Hydrolysis of:[a]		
		Phospholipids		1-Acyl lysophospholipids (^{14}C release)
		^{3}H release	^{14}C release	
8	400	38.0	24.6	61.1
17	400	35.9	12.9	10.9
28	20,000	0.8	0.5	2.6
63	20	3.8	1.0	7.7

[a] Samples (0.1 ml) of the fractions were diluted with 0.9 ml of PBS and were incubated with the substrates for 1 h at 37°C.

[b] Determined against mouse erythrocytes by the tube assay of Bernheimer and Schwartz (6).

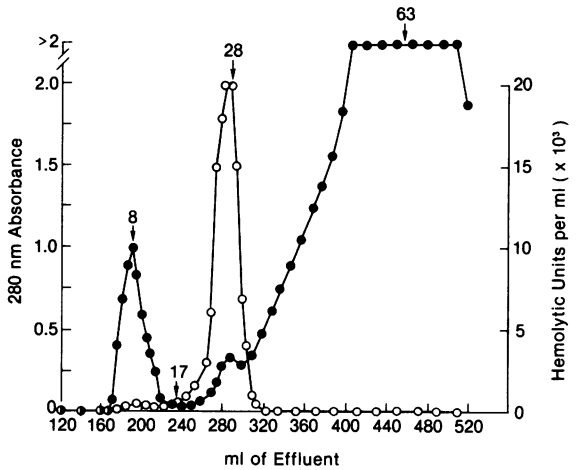

FIG. 2. Gel filtration of partially purified *V. vulnificus* cytolysin preparation with Sephadex G-75. Fractions (5 ml) were assayed for cytolytic activity against mouse erythrocytes by the tube assay (○) and for absorbance at 280 nm (●). In addition, samples (0.1 ml) of the four fractions indicated with numbered arrows were diluted with 0.9 ml of PBS and assayed for phospholipase activity against a mixture of ^{3}H-^{14}C-labeled phospholipids and for lysophospholipase activity against a mixture of ^{14}C-labeled 1-acyl lysophospholipids (Table 4), as described in the text. The column void volume was ca. 180 ml.

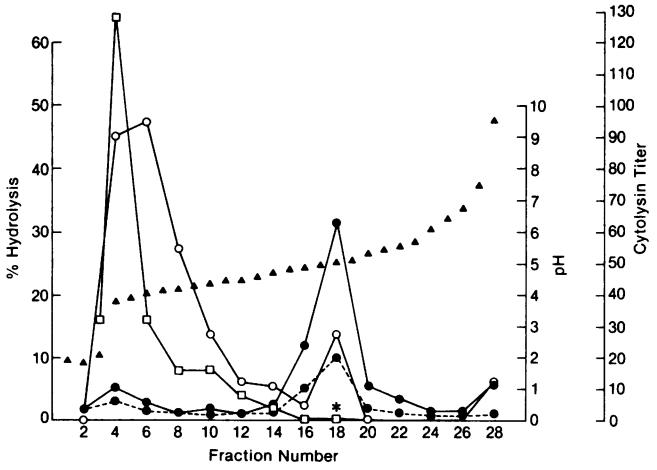

FIG. 3. Isoelectric focusing of the *V. vulnificus* culture filtrate concentrate in a pH 3.5 to 5.0 glycerol density gradient. The pH (▲) of each fraction (4 ml) was determined at 4°C. The fractions were assayed for cytolytic activity against sheep erythrocytes by the microtiter plate method (□). The cytolysin titers of fractions 1 and 2 were not determined because of nonspecific hemolysis caused by the highly acidic pH of the fractions. In addition, samples (0.1 ml) of the fractions were diluted with 0.9 ml of PBS and assayed for phospholipase and lysophospholipase activity (1 h at 37°C) as described in the text. Symbols: ●, phospholipase activity (solid line indicates release of ^{3}H-labeled fatty acids and dashed line indicates release of ^{14}C-labeled fatty acids); ○, lysophospholipase activity (release of ^{14}C-labeled fatty acids from 1-acyl phospholipid substrate preparation); *, activity of fraction 18 against 1-acyl phospholipid substrate preparation after heating the fraction sample at 56°C for 30 min.

neither the CFC nor the phospholipase purified by isoelectric focusing were active against *p*-nitrophenylphosphorylcholine, and the purified phospholipase was not active against labeled diglycerides. In addition, the observation that heating the CFC at 56°C for 30 min abolished the activity of the preparation against ^{3}H-^{14}C-labeled phospholipids (Table 1) but not against ^{14}C-labeled, 1-acyl lysophospholipids (Table 2) suggested that hydrolysis of both acyl ester bonds by the CFC was caused by the sequential action of a phospholipase A_2 and a lysophospholipase rather than by the combined action of a phospholipase A_1 and a phospholipase A_2 or by the action of a single enzyme similar to the phospholipase B of *Penicillium notatum* (31). Identification of the phospholipase as a phospholipase A_2 was confirmed with a purified ^{32}P-^{3}H-labeled PC substrate preparation. The only reaction products detected after incubation of the substrate with the phospholipase purified by isoelectric focusing were ^{3}H-labeled fatty acids and ^{32}P-labeled lysophospholipids (Table 3).

A small amount of cytolytic activity against erythrocytes was detected in the void volume peak obtained by gel filtration of a partially purified cytolysin preparation with Sephadex G-75. Although the fraction contained large amounts of phospholipase A_2 and lysophospholipase activity, the observation that culture filtrate preparations freed of phospholipase A_2 activity by heating (Table 1) and phospholipase A_2 preparations freed of lysophospholipase activity by isoelectric focusing (Fig. 3) lacked hemolytic activity shows that neither of the enzymes alone is cytolytic for erythrocytes. At the present time, it is not known whether the hemolytic activity detected in the void volume peak is a property of the combined or synergistic action of the enzymes, or is caused by contamination of the fraction with a minor cytolysin or an aggregate of the major cytolysin. The inability of the *V. vulnificus* phospholipase A_2 to lyse mammalian erythrocytes is not unique. The phospholipase A_2-possessing glycerophospholipid:cholesterol acyltransferase of *A. hydrophila* is nonhemolytic (26), and the phospholipase A of *V. parahaemolyticus* induces hemolysis only in the presence of exogenous PC (37). On the other hand, partially purified preparations obtained from the culture supernatant fluids of *Fusobacterium necrophorum* and *Flavobacterium aquatile* have been reported to possess phospholipase A and lysophospholipase activities and to be lytic for erythrocytes (1, 35). However, the fact that homogeneity of the preparations was not demonstrated makes it difficult, at the present time, to rigorously ascribe hemolytic activity to the enzyme(s) rather than to contaminating, nonenzymatic cytolysins.

At the present time, it is not known whether the extracellular phospholipase A_2 or lysophospholipase of *V. vulnificus* plays a role in the production of disease by the bacterium. We have observed, however, that phospholipase activity in culture filtrates of a weakly virulent strain of *V. vulnificus* (Centers for Disease Control strain A1402) (23) is only about one-third of that in culture filtrates of the virulent strain used in the present communication (unpublished observation). Additional studies are needed to further characterize the enzymes and to determine their possible importance in the metabolism of the bacterium and in its ability to cause disease.

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LITERATURE CITED

1. **Abe, P. M., J. Kendall, L. R. Stauffer, and J. W. Holland.** 1979. Hemolytic activity of *Fusobacterium necrophorum* culture supernatants due to the presence of phospholipase A and lysophospholipase. Am. J. Vet. Res. **40**:92–96.
2. **Baumann, P., and L. Baumann.** 1977. Biology of the marine enterobacteria: genera *Beneckea* and *Photobacterium*. Annu. Rev. Microbiol. **31**:39–61.
3. **Baumann, P., L. Baumann, S. S. Bang, and M. J. Woolkalis.** 1980. Reevaluation of the taxonomy of *Vibrio, Beneckea,* and *Photobacterium*: abolition of the genus *Beneckea*. Curr. Microbiol. **4**:127–132.
4. **Baumann, P., L. Baumann, and B. G. Hall.** 1981. Lactose utilization by *Vibrio vulnificus*. Curr. Microbiol. **6**:131–135.
5. **Beckman, E. N., G. L. Leonard, L. E. Castillo, C. F. Genre, and G. A. Pankey.** 1981. Histopathology of marine vibrio wound infections. Am. J. Clin. Pathol. **76**:765–772.
6. **Bernheimer, A. W., and L. L. Schwartz.** 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. **30**:455–468.
7. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine vibrio. Clinical characteristics and epidemiology. N. Engl. J. Med. **300**:1–5.
8. **Blake, P. A., R. E. Weaver, and D. G. Hollis.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34**:341–367.
9. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
10. **Brayton, P. R., P. A. West, E. Russek, and R. R. Colwell.** 1983. New selective plating medium for isolation of *Vibrio vulnificus* biogroup 1. J. Clin. Microbiol. **17**:1039–1044.
11. **Buckley, J. T., L. N. Halasa, and S. MacIntyre.** 1982. Purification and partial characterization of a bacterial phospholipid:cholesterol acyltransferase. J. Biol. Chem. **257**:3320–3325.
12. **Castillo, L. E., D. L. Winslow, and G. A. Pankey.** 1981. Wound infection and septic shock due to *Vibrio vulnificus*. Am. J. Trop. Med. Hyg. **30**:844–848.
13. **Craig, D. B., and D. L. Stevens.** 1980. Halophilic vibrio sepsis. South. Med. J. **73**:1285–1287.
14. **Daniel, L. W., L. King, and M. Waite.** 1981. Source of arachidonic acid for prostaglandin synthesis in Madin-Darby canine kidney cells. J. Biol. Chem. **256**:12830–12835.
15. **Daniel, L. W., M. Waite, L. S. Kucera, L. King, and I. Edwards.** 1981. Phospholipid synthesis in human embryo fibroblasts infected with herpes simplex virus type 2. Lipids **16**:655–662.
16. **Ellington, E. P., J. G. Wood, and E. O. Hill.** 1982. Disease caused by a marine vibrio–*Vibrio vulnificus*. N. Engl. J. Med. **307**:1642.
17. **Farmer, J. J., III.** 1979. *Vibrio* (*"Beneckea"*) *vulnificus*, the bacterium associated with sepsis, septicaemia, and the sea. Lancet **ii**:903.
18. **Ghosh, H. K., and T. E. Bowen.** 1980. Halophilic vibrios from human tissue infections on the Pacific coast of Australia. Pathology **12**:397–402.
19. **Hollis, D. G., R. E. Weaver, C. N. Baker, and C. Thornsberry.** 1976. Halophilic *Vibrio* species isolated from blood cultures. J. Clin. Microbiol. **3**:425–431.
20. **Johnston, J. M., W. A. Andes, and G. Glasser.** 1983. *Vibrio vulnificus*. A gastronomic hazard. J. Am. Med. Assoc. **249**:1756–1757.
21. **Kelly, M. T., and D. M. Avery.** 1980. Lactose-positive *Vibrio* in seawater: a cause of pneumonia and septicemia in a drowning victim. J. Clin. Microbiol. **11**:278–280.

22. **Kelly, M. T., and W. F. McCormick.** 1981. Acute bacterial myositis caused by *Vibrio vulnificus*. J. Am. Med. Assoc. **246**:72–73.
23. **Kreger, A., and D. Lockwood.** 1981. Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. Infect. Immun. **33**:583–590.
24. **Kurioka, S., and M. Matsuda.** 1976. Phospholipase C assay using *p*-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal. Biochem. **75**:281–289.
25. **Lundin. H. S., S.-G. Hjalmarsson, H. Davies, and H. Perlmutter.** 1975. High-speed electrofocusing with the LKB 8100 ampholine electrofocusing columns using constant power. LKB Application Note 194. LKB-Produkter AB, Bromma, Sweden.
26. **MacIntyre, S., and J. T. Buckley.** 1978. Presence of glycerophospholipid-cholesterol acyltransferase and phospholipase in culture supernatant of *Aeromonas hydrophila*. J. Bacteriol. **135**:402–407.
27. **MacIntyre, S., T. J. Trust, and J. T. Buckley.** 1979. Distribution of glycerophospholipid:cholesterol acyltransferase in selected bacterial species. J. Bacteriol. **139**:132–136.
28. **Matsuo, T., S. Kohno, T. Ikeda, K. Saruwatari, and H. Ninomiya.** 1978. Fulminating lactose-positive *Vibrio* septicemia. Acta Pathol. Jpn. **28**:937–948.
29. **Mertens, A., J. Nagler, W. Hansen, and E. Gepts-Friedenreich.** 1979. Halophilic, lactose-positive *Vibrio* in a case of fatal septicemia. J. Clin. Microbiol. **9**:233–235.
30. **Misaki, H., and M. Matsumoto.** 1978. Purification of lysophospholipase of *Vibrio parahaemolyticus* and its properties. J. Biochem. **83**:1395–1405.
31. **Okumura, T., S. Kimura, and K. Saito.** 1980. A novel purification procedure for *Penicillium notatum* phospholipase B and evidence for a modification of phospholipase B activity by the action of an endogenous protease. Biochim. Biophys. Acta **617**:264–273.
32. **Pollak, S. J., E. F. Parrish III, T. J. Barrett, R. Dretler, and J. G. Morris, Jr.** 1983. *Vibrio vulnificus* septicemia. Isolation of organism from stool and demonstration of antibodies by indirect immunofluorescence. Arch. Intern. Med. **143**:837–838.
33. **Rouser, G., A. N. Siakotos, and S. Fleischer.** 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. Lipids **1**:85–86.
34. **Tison, D. L., and R. J. Seidler.** 1981. Genetic relatedness of clinical and environmental isolates of lactose-positive *Vibrio vulnificus*. Curr. Microbiol. **6**:181–184.
35. **Tsubura, E., and S. Yasuoka.** 1975. Cytolytic substance produced by *Flavobacterium aquatile* B5X. Toxicon **13**:127.
36. **Winter, A., and C. Karlsson.** 1976. Preparative electrofocusing in density gradients. LKB Application Note 219. LKB-Produkter AB, Bromma, Sweden.
37. **Yanagase, Y., K. Inoue, M. Ozaki, T. Ochi, T. Amano, and M. Chazono.** 1970. Hemolysins and related enzymes of *Vibrio parahaemolyticus*. I. Identification and partial purification of enzymes. Biken J. **13**:77–92.