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HEMOLYSINS AND RELATED ENZYMES OF *VIBRIO PARAHAEMOLYTICUS*

I. IDENTIFICATION AND PARTIAL PURIFICATION OF ENZYMES[1]

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SUMMARY *Vibrio parahaemolyticus* produces a phospholipase A, a lysophospholipase, a glycerophosphorylcholine (GPC) diesterase and two kinds of direct hemolytic factors (DLF's), one of which is heat-stable (DLF-S) and the other heat-labile (DLF-L). The kind of DLF produced depends mainly upon the cultural conditions of this organism.

A phospholipase A-negative mutant strain of this organism was obtained and used for isolation and characterization of DLF and other enzymes.

INTRODUCTION

Since the discovery of *Vibrio parahaemolyticus* by Fujino and others (1951, 1953) hemolysis by this organism has been observed by many investigators. Recently it was reported that pathogenic strains of this organism possessed a heat-stable hemolysin while nonpathogenic strains caused no hemolysis when cultured in medium containing blood (Kanagawa phenomenon) (Miyamoto *et al.*, 1969). From its heat-stability we thought that the hemolysin might be a phospholipase A.

This paper shows that *V. parahaemolyticus* can produce various enzymes causing hemolysis, namely a phospholipase A, a lysophospholipase and two kinds of direct hemolysins (DLF), one of which is heat-stable and the other heat-labile. In addition glycerophosphorylcholine diesterase (GPC diesterase) was isolated from the culture filtrate of this or-

1. Presented in part at the 20th Kansai Meeting of the Japanese Bacteriological Society in Kyoto on October 1, 1967, at the 42nd General Meeting of the Japanese Bacteriological Society in Tokushima on April 6, 1969, and at the 16th Symposium on Toxins in Hakone on July 15, 1969.
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ganism.

MATERIALS AND METHODS

1. *Bacterial strains*

Vibrio parahaemolyticus, strain 2597 (K3 : O2) (parent strain), isolated from a patient suffering from food-poisoning, by Dr. Maeshima and his associates of the Public Health Research Institute of Kobe City, Kobe, Hyogo, Japan, was kindly supplied by them.

A phospholipase A-negative mutant, strain 1-1, was obtained from the parent strain by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (1965). Briefly, an overnight culture of the parent strain in medium I (*vide infra*) at 37 C was diluted to 1/100 with the same medium. It was cultured in an L-tube at 37 C with continuous shaking until the optical density at 550 mμ reached 0.20, measured in a cuvette of 19 mm diameter in a Coleman Junior spectrophotometer. Then the culture was centrifuged, washed with 2.0% NaCl containing M/75 phosphate buffer, pH 6.2, and resuspended in the same buffer at half the previous volume. Then 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (final concentration) was added, and the bacterial suspension was incubated at 37 C for 60 min. The bacteria were centrifuged, washed with medium I and inoculated on 90 mm plates of medium I-agar containing egg yolk lecithin at a concentration of 50 μg/ml. The plates were incubated at 37 C overnight, and then overlayed with 3 ml of 0.5% agar containing 1% NaCl and washed rabbit erythrocytes at a concentration of 1.0×10^8 cells/ml. The plates were incubated at room temperature for 4 hr and then in a refrigerator overnight. Strain 1-1 was isolated as one of the colonies without a surrounding hemolytic zone. If the mutant strain was cultured longer, a small hemolytic zone appeared as shown later.

V. parahaemolyticus, strain 4750 (K3 : O2), which gives a strong positive reaction in the " Kanagawa Phenomenon " (Miyamoto et al., 1969), was obtained by courtesy of Dr. Miyamoto of the Kanagawa Prefectural Public Health Laboratory.

2. *Culture media*

1) *Medium I* contained 10 g of Polypeptone, 20 g of NaCl, 5 g of yeast extract and 5 g of $Na_2HPO_4 \cdot 12H_2O$ in 1,000 ml of distilled water, pH 8.5, and for solid medium 1.5% agar was added.

2) *Modified Syncase medium* (Finkelstein et al., 1966) contained dialysate from 10 g of Casamino acid, Certified, dialysate from 5 g of yeast extract, 25 g of NaCl, 5 g of $Na_2HPO_4 \cdot 12H_2O$, 5 g of K_2HPO_4, 1.118 g of NH_4Cl, 89 mg of Na_2SO_4, 42 mg of $MgCl_2 \cdot 6H_2O$, 4 mg of $MnCl_2$, 5 mg of $FeCl_3 \cdot 6H_2O$ and 5 g of sucrose in 1,000 ml of distilled water, pH 8.5.

3) *Modified peptone medium* (Evans and Richardson, 1968) contained dialysate from 30 g of Bactopeptone, dialysate from 3 g of yeast extract, 30 g of NaCl and 0.1 M Tris-HCl buffer in 1,000 ml of distilled water, pH 6.5.

3. *Antiserum*

Rabbit antiserum against O2 antigen of *V. parahaemolyticus* was kindly given by Dr. M. Torii of this Institute.

4. *Chemicals*

Egg yolk lecithin was prepared and purified by alumina column chromatography as described by Singleton et al. (1965). The suspension was prepared by quick injection of a 1% solution in ethanol into diluent. Lysolecithin was prepared from the lecithin by phospholipase A of " Habu " (*Trimeresurus flavoviridis*) venom according to the method of Hanahan (1952). Glycerophosphorylcholine was prepared from the lecithin by methanolic potassium hydroxide according to Dawson's method (1960).

N-methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from the K & K Laboratory, New York, N.Y. It was dissolved in water just before use and sterilized by passage through a Millipore filter membrane, type HA (Millipore Filter Corp., Bedford, Mass.). Ethylenediaminetetraacetate, disodium and tetrasodium salts (EDTA), CaEDTA and MgEDTA were obtained from Dojin Chemicals Co., Osaka, Japan. Silica Gel H for thin-layer chromatography was obtained from Merck A. G., Darmstadt, Germany. Diethylaminoethyl (DEAE) and carboxymethyl (CM) cellulose were obtained from Brown Co., Berlin, N. H.

Polypeptone was obtained from Daigo Eiyo Co., Ltd., Osaka, Japan. Casamino acid, Certified, Bacto-Yeast Extract and Bactopeptone were obtained from Difco Laboratories, Detroit, Michigan.

A lyophilized preparation of " Habu " (*Trimeresurus flavoviridis*) venom was kindly given by Dr. Kubo of this Institute, and the phospholipase A was purified from it by heat treatment of acidified venom

at 100 C for 10 min and chromatography on CM-cellulose column followed by dialysis and lyophilization.

5. *Measurement of ionic strengths*

The ionic strengths of solutions used for chromatography were checked by measuring their conductivity with a Serfass Conductivity Bridge, model RCM 15B1 (A. H. Thomas Co., Philadelphia, Pa.).

6. *Thin-layer chromatography (TLC)*

Reaction mixtures were extracted with chloroform-methanol by the method of Bligh and Dyer (1959). Phospholipid samples were applied on a Silica Gel H plate and developed with a mixture of either a) chloroform-acetone-methanol-acetic acid-water (10 : 4 : 2 : 2 : 1, v/v/v/v) or b) chloroform-methanol-water (65 : 25 : 4, v/v/v). For detecting phospholipids and their breakdown products, the following reagents were used: iodine vapor for lipids (Stahl, 1967), ninhydrin for amino compounds (Stahl, 1967) and molybdic acid for phosphate compounds (Dittmer, 1964).

7. *Assays of enzyme activities*

Activities of phospholipase A and lysophospholipase were measured as decreases of acyl ester contents by the method of Shapiro (1953) using egg yolk lecithin and lysolecithin, respectively, as substrates.

Activity of glycerophosphorylcholine (GPC) diesterase was estimated by measuring free choline released from GPC by the method of Appleton et al. (1953).

In semiquantitative titrations of these enzymes and of direct hemolysins (DLF), Microtiter techniques were used. Isotonic saline containing 0.005 M tris(hydroxymethyl)amino methane (Tris)-HCl buffer, pH 7.5 and 0.01 M $CaCl_2$ (TBS-Ca^{++}) was used as diluent.

Phospholipase A: Drops (ca. 0.025 ml) of serially diluted sample in TBS-Ca^{++} were mixed with 1 drop of washed rabbit erythrocytes at a concentration of 1.0×10^8 cells/ml and 1 drop of 50 μg/ml egg yolk lecithin in a cup of a Microtiter plate. The plate was incubated at 37 C for 2 hr and then overnight in a cold room. The degrees of lysis were recorded as 4 (complete lysis) to 0 (no lysis). The highest dilution giving lysis of about half the erythrocytes (score 2) was taken as the titer of this sample.

Lysophospholipase: One drop of a sample diluted serially was mixed with 1 drop of 40 μg/ml lysolecithin, and incubated at 37 C for 1 hr. Then 1 drop of washed rabbit erythrocytes at a concentration of 1.0×10^8 cells/ml, was added and the mixture was incubated further at 37 C for 10 min. Then degree of lysis was read after centrifugation. The highest dilution giving about half the erythrocytes left (score 2) was taken as the titer of the sample.

Direct Lytic Factor (DLF): Drops of serially diluted sample were mixed with 1 drop of diluent and 1 drop of washed rabbit erythrocytes at a concentration of 1.0×10^8 cells/ml. The mixture was incubated at 37 C for 2 hr and then overnight in a cold room.

RESULTS

1. *Lysis of rabbit erythrocytes by V. parahaemolyticus*

When *V. parahaemolyticus*, strain 2597 (parent strain) was inoculated by stabbing into medium I agar plates containing washed rabbit erythrocytes at a concentration of about 5×10^8 cells/ml, and incubated at 37C for 40 hr, a clear narrow (about 1mm wide) hemolytic zone appeared surrounding the colony (Fig. 1a). On the other hand, if the organism was first inoculated into a plate containing no erythrocytes, incubated at 37C for 40 hr and then overlayed with agar in Tris-HCl-buffered saline with added Ca^{++} (TBS-Ca^{++}) containing washed rabbit erythrocytes, a faint hemolytic zone of about the same size appeared on the colony (Fig. 1b).

However, when the parent strain was inoculated into medium I agar containing washed erythrocytes and 50 μg/ml of egg yolk lecithin and incubated at 37C for 24 hr, a clear wide hemolytic zone (about 20 mm wide) appeared around the colony (Fig. 1c). If the organism was first inoculated into medium I agar containing 50 μg/ml lecithin, incubated at 37C for 24 hr and then overlayed with erythrocyte agar, a triple concentric hemolytic zone appeared, *i.e.*, a central clear narrow zone, a middle turbid zone and a peripheral clear zone (Fig. 1d). These results suggested that this organism possesses at least three factors related with hemolysis, *i.e.*, a hemolysin (direct lytic

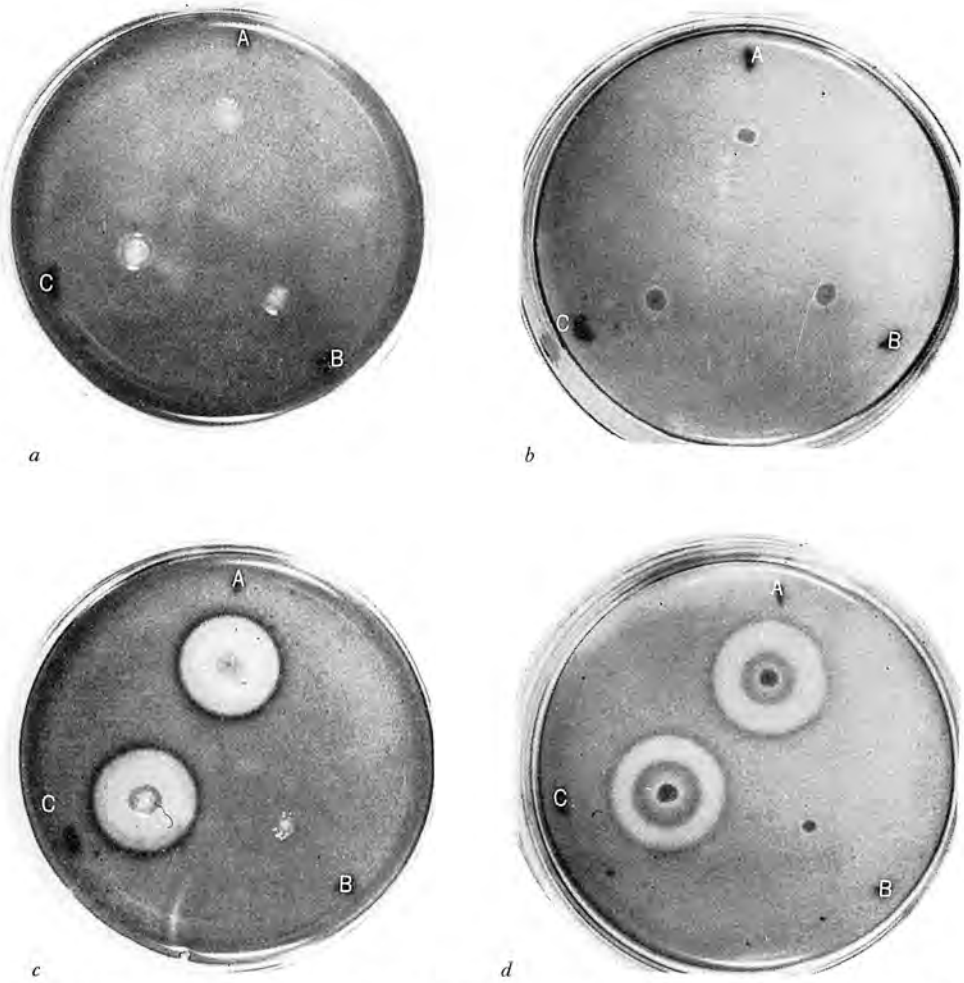


FIGURE 1. *Hemolysis by V. parahaemolyticus, strain 2587 (parent strain), its phospholipase A-negative mutant, strain 1-1, and strain 4750, in various conditions.*

a) 40 hr culture at 37 C on medium I-agar containing washed rabbit erythrocytes; b) 40 hr culture at 37 C on medium I-agar, followed by overlay with agar containing washed rabbit erythrocytes; c) 24 hr culture at 37 C on medium I-agar containing washed rabbit erythrocytes and lecithin; d) 24 hr culture at 37 C on medium I-agar containing lecithin, followed by overlay with agar containing washed erythrocytes.

A: strain 2597, B: strain 1-1, C: strain 4750.

factor, DLF) which can lyze erythrocytes in the absence of added phospholipid, a phospholipase A which causes enlargement of the hemolytic zone in the presence of lecithin, and a factor destroying lysolecithin formed by the phospholipase A, possibly a lysophospholipase which is responsible for the middle turbid zone observed when the colony grown in the presence of lecithin was overlayed with erythrocyte agar.

2. *Fractionation of hemolytic factors of V. parahaemolyticus*

The parent strain was cultured at 37 C for 18 hr

in Roux bottles, containing 200 ml of medium I. Then each culture was centrifuged, and the supernatant obtained was then sterilized by filtration through a Millipore filter membrane, type HA. Solid ammonium sulfate was added to the filtrate to saturation. After standing the mixture overnight in a cold room, the precipitate was collected by centrifugation at 9,000 rev/min for 30 min. It was then dissolved in a small volume of 0.1 M Tris-HCl buffer, pH 7.5, and dialyzed against 0.01 M Tris-HCl buffer, pH 7.5. The dialyzed material was fractionated stepwise with ammonium sulfate and precipitates were obtained at 0.2, 0.4, 0.6 and 1.0 saturations. Each precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 7.5, and dialyzed against 0.01 M Tris-HCl buffer, pH 7.5. The dialyzed fractions, each in 1/50 of the volume of the original filtrate, were then centrifuged at 10,000 × g for 60 min to remove insoluble material.

The hemolytic activities of the fractions obtained were assayed in the presence of lecithin,

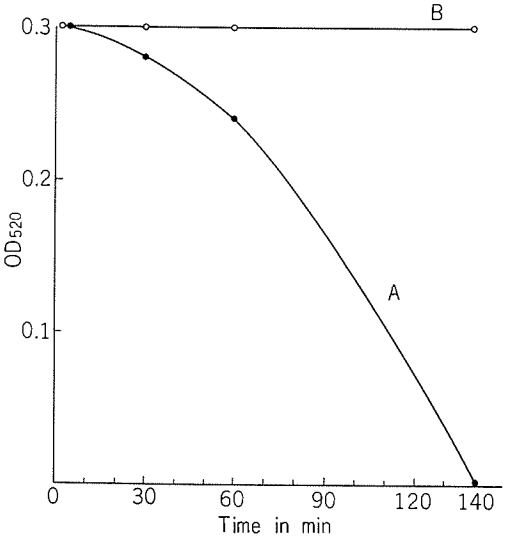


FIGURE 2. *Phospholipase activity of the Fraction A from the culture filtrate of the parent strain grown in medium I, measured by the decrease in acyl ester content of lecithin.*

Reaction mixture contained 2.0 ml of the test fraction (Fraction A) (A) or distilled water (B) and 1.0 mg/ml lecithin in 10.0 ml of 0.005 M $CaCl_2$ in 0.03 M Tris-HCl buffer, pH 7.5. Temperature: 37 C. Acyl ester was measured by the method of Shapiro (1953).

and only the fraction precipitating between 0.2 and 0.4 saturation of ammonium sulfate (Fraction A) caused lysis of rabbit erythrocytes. This suggested that this fraction might contain phospholipase A. In fact, as shown in Fig. 2, this fraction caused a decrease in acyl ester from egg yolk lecithin when assayed by the method of Shapiro (1953). However, on comparison with phospholipase A of "Habu" venom, this fraction hydrolyzed almost all the acyl esters of lecithin, while the latter enzyme hydrolyzed only about half, as shown in Fig. 3. Therefore, Fraction A might liberate two moles of fatty acids from one mole of lecithin, while the snake venom phospholipase A liberates only one fatty acid at the β-position.

Fraction A also decreased the acyl ester content of lysolecithin, as shown in Fig. 4.

Ca^{++} ion is an activator of the phospholipase A's from many sources. Preliminary experiments showed that addition of $CaCl_2$ at a final concentration of 0.005 M to the reaction mixture did not activate the decrease in the acyl ester content of either lecithin or lysolecithin by Fraction A. Moreover, 0.005 M EDTA did not inhibit these reactions. However, before

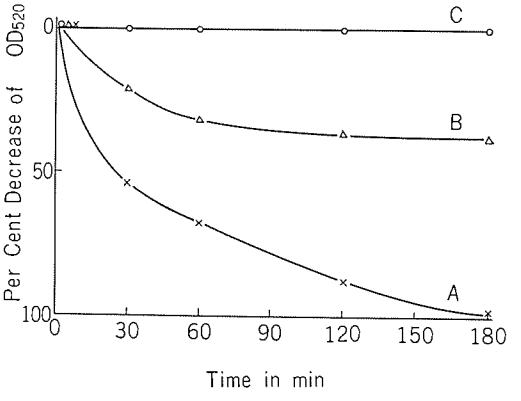


FIGURE 3. *Comparison of the activities of Fraction A from the culture filtrate of the parent strain and of "Habu" venom phospholipase A.*

A: Fraction A from the culture filtrate of the parent strain grown in medium I, B: "Habu" venom phospholipase A, C: control without enzyme. Experimental conditions were as those for Fig. 2.

any conclusions on the effects of divalent metal ions on these enzymes can be reached, more purified enzyme preparations are necessary.

Fraction A can liberate all the fatty acids from either lecithin or lysolecithin. Thus this fraction must contain either a phospholipase B, which can hydrolyze acyl esters at both the β- and α'-positions of phospholipids, as suggested for the enzyme of *Penicillium notatum* (Dawson, 1958) and of rat intestinal mucosa (Epstein and Shapiro, 1959), or a phospholipase A, which hydrolyzes only the acyl ester at the β-position of phospholipids, and a lysophospholipase, which liberates fatty acid from lysophospholipid.

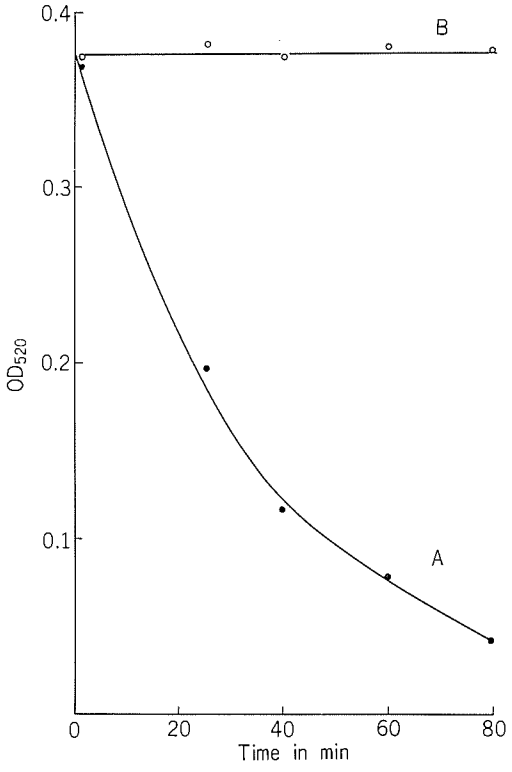


FIGURE 4. *Lysophospholipase activity of the fraction A from the culture filtrate of the parent strain grown in medium I.*

A: Fraction A from the culture filtrate of the parent strain, B: control without enzyme. Experimental conditions were as those for Fig. 2, except that 2.5 mg/ml lysolecithin was used instead of lecithin.

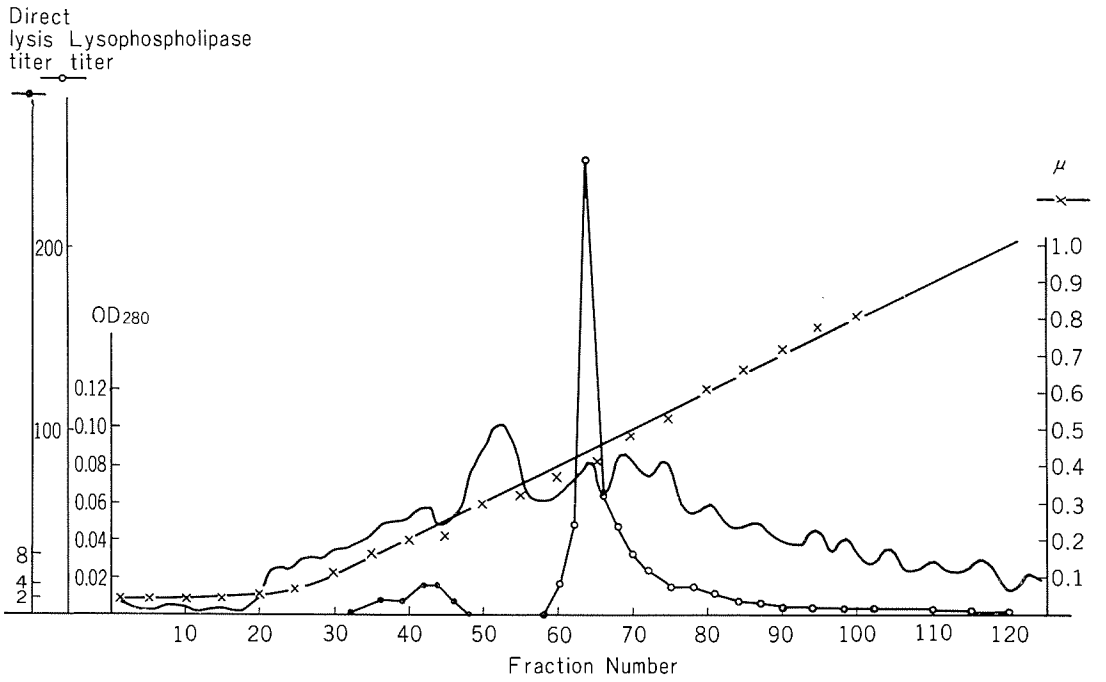
The other fraction, obtained by precipitation between 0 to 0.2 saturation of ammonium sulfate from the parent strain grown in medium I, however, liberated fatty acid from lysolecithin but not from lecithin. Therefore, it was concluded that this fraction contained no phospholipase A but only lysophospholipase which could not act on lecithin. The separation of these enzymes from the Fraction A was attempted by chromatography on a DEAE-cellulose column.

Twenty ml of Fraction A of the culture filtrate of the parent strain grown in medium I were dialyzed against 0.05 M NaCl in 0.005 M Tris-HCl buffer, pH 7.5, and applied on the DEAE-cellulose column (30 × 520 mm) equilibrated with the same buffer. The column was eluted with 2,000 ml of a linear gradient from 0.05 M to 1.0 M NaCl in 0.005 M Tris-HCl buffer, pH 7.5. Fifteen ml fractions were collected in an automatic fraction collector. Chromatography was carried out in a cold room maintained at 2 to 4 C. Phospholipase A, lysophospholipase and direct hemolysin activities were assayed as described in Materials and Methods.

As shown in Fig. 5, a direct hemolytic factor (DLF) was well separated from lysophospholipase activity. Phospholipase A activity, however, could not be found in any of the fractions. Screening assay for this enzyme, using washed rabbit erythrocytes and added lecithin, showed that the hemolytic activity was in the same fractions as DLF activity, and addition of lecithin did not enhance the lytic activity of these fractions. Therefore, this DLF corresponded to DLF-S described later.

3. *Lysophospholipase of a phospholipase A-negative mutant*

To confirm the presence of DLF a phospholipase A-negative mutant, strain 1–1, was isolated by treating the parent strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described in Materials and Methods. When this strain was inoculated into medium I agar containing washed rabbit erythrocytes and incubated at 37C for 40 hr, a clear narrow hemolytic zone appeared just as with the parent



FIGGURE 5. *Chromatography on a DEAE-cellulose column of Fraction A from the culture filtrate of the parent strain grown in medium I.*

strain (Fig. 1a). However, only a small faint hemolytic zone appeared after 24 hr incubation even when lecithin was added to the plate (Fig. 1c). A similar small faint hemolytic zone was observed when a colony of this strain grown on medium I agar, with or without added lecithin, was overlayed with erythrocyte agar (Fig. 1b and 1c). The formation of a hemolytic zone by this strain again suggested the existence of a direct hemolytic factor (DLF) as discussed later.

The mutant strain 1-1 was grown in medium I at 37 C for 18 hr as described in the preceding paragraph for the parent strain. The supernatant was collected by centrifugation and filtered through a Millipore filter membrane. The filtrate was saturated with $(NH_4)_2SO_4$. The precipitate obtained was dissolved in Tris-HCl buffer and dialyzed as in the case of the parent strain.

The fraction, obtained by 0 to 1.0 saturation with $(NH_4)_2SO_4$, was tested for phospholipase A and lysophospholipase activities with egg yolk lecithin and lysolecithin, respectively, substrates. As shown in Fig. 6, it liberated fatty acid from lysolecithin, but not from lecithin. This indicates that the mutant strain 1–1 possessed lysophospholipase but not phospholipase A. It was also suggested that the results obtained with Fraction A from the culture filtrate of the parent strain can be explained by supposing that this fraction contains both phospholipase A and lysophospholipase. The purification and characterization of lysophospholipase from this mutant strain are now in progress.

4. *Existence of Glycerophosphorylcholine (GPC) diesterase in the culture filtrate of V. parahaemolyticus*

It seemed possible that the culture filtrate of *V. parahaemolyticus* might contain GPC since this organism could metabolize lecithin to GPC through lysolecithin. This was tested using fractions obtained with $(NH_4)_2SO_4$ from the culture filtrate of the parent strain and GPC

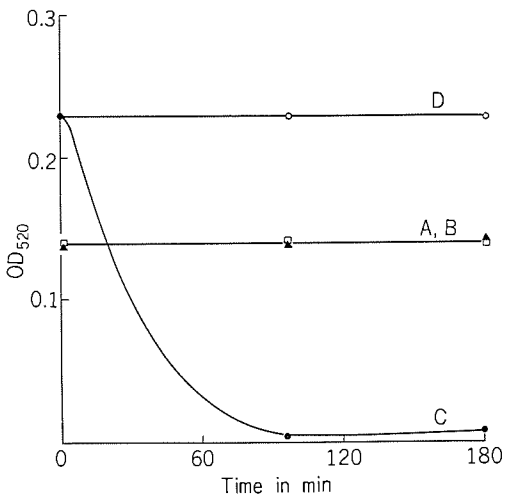


FIGURE 6. *Lysophospholipase A activity of the fraction precipitated by 0 to 1.0 saturation of ammonium sulfate from the culture filtrate of the phospholipase A-negative mutant strain 1-1 grown in medium I.*

A, C: fraction tested; B, D: controls without enzyme; A, B: with lecithin as substrate; C, D: with lysolecithin as substrate.

The experimental conditions were as described in the legends of Fig. 2 and 4.

prepared by the method of Dawson (1960) as substrate.

Reaction mixture contained 0.1 ml of 0.16% GPC, 0.1 ml of 0.1 M $CaCl_2$, 0.7 ml of 0.1 M Tris-HCl buffer, pH 7.5 and 0.1 ml of test fraction. Controls, containing neither substrate nor test fraction were also included. The reaction was carried out at 37 C, and free choline liberated was measured by the method of Appleton (1953).

As shown in Fig. 7, only the fraction obtained with 0.4 to 0.6 saturation of $(NH_4)_2SO_4$ liberated choline from GPC, and this fraction contained neither phsopholipase A nor lysophospholipase as judged by its failure to decrease the acyl ester content of either lecithin or lysolecithin.

GPC diesterase activity was also found in a a similar fraction obtained from the phospholipase A-negative mutant strain 1–1, and was partially purified by chromatography on a DEAE-cellulose column.

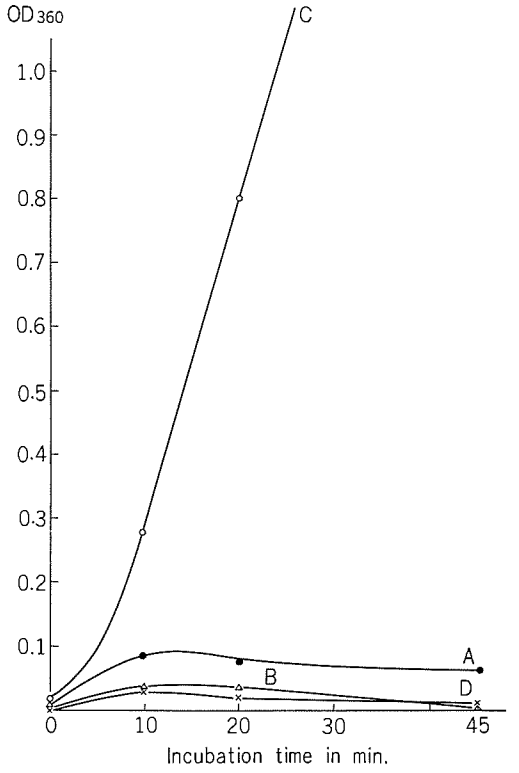


FIGURE 7. *Liberation of choline from glycerophosphorylcholine by fractions obtained by step-wise addition of ammonium sulfate to the culture filtrate of the parent strain grown in medium I.*

Fraction precipitated by A: 0 to 0.2 saturation, B: 0.2 to 0.4 saturation, C: 0.4 to 0.6 saturation, D: 0.6 to 1.0 saturation. For experimental conditions, see text.

The fraction obtained by 0.4 to 0.6 saturation of $(NH_4)_2SO_4$ from a culture filtrate of the mutant strain 1-1 grown in medium I was dialyzed against 0.1 M NaCl in 0.005 M Tris-HCl buffer, pH 7.5 (starting buffer). Fourteen ml of the material were applied on a column of DEAE-cellulose (30 × 520 mm) equilibrated with the starting buffer, and eluted with 2,000 ml of a linear gradient from 0.1 M to 0.8 M NaCl in Tris-HCl buffer, pH 7.5. The GPC diesterase activity was assayed by measuring free choline released from GPC by the method of Appleton et al. (1953).

As shown in Fig. 8, GPC diesterase activity was eluted around μ=0.35. Fractions 118

to 122 were pooled and used for characterization of this enzyme. It was shown that this enzyme required Ca^{++} ion for activity, and Mg^{++} ion could not replace Ca^{++}. The pH optimum was about 8.6 and no activity was observed at below pH 6.

5. *Heat-stable direct hemolytic factor (DLF-S) of V. parahaemolyticus, strain* 4750

As described above, the parent strain, strain 2597, possesses at least two factors causing hemolysis, *i.e.*, a direct hemolysin and a phospholipase A. Miyamoto et al. (1969) reported that pathogenic strains of *V. parahaemolyticus* isolated from patients suffering from food-poisoning generally possess a heat-stable proteinous hemolysin, unlike almost all the nonpathogenic strains obtained from sea water or fish (Kanagawa phenomenon). We obtained strain 4750, a representative strain giving a positive "Kanagawa phenomenon," by courtesy of Dr. Miyamoto of the Kanagawa Prefectural Public Health Laboratory. It was observed that this strain produced hemolytic factors and related enzymes similar to those produced by the parent strain.

Strain 4750 was grown at 37 C for 18 hr in Roux bottles containing 250 ml of modified Syncase medium. Each culture was then centrifuged at 12,000 $\times g$ for 30 min, and the supernatant was filtered through a Millipore filter membrane. Five hundred ml of the filtrate were diluted 1/20 with distilled water and applied on a column of DEAE cellulose (30 × 500 mm). The column was washed with 2,000 ml of 0.005 M Tris-HCl buffer, pH 7.5, and then eluted with 4,000 ml of a linear gradient from 0 to 0.6 M NaCl in the same buffer.

As shown in Fig. 9, DLF-S was eluted around μ=0.1 and lysophospholipase at between 0.17 and 0.49. Neither the DLF-S nor the lysophospholipase fractions contained somatic antigen precipitable with rabbit anti-O2-antiserum, or activities of phospholipase

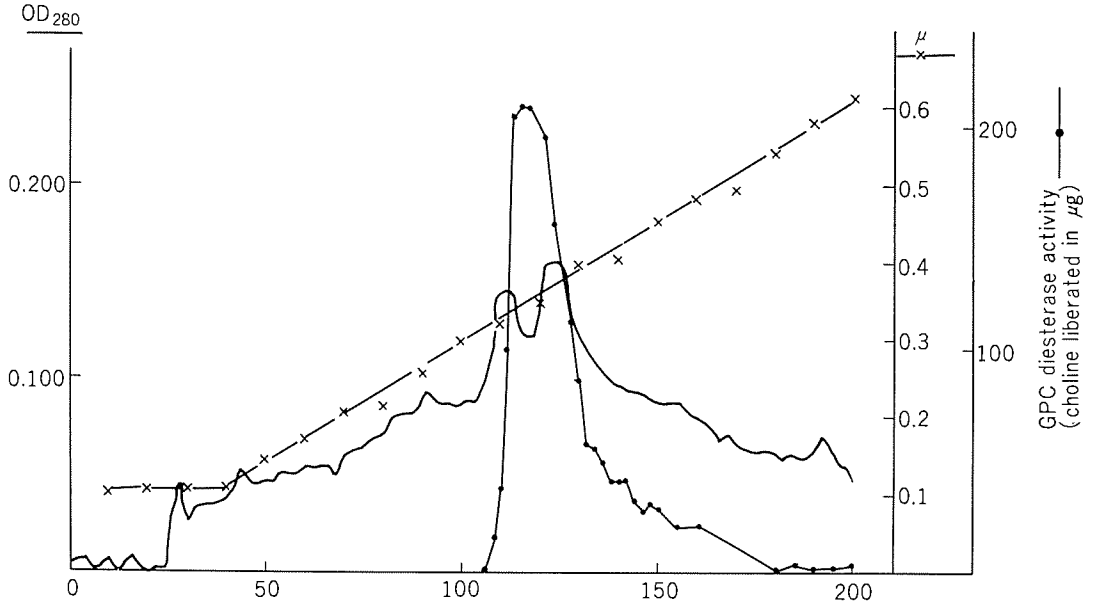


FIGURE 8. *Chromatography on a DEAE-cellulose column of the fraction precipitated by 0.4 to 0.6 saturation of ammonium sulfate from a culture filtrate of the phospholipase A-negative mutant strain 1-1 grown in medium I.*

GPC diesterase activity is expressed as the amount of choline liberated from GPC per ml of fraction per 60 min. Experimental conditions those as were for Fig. 7.

A or GPC diesterase.

Fraction Nos. 82 to 106 obtained from the column shown in Fig. 9, containing DLF-S activity, were pooled. The pooled fractions were diluted and rechromatographed on two successive columns of DEAE-cellulose.

As shown in Fig. 10, on progressive purification the DLF-S activity tended to be eluted at lower ionic strength, and it was eluted from the third column around μ=0.065 coinciding with the protein peak.

DLF-S was also isolated from the culture filtrate of the phospholipase A-negative mutant strain 1–1 grown in modified Syncase medium, as from strain 4750. The properties of the DLF-S preparations from the two strains were the same (requiring Ca^{++} ion for activity, heat stable, not activated by lecithin, etc.).

6. *Requirement of Ca^{++} ion for the activity of DLF-S*

DLF-S was isolated and purified as in the previous experiment from culture filtrate of phospholipase A-negative mutant strain 1-1. The fraction showing peak activity (Fr. No. 44) from the third DEAE-cellulose column was used as the DLF-S preparation. It was diluted serially in Tris-HCl-buffered saline (TBS). One tenth ml of each dilution or TBS (as a control) was mixed with 0.1 ml 0.01 M $CaCl_2$ (reaction series 1), 0.01 M $MgCl_2$ (2), 0.01 M $CaCl_2$ plus 0.01 M Na_3 EDTA (3), 0.01 M $CaCl_2$ plus 0.01 M Ca EDTA (4), or 0.01 M $CaCl_2$ plus 0.01 M Mg EDTA, each dissolved in TBS. Reaction series 6 received 0.1 ml of TBS instead of meal ion or chelator. Then 0.1 ml of rabbit erythrocytes at a concentration of 1.0×10^8 cells/ml was added to each reaction mixture. The mixtures were incubated at 37 C for 2 hr and then in a cold room overnight.

From the results shown in Table 1 it is evident that Ca^{++} ion is essential to the activity of DLF-S. Mg^{++} ion cannot substitute for Ca^{++} ion, since the addition of $MgCl_2$, or $CaCl_2$ plus an equimolar amount of Mg DETA, does not activate the DLF-S activity.

The same DLF-S preparation, as used in the preceding experiment, in 0.06 M NaCl in 0.005 M Tris-HCl buffer, pH 7.5, was heated in a boiling water

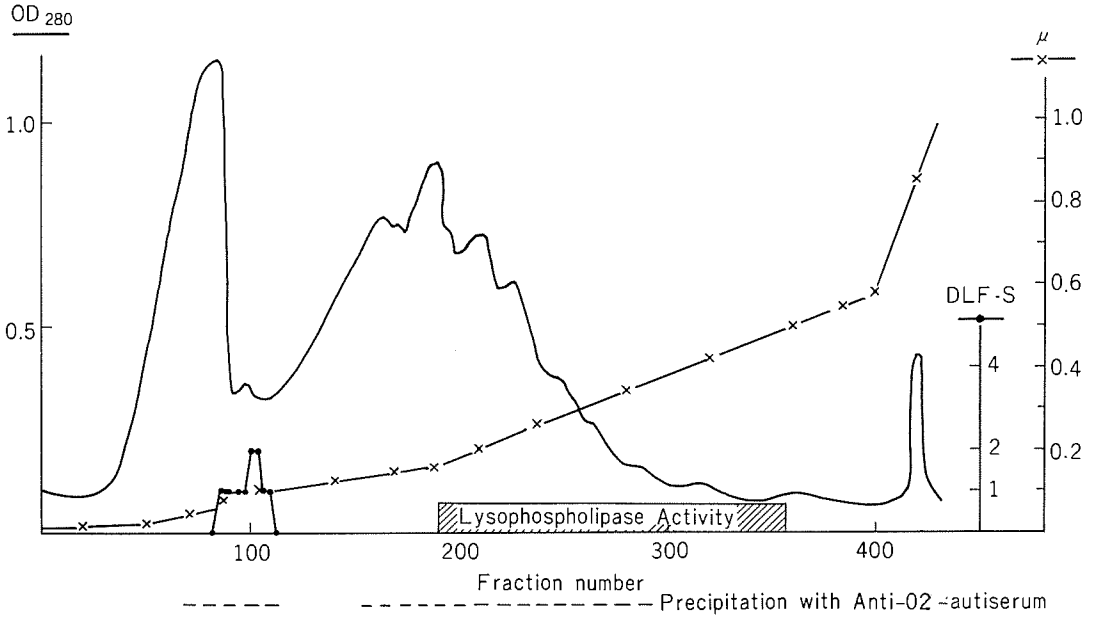


FIGURE 9. *Chromatography on a DEAE-cellulose column of the culture filtrate of V. parahaemolyticus, strain 4750, grown in modified Syncase medium.*

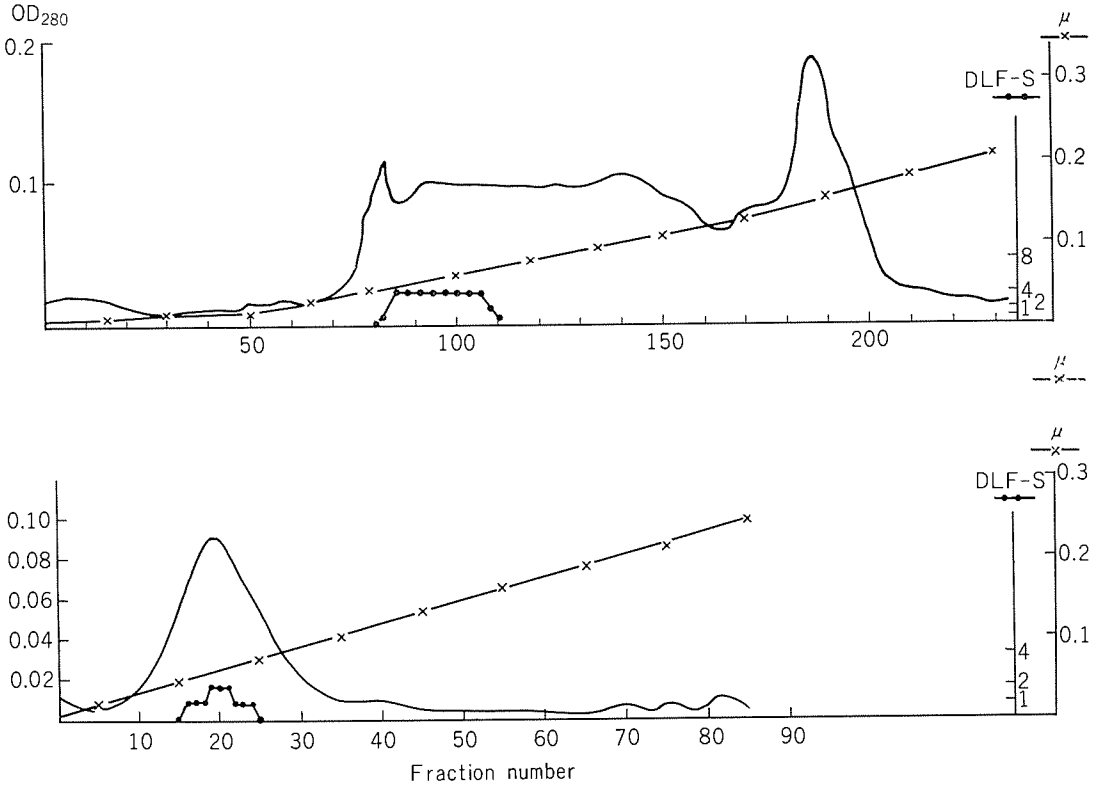


FIGURE 10. *Rechromatographies of DLF-S on DEAE-cellulose columns.*

bath for 10 min, and then cooled in an ice bath. It was titrated for hemolytic activity on a Microtiter plate.

As shown in Table 2, heat treatment did not destory the hemolytic activity of DLF-S, but enhanced it somewhat. This enhancement by heat-treatment may be due to destruction of an inhibitor of DLF-S.

7. *Heat-labile direct hemolytic factor (DLF-L) of V. parahaemolyticus grown in modified peptone medium*

Evans and Richardson (1968) used culture medium containing 3% peptone for production of choleragen and skin permeability factor by *Vibrio cholerae*. We employed a similar medium containing a higher concentration of NaCl, for halophilic *V. parahaemolyticus*, and a dialysate of peptone instead of peptone, to avoid contamination with macromolecular materials from the growth medium. A dialysate of yeast extract was also added.

Phospholipase A negative mutant 1-1 was cultured in modified peptone medium at 37 C for 4 hr on a mechanical shaker. The culture was centrifuged and the supernatant was filtered through a Millipore filter membrane. This filtrate (270 ml) was diluted with distilled water to an ionic strength $\mu=0.025$, and applied to a column of DEAE-cellulose (30× 500 mm) equilibrated with 0.005 M Tris-HCl buffer, pH 7.5 (starting buffer). After washing with 1,000 ml on the starting buffer, the column was eluted with 4,000 ml of a linear gradient from 0 to 0.6 M NaCl in the starting buffer. Fractions of 9 ml were collected in an automatic fraction collector. As shown in Fig. 11a, the direct hemolytic activity appeared around $\mu=0.3$. The fractions Nos. 183 to 246 were pooled

TABLE 1. *Effects of Ca^{++} and Mg^{++} ions and of EDTA on the activity of DLF-S*

Exp. series	Ion added	Dilution of DLF-S								Control without DLF-S
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
1	$CaCl_2$	4	4	4	0	0	0	0	0	0
2	$MgCl_2$	0	0	0	0	0	0	0	0	0
3	$CaCl_2$+Na_3EDTA	0	0	0	0	0	0	0	0	0
4	$CaCl_2$+CaEDTA	4	4	2	0	0	0	0	0	0
5	$CaCl_2$+MgEDTA	1	0	0	0	0	0	0	0	0
6	None	0	0	0	0	0	0	0	0	0

TABLE 2. *Heat stability of direct hemolytic factors (DLF-S and DLF-L) and effect of lecithin on their activities*

DLF	Treatment	Washed erythrocytes									Washed erythrocytes+Lecithin								
		Dilution of DLF									Dilution of DLF								
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
DLF-S	None	4	4	4	1	0	0	0	0	0	4	4	4	0	0	0	0	0	0
	100°C, 10 min	4	4	4	2	1	0	0	0	0	4	4	4	2	0	0	0	0	0
DLF-L	None	4	2	0	0	0	0	0	0	0	4	4	4	4	2	0	0	0	0
	56°C, 10 min	0	0	0	0	0	0	0	0	0	4	3	2	1	1	1	1	0	0
	100°C, 10 min	0	0	0	0	0	0	0	0	0	4	2	2	1	1	1	1	0	0

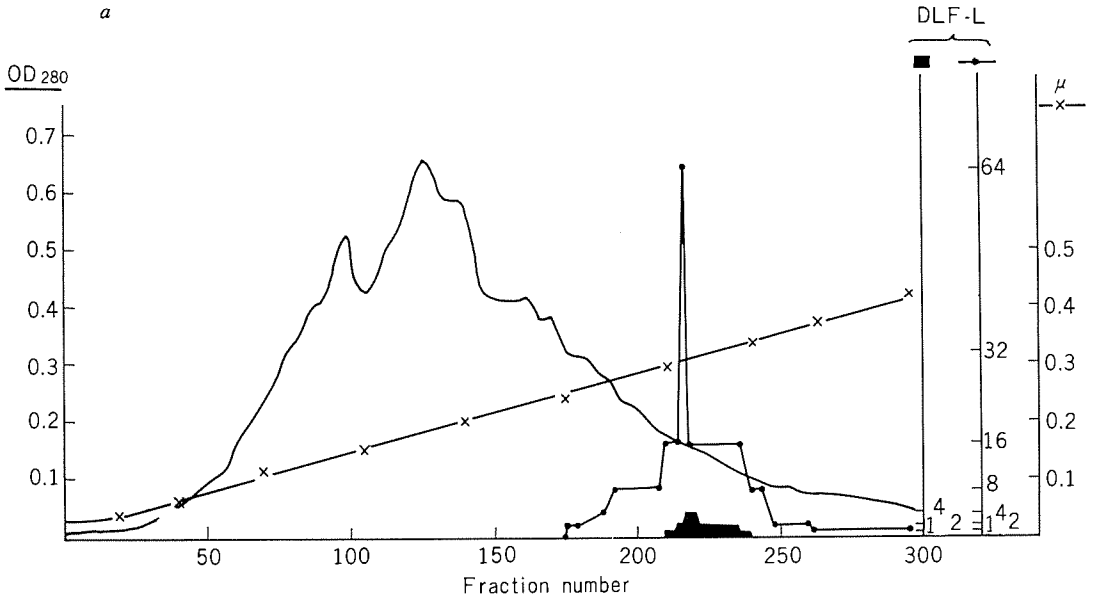
and diluted with 2 volumes of distilled water and applied on the second column of DEAE-cellulose (13×280 mm) equilibrated with 0.005 M Tris-HCl buffer, pH 7.5. After all the applied material had sunk into the column, the column was washed with 200 ml of 0.2 M NaCl in 0.005 M Tris-HCl buffer, pH 7.5, and then eluted with 400 ml of a linear gradient from 0.2 M to 0.5 M NaCl in 0.005 M Tris-HCl buffer, pH 7.5. Four-ml fractions were collected (Fig. 11b).

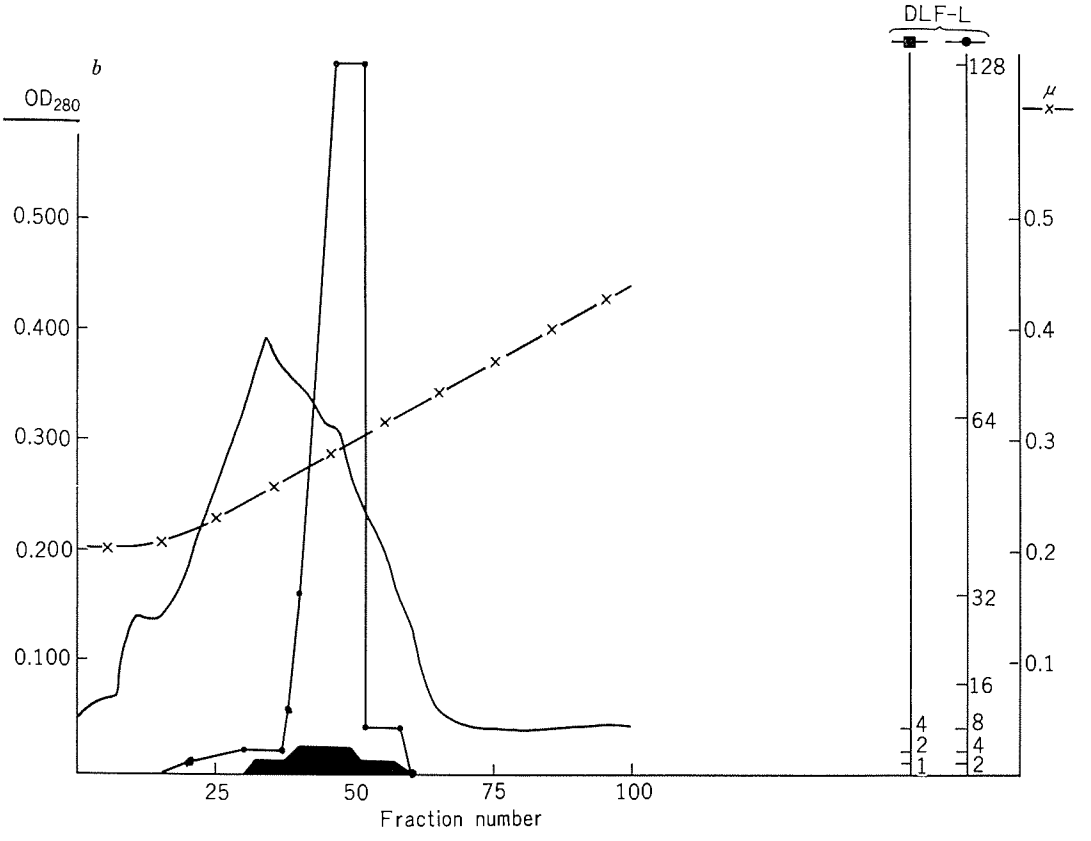
As shown in Fig. 11, the hemolytic activity was eluted from the second column at from μ=0.25 to 0.32. This direct hemolytic factor (DLF-L) was obtained from the same strain 1–1 as the direct hemolytic factor (DLF-S) described in the preceding paragraph. DLF-S was obtained from the culture filtrate of the organism in modified Syncase medium. The direct hemolytic factor (DLF-L) obtained from modified peptone medium was heat-labile and its activity decreased even on heating at 56C for 10 min (Table 2). Moreover, the hemolytic activity of DLF-L was enhanced in the presence of lecithin in the reaction mixture as shown in Table 2 and Fig. 11. Both the direct hemolytic factors (DLF-S and DLF-L) used in the experiment shown in Table 2 were obtained from the phospholipase A-negative mutant strain 1–1, and the deficiency of this mutant strain in phospholipase A activity has

▶

FIGURE 11. *Chromatography on a DEAE-cellulose column of the culture filtrate of the phospholipase A-negative mutant strain 1–1 grown in modified peptone medium.*

DLF-L activity was measured either with (—•—) or without (shaded) lecithin.


a
DLF-L
OD 280
μ
0.7
0.6
0.5
0.4
0.3
0.2
0.1
64
32
16
8
4
2
1
0.5
0.4
0.3
0.2
0.1
50
100
150
200
250
300
Fraction number


b
DLF-L
OD280
μ
0.500
0.400
0.300
0.200
0.100
128
64
32
16
8
4
2
1
0.5
0.4
0.3
0.2
0.1
25
50
75
100
Fraction number

been repeatedly confirmed. However, the DLF-L activity was observed to be enhanced by the addition of lecithin, while the DLF-S activity was not. The effect of lecithin on DLF-L was more obvious when added to heat-treated DLF-L, which had apparently lost its lytic activity (Table 2). This enhancing effect of lecithin was further confirmed using lecithin-treated erythrocytes.

Rabbit erythrocytes were obtained by centrifugation of fresh blood. They were washed three times with TBS-Ca^{++} and resuspended in the same buffer at a concentration of 1.0×10^8 cells/ml. Part of the suspension was mixed with an equal volume of 50 μg/ml lecithin in TBS-Ca^{++}. The mixture was incubated at 37 C for 30 min. It was then centrifuged and the cells were washed three times with TBS-Ca^{++} and resuspended in the same buffer at a concentration of 1.0×10^8 cells/ml. Washed erythrocytes before and after treatment with lecithin were tested for DLF-L and DLF-S lytic activities.

As shown in Table 3, DLF-L activity was raised after lecithin treatment, while DLF-S activity was the same before and after lecithin-treatment.

The increase in DLF-L activity on lecithin treatment was not due to phospholipase A activity of the preparation, because a) the direct hemolytic factor used in the experiments shown in Tables 2 and 3 was obtained from the phospholipase A-negative mutant strain (strain 1–1), b) the presence of a large excess of lysophospholipase in the reaction mixture did not inhibit the effect of lecithin, c) when lecithin was incubated with the DLF-L preparation at 37C for 2 hr, and then chromatographed by TLC, a spot corresponding to lecithin was found, but none corresponding to lysolecithin. Recently, Fujino *et al.* reported the existence of a heat-labile hemolysin in *V. parahaemolyticus*, but the relationship between their hemolysin and the DLF-L described here is not clear yet. The DLF-L of the parent strain is now under study. Further purification and characterization of both DLF-S and DLF-L are now in progress.

DISCUSSION

This paper shows that *V. parahaemolyticus* can produce a phospholipase A, a lysophospholipase and glycerophosphorylcholine (GPC) diesterase as well as two kinds of direct hemolysin (DLF). The existence of both phospholipase A and lysophospholipase was confirmed by the fact that a phospholipase A-negative mutant obtained from this organism still possesses the lysophospholipase. The presence of these enzymes explains the complex hemolytic zone surrounding colonies of this organism in various cultural conditions (Fig. 1).

Hayaishi and Kornberg isolated a phospholipase A, a lysophospholipase and GPC diesterase from *Serratia plymuthicum* (1954). In the present work isolation and partial purification of these enzymes of *V. parahaemolyticus* were attempted. On fractionation with ammonium sulfate the phsopholipase A was

TABLE 3. *Activities of DLF-S and DLF-L on untreated or lecithin-treated erythrocytes*

DLF	Untreated erythrocytes									Lecithin-treated erythrocytes								
	Dilution of DLF									Dilution of DLF								
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/1	1/2	1/4	1/8	1/16	1/32	1/62	1/128	1/256
DLF-S	4	4	4	2	1	1	0	0	0	4	4	4	2	0	0	0	0	0
DLF-L	4	4	4	2	1	1	1	0	0	4	4	4	4	4	3	2	1	0

obtained in the fraction precipitating between 0.2 and 0.4 saturation, the lysophospholipase in fractions precipitating at both 0 to 0.2 and 0.2 to 0.4 saturation, and GPC diesterase in the fraction precipitating with 0.4 to 0.6 saturation. The lysophospholipase and GPC diesterase were purified further by column chromatography, but the phospholipase A activity was not recovered from the column. It is not certain if the phospholipase A consists of more than one component, like the enzyme in *Serratia* (Hayaishi and Kornbert, 1954), and if so whether these components can be separated by chormatography.

In addition to these enzymes two kinds of direct hemolysin (DLF) were isolated from the culture filtrate of *V. parahaemolyticus*. The kind of DLF produced depended upon the culture conditions. One of these hemolysins (DLF-S) is heat-stable and its activity is not enhanced by the presence of lecithin, while the other (DLF-L) is heat-labile and its activity is enhanced by addition of lecithin to the reaction mixture. This effect of lecithin on DLF-L is not due to the existence of phospholipase A in the preparation, but seems to be due to some change in the micelle structure of the surface membrane of the erythrocyte caused by lecithin, making it more accessible to DLF-L. This explanation was supported by the fact that erythrocytes which had been treated with lecithin and then thoroughly washed were much more susceptible to DLF-L than untreated erythrocytes. Moreover, DLF-L can be produced by the phsopholipase A-negative mutant. The addition of a large excess of lysophospholipase to the reaction mixture did not inhibit the enhancing effect of lecithin on this lytic factor. When lecithin was incubated with DLF-L, no lysolecithin was produced, as judged by thin-layer chromatography. Further purification and characterization of these direct hemolytic factors are now in progress.

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