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STUDIES ON THE ROLE OF PLAKIN XI
DEMONSTRATION OF PHOSPHOLIPASE A IN PLAKIN

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SUMMARY The phospholipids of *Bacillus megaterium* were fractionated by thin layer chromatography into four fractions: PL1 (suspected to be polyglycerophosphatides), PL2 (phosphatidylethanolamine), PL3 and PL4 (both sugar containing phospholipids). Plakin was active on all four phospholipids.

Since the main enzyme of heated " Habu " venom is phospholipase A, comparative studies were performed using the above bacterial phospholipids as substrates. Plakin was proved to contain phospholipase A.

Fatty acids liberated from phospholipids by plakin or heated " Habu " venom consisted mainly of those shorter than C-14 acids, while lysoderivertives contained those longer than C-15 acids.

INTRODUCTION

Studies on plakin have been carried out in this laboratory over the last 10 years. Plakin is an antibacterial protein found in extracts of blood platelets of rats, rabbits and horses (GRUBER and FUTAKI, 1907) and active upon *Bacillus anthracis* and other related bacteria. Important information on the nature and mode of action of plakin has been gained using crude extracts of rabbit or horse blood platelets. Thus, it was found that (1) in addition to *B. anthracis*, *Bacillus megaterium* and some strains of *Bacillus subtilis* are sensitive to plakin (AMANO *et al.*, 1952, 1953a); (2) oxygen uptake with glutamate, succinate or malate as substrate instantaneously stopped upon addition of sufficient plakin (AMANO *et al.*, 1952); (3) the permeability barrier of bacteria was damaged, concomitantly with this cessation of oxygen uptake (AMANO *et al.*, 1953b, 1956); (4) definite cytoplasmic lysis could be demonstrated only after the permeability barrier and oxygen uptake had been affected (AMANO *et al.*, 1952, 1953d); (5) for activity against *B. anthracis*, but not against *B. megaterium* and *B. subtilis*, plakin needed two co-factors present in normal serum (bicarbonate ion and a serum protein fraction migrating with albumin or α-globulin) (AMANO *et al.*, 1957); (6) Ca^{2+}, Mg^{2+} or Mn^{2+} was found to be indispensable for the activity of plakin, (KATO *et al.*, 1954); (7) protoplasts of *B. megaterium*, prepared by treatment with lysozyme in a sucrose medium, which were otherwise stable, were lysed by plakin (AMANO *et al.*, 1956).

Recently, the purification of plakin was achieved, after removing the very labile lipoprotein from the crude extract, by salting out with ammonium sulfate followed by hydroxylapatite column chromatography. The purity was increased about 250 fold over the crude preparation although the purified preparation was still impure (HIGASHI *et al.*, 1963). Using this partially purified preparation, the following additional information was obtained: (1) in kinetic studies, plakin itself was shown to have no lytic activity on the protoplasts of *B. megaterium* and "real" lysis was evoked by some lytic agent liberated through the action of plakin; (2) activity of plakin was reduced under anaerobic conditions or on addition of cytochrome oxidase inhibitors ("oxygen effect"); (3) cessation of oxygen uptake occurred in parallel with the "real" lysis of protoplasts and one did not precede the other; (4) the site of this blockage of the respiratory chain caused by plakin was found to be between succinic dehydrogenase and the cytochrome system. However, this blockage could be regarded as a secondary effect due to lysis, because the same damage was observed in protoplasts lysed osmotically; (5) evidence was obtained suggesting that plakin is a kind of phospholipase. To investigate the effect of plakin on phospholipids, protoplast membranes containing ^{32}P labelled phospholipids or unlabelled phospholipids were extracted with chloroform-methanol (1 : 2) and used as substrates. In both types of experiment, the amount of P-compounds which were soluble in methanol-water increased with the incubation time, and the intact phospholipids in the chloroform fraction decreased. The optimal pH of plakin was found to be about 8.5 (HIGASHI *et al.*, 1963).

Further studies showed that plakin is a phospholipase and this paper presents experimental results suggesting the presence of phospholipase A activity in the plakin preparation.

MATERIALS AND METHODS

1. *Bacterial strain used*

Bacillus megaterium strain KM was grown at 37°C with mechanical shaking in Penassay broth or in "C" medium, which consists of Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (3 g), NH_4Cl (2 g), $MgSO_4$ (40 mg), Na_2SO_4 (110 mg), ferric citrate (34 mg), glucose (10 g) and sodium glutamate (5 g) per liter. When ^{32}P-labelled phospholipid was required, the phosphate in the "C" medium was replaced by tris-(hydroxymethyl)aminomethane hydrochloride (M/20 final concentration, at pH 7.2) and ^{32}P phosphate was added at a level of 1 μc per ml.

2. *Protoplasts*

The bacterial cells were harvested from actively growing cultures and suspended in phosphate buffer (pH 7.4) containing 15 per cent sucrose and 2×10^{-3}M $MgSO_4$, and then treated with lysozyme.

3. *Bacterial phospholipid*

The bacterial cells harvested from the above medium were extracted by chloroform-methanol (1 : 2) and then further extracted into chloroform by adding equal volumes of chloroform and 1 per cent KCl solution. The chloroform layer was then washed and evaporated off. The residue was washed twice with cold acetone to remove neutral fat and then dried.

4. *Phosphatidylethanolamine*

This was a gift from Dr. K. Saito, who extracted it from a species of the genus *Bacillus*.

5. *Lecithin and lysolecithin*

Lecithin was prepared from egg yolk phospholipids by alumina column chromatography as described by SINGLETON *et al.* (1964). Lysolecithin was prepared according to the method of Hanahan (1952).

6. *Glycerophosphorylethanolamine*

This was prepared from phosphatidylethanolamine by methanolic potassium hydroxide according to Dawson's method (1960).

7. *Plakin preparation*

The platelets from 16 l of citrated horse blood were obtained by differential centrifugation, suspended in 500 ml of 1 M NaCl solution and treated in a Kubota sonic oscillator (10 Kc) for 4 minutes.

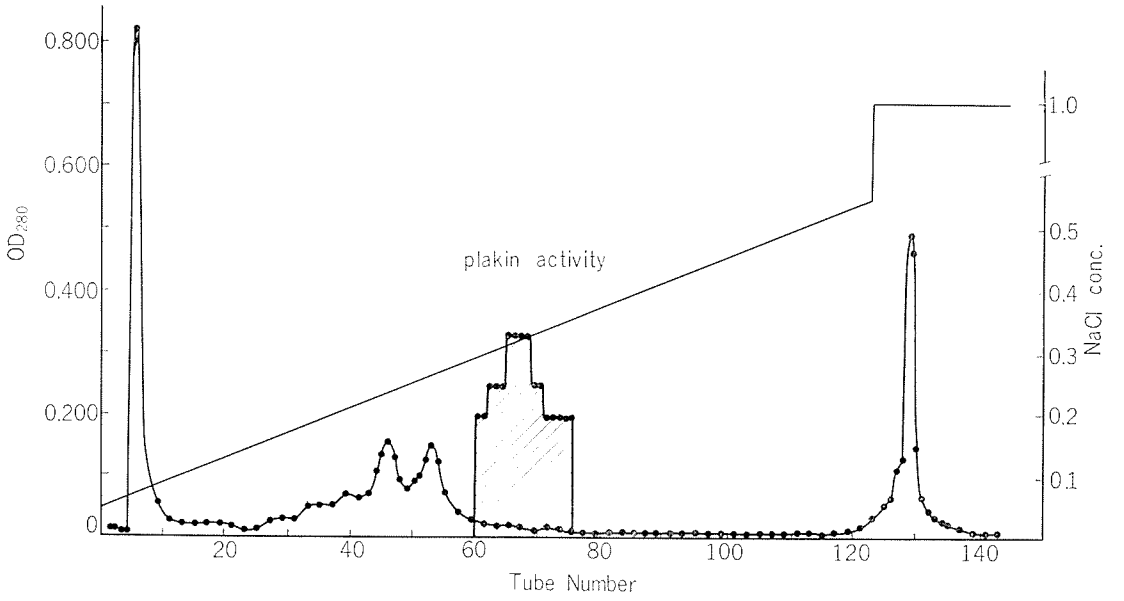

FIGURE 1 DEAE cellulose column chromatography of plakin.
•——• OD_{280} ——— NaCl concentration

The mixture was centrifuged and the supernatant was adjusted to pH 2.5 with 1N HCl. The resulting precipitate was removed by centrifugation. The pH of the supernatant was adjusted to 7.0, and plakin was fractionated with ammonium sulfate, precipitating between 0.4 and 0.8 saturation. This precipitate was dissolved in 0.1M phosphate buffer at pH 6.8 and dialyzed against saline.

DEAE cellulose column chromatography was used rather than hydroxylapatite column chromatography (HIGASHI *et al.*, 1963) as follows. To a column (2×50 cm) of DEAE cellulose equilibrated with 0.05M saline containing 0.05M borate buffer at pH 8.0, were applied 10 ml of plakin preparation, which had been previously salted out with ammonium sulfate and dialyzed against the same borate-buffered saline. The column was eluted with a linear concentration gradient of sodium chloride (up to 0.55M) buffered with 0.05M borate at pH 8.0. The results are shown in Fig. 1. Plakin activity was detected after the third major peak of material absorbing at 280 mμ. The active fractions were pooled. The specific activity based on the protein N was about 250 fold more than that of the crude platelet extract, and the yield (30 per cent) was higher than that obtained by hydroxylapatite column chromatography (10 per cent). However, this preparation was still impure. Further purification was abandoned because of the low yield of total protein in the active fraction.

8. *Heated "Habu" venom*

A dried preparation of venom of "Habu" (*Trimeresurus flavoviridus*) was kindly given by Dr. T. KUBO of this Institute. A solution of 5 mg per ml of this dissolved in M/100 phosphate buffer, pH 6.0, was heated in a boiling water bath for 10 minutes and centrifuged and the supernatant was used.

9. *Lysophospholipase*

Pancreatic lysophospholipase was purified according to the procedure described by SHAPIRO (1953).

10. *Estimation of plakin*

To tubes containing 5.0 ml of protoplast suspension in 15 per cent sucrose solution, a mixture of 0.3 ml of 50 per cent sucrose solution, 0.2 ml of M/15 phosphate buffer (pH 7.4) and 0.5 ml of varying dilutions of plakin preparation were added, and the tubes were incubated at 37°C. The tubes were mixed by inversion at 10 minute intervals and, after mixing, the optical densities were read at 550 mμ in a Coleman universal spectrophotometer.

It was found that the process of protoplast-lysis showed a lag period during which no decrease in optical density occurred. As the duration of the lag period was inversely proportional to the dilution of plakin, the plakin concentration could be assayed by determining the duration of the lag period (HIGASHI *et al.*, 1963).

11. *Enzymic digestion of phospholipids and their derivatives*

Substrates were suspended in water and 0.2 ml of these suspensions were added to reaction tubes. The other constituents of the tubes are given in the " Results ".

12. *Thin layer chromatography (TLC)*

Silica gel G plates were used for TLC. Phospholipid samples were applied to the plates and chromatography was carried out by development with solvent I: diisobutylketone-acetic acid-water (8 : 5 : 1) or solvent II: phenol-water-acetic acid-ethanol (8 : 2 : 1 : 1). To detect the phospholipids and their breakdown products the following reagents were used: iodine vapor for lipids, ninhydrin for amino compounds, molybdic acid for phosphate compounds, ammoniacal silver nitrate for reducing sugars and glycerol compounds, periodic acid and fuchsin-sulfurous acid for glycol compounds (periodate-Schiff reagent) and hydroxylamine-ferric chloride for acyl-esters (SKIDMORE, 1962).

13. *Autoradiography*

This was performed using Fuji X-ray films.

14. *Gas chromatography of fatty acids*

Analyses were kindely performed by Drs. T. YAMAKAWA and N. UEDA of the Institute for Infectious Diseases, Tokyo University, using a Hitachi-Perkin Elmer model F-6 gas chromatograph.

RESULTS

1. *Effect of purified plakin on bacterial phospholipids*

The purified preparation of plakin had been demonstrated to contain a kind of phospholipase which hydrolyzes the membrane phospholipids of sensitive bacteria (HIGASHI *et al.*, 1963). Therefore, further studies were made on the nature of this phospholipase.

First, the effect of plakin on the acyl-ester linkages of the crude bacterial phospholipids was investigated by estimating the acyl-ester content according to the method of Shapiro (1953). The constitution of each reaction mixture for this estimate is shown in Table 1 and the results in Fig. 2. As can be seen, the acyl-ester content decreased with the incubation period, and hence the effect of plakin was shown to be due to a phospholipase which hydrolyzed ester linkages between hydroxyl groups and fatty acids.

It is also shown in Fig. 2 that the phospholipase activity was greatly activated by Ca^{2+} and to a lesser degree by Mg^{+2} while it was completely inhibited by EDTA. This indicates that the phospholipase of plakin requires Ca^{2+} as a co-factor, and it is interesting that Ca^{2+} is also required for the antibacterial activity of plakin (KATO *et al.*, 1954).

TABLE 1 *The compositions of the reaction tubes in Fig. 2.*

Tube	A	B	C	D	E
	ml.	ml.	ml.	ml.	ml.
Emulsion of bacterial phospholipid (10 mg/ml)	0.2	0.2	0.2	0.2	0.2
Plakin	0.2	0.2	0.2	0.2	—
50 mM $MgSO_4$	0.1	—	—	—	—
50 mM $CaCl_2$	—	0.1	—	—	—
50 mM EDTA	—	—	0.1	—	—
50 mM Tris buffer (pH 8.0)	0.5	0.5	0.5	0.5	0.5
Water	—	—	—	0.1	0.3

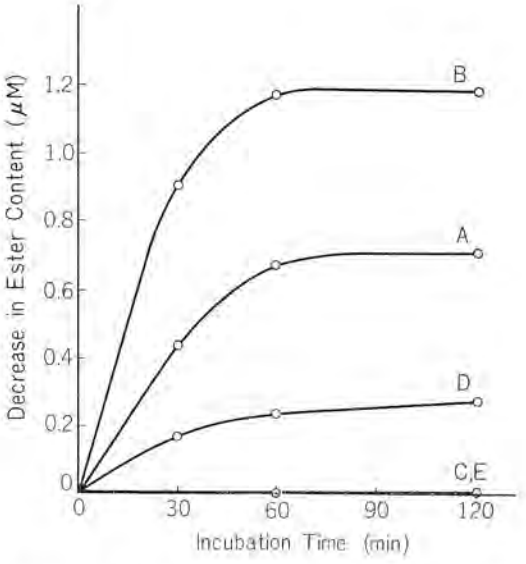

FIGURE 2 Effect of plakin on acyl-ester content of bacterial phospholipids.

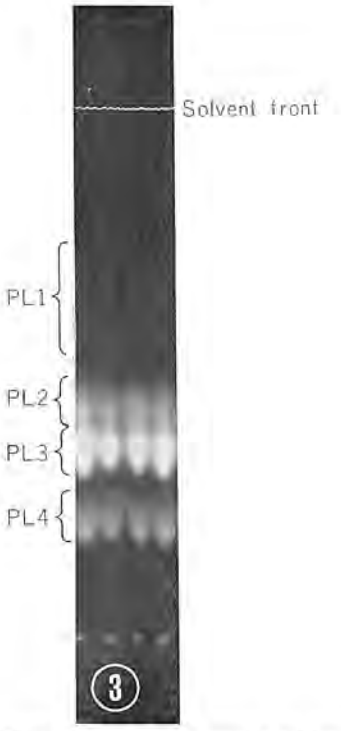

FIGURE 3 Thin layer chromatography of bacterial phospholipids.

2. *Fractionation of bacterial phospholipids and effect of plakin on each fraction*

To test the substrate specificity of the phospholipase of plakin, ^{32}P-labelled phospholipids prepared from *Bacillus megaterium* were fractionated by thin layer chromatography on silica gel G plates using diisobutylketone-acetic acid-water (8 : 5 : 1) as developing solvent. An autoradiograph was then taken. It showed four main spots of phospholipids (Fig. 3) and each was eluted with chloroform-methanol (1 : 2). These fractions were called PL1, PL2, PL3 and PL4, in order of increasing Rf value. The Rf value and colour reactions of each fraction are shown in Table 2.

PL2 gave a positive ninhydrin reaction and was proved, by comparison with an authentic sample, to be phosphatidylethanolamine (Figs. 7 and 8). PL3 and PL4 were shown to contain reducing sugar because of their very strong reactions with ammoniacal silver nitrate.

WAKUI *et al.*, (1959) have shown that of the phospholipases only phospholipase A is found in " Habu " venom after heating at 100°C for 10 minutes. Therefore, the effects of plakin on these four fractions were compared with those of heated " Habu " venom. The constitutions of the reaction mixtures are shown in Table 3. The tubes were incubated at 37°C for 2 hours and then 3.75 ml of chloroform-methanol (1 : 2) were added to each. The mixtures were centrifuged to remove the precipitated protein. The supernatants were dried *in vacuo* and each residue was dissolved

TABLE 2 *Color reaction and Rf values of bacterial phospholipids*

Phospholipid	Rf Values	Color Reaction			
		P^{32}	I_2	Nin	Ag
PL 1	0.59	+	+	−	+
PL 2	0.46	+	+	+	+
PL 3	0.39	+	+	−	++
PL 4	0.28	+	+	−	++

TABLE 3 *The constitutions of the reaction mixtures in Fig. 4*

Tube		1	2	3	4	5	6	7	8	9	10	11	12
		ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Emulsion of phospholipid	PL-1	0.2	0.2	0.2									
	PL-2				0.2	0.2	0.2						
	PL-3							0.2	0.2	0.2			
	PL-4										0.2	0.2	0.2
Heated "Habu" venom		—	0.1	—	—	0.1	—	—	0.1	—	—	0.1	—
Plakin		—	—	0.2	—	—	0.2	—	—	0.2	—	—	0.2
0.2 M borate buffer (pH 8.0)		0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
50 mM $CaCl_2$		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Water		0.5	0.4	0.3	0.5	0.4	0.3	0.5	0.4	0.3	0.5	0.4	0.3

TABLE 4 *Lysophospholipase treatment of PL2-Pk and PL2-Sv*

	ml.	ml.	ml.	ml.	ml.
PL2-Pk	0.3	0.3	—	—	—
PL2-Sv	—	—	0.3	0.3	—
	adjusted to pH 6.0				
Lysophospholipase	—	0.3	—	0.3	0.3
Water	0.3	—	0.3	—	0.3
	incubated for 30′ at 30°C				

in a small amount of chloroform-methanol-water (5 : 10 : 4), and thin layer chromatography was carried out on these samples followed by autoradiography. Fig. 4 shows the autoradiograph. The four phospholipids were found to be hydrolyzed by plakin and the reaction products were designated as PL1-Pk, PL2-Pk, PL3-Pk and PL4-Pk. In addition, the Rf values of PL1-Pk, PL2-Pk, PL3-Pk and PL4-Pk were the same as those of the lysoderivatives of the four phospholipids obtained by treatment with heated "Habu" venom (PL1-Sv, PL2-Sv, PL3-Sv and PL4-Sv), respectively.

3. *Identification of reaction products of bacterial phospholipids wtih plakin as lysophospholipids*

Studies were made of whether PL2-Pk was really lysoglycerophosphorylethanolamine. For this purpose, PL2-Pk and PL2-Sv were incubated with lysophospholipase, and attempts were made to identify each reaction product with glycerophosphorylethanolamine (GPE).

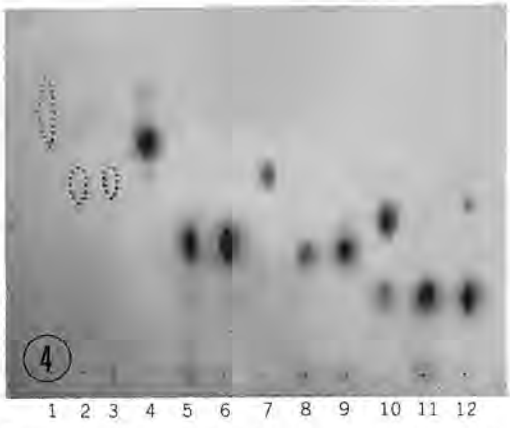

FIGURE 4 Thin layer chromatography of plakin and heated "Habu" venom treated phospholipids.

PL2-Pk or PL2-Sv was incubated at 37°C for 30 minutes with a partially purified preparation of bovine pancreatic lysophospholipase, prepared according to the method of Shapiro (1953). The constitution of each reaction mixture is shown in Table 4. After incubation, 2.25 ml of chloroform-methanol (1 : 2) were added and the mixtures were centrifuged. The deproteinized supernatants were dried *in vacuo* and the residues were dissolved in a small amount of chloroform-methanol-water (5 : 10 : 4). Thin layer chromatography was carried out on these samples using solvent I: diisobutylketone-acetic acid-water (8 : 5 : 1) and solvent II: phenol-water-acetic acid-ethanol (8 : 2 : 1 : 1), and then autoradiography and

FIGURE 5 TLC of PE, PL2 and their breakdown products. (Autoradiography, Solvent I)

FIGURE 6 TLC of PE, PL2 and their breakdown products. (Autoradiography, Solvent II)

color reactions were performed. The results of the autoradiography are presented in Figs. 5 (solvent I) and 6 (solvent II). As can be seen, the reaction products (PL2-Pk-Lp and PL2-Sv-Lp) of PL2-Pk and PL2-Sv with lysophospholipase had identical Rf values: both PL2-Pk-Lp and PL2-Sv-Lp were found at the origine after chromatography with solvent I and at a position with an Rf of 0.27 with solvent II.

On other plates, PL2-Pk-Lp and PL2-Sv-Lp were compared by the ninhydrin reaction with glycerophosphorylethanolamine (GPE) prepared from a sample of phosphatidylethanolamine (PE) by treatment with methanolic potassium hydroxide according to Dawson's method (1960). These plates, included PE, PE treated with heated "Habu" venom (PE-Sv), PE treated with plakin (PE-Pk), PL2, PL2-Sv, PL2-Pk, ethanolamine (E) as a reference and a sample (Lp) without added substrates as a control (Table 4). As can be seen in Figs. 7 (solvent I) and 8 (solvent II), PL2-Pk-Lp and PL2-Sv-Lp gave several ninhydrin-positive spots other than that corresponding to GPE; however, those spots not

FIGURE 7 TLC of PE, PL2 and their breakdown products. (Ninhydrin, Solvent I)

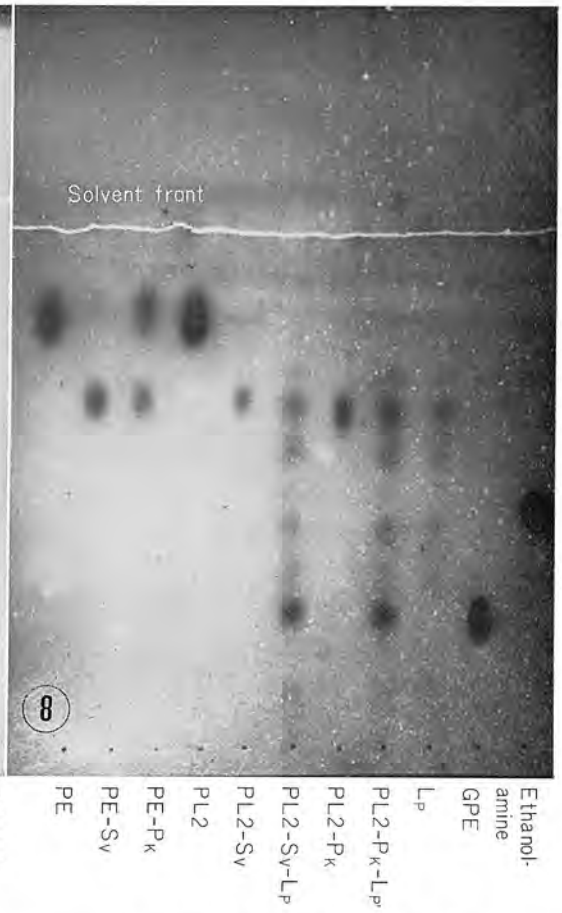

FIGURE 8 TLC of PE, PL2 and their breakdown products. (Ninhydrin, Solvent II)

corresponding to GPE appear to be derived from the preparation of lysophospholipase, since the Lp control gave the same spots. Although the chromatograph stained with ninhydrin shown in Fig. 7 did not give clear spots of GPE from samples PL2-Pk-Lp or PL2-Sv-Lp, the autoradiographs clearly show spots corresponding to the ninhydrin-positive spot of GPE. The identity of the main spot of PL2-Pk-Lp or PL2-Sv-Lp in the ninhydrin reaction with GPE is more clearly demonstrated in Fig. 8, in which the main spots of PL2-Pk-Lp and PL2-Sv-Lp and the spot of GPE were all located at Rf 0.27. These spots were shown to contain phosphate by the ammonium molybdate reaction. Fig. 8, however, shows a spot given by PL2-Pk-Lp or PL2-Sv-Lp with the same Rf value as PL2-Pk or PL2-Sv but which cannot be seen in Figs. 5, 6 and 7. This spot did not contain ^{32}P (Fig. 6), which excludes the possibility that it was the substrate, PL2-Pk or PL2-Sv. Hence it can reasonably be considered that some ninhydrin positive substance, derived from the preparation of lysophospholipase, happened to appear as a spot having the same Rf value as PL2-Pk or PL2-Sv

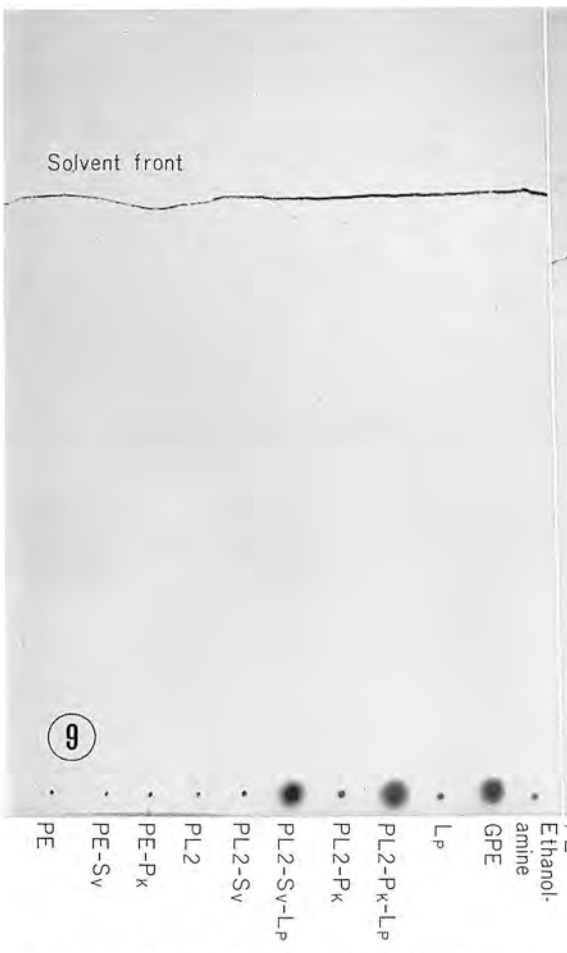

FIGURE 9 TLC of PE, PL2 and their breakdown products. (Periodate-Schiff, Solvent I)

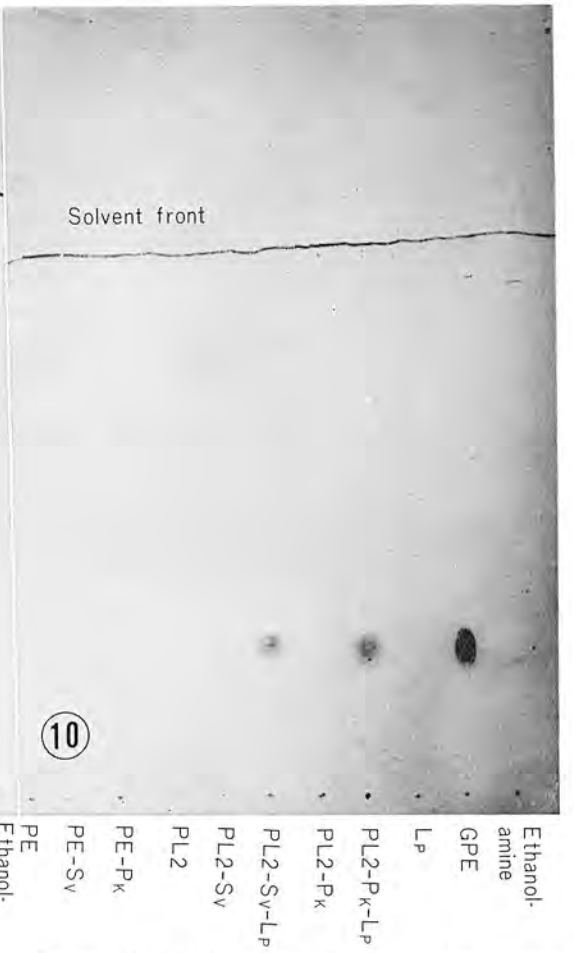

FIGURE 10 TLC of PE, PL2 and their breakdown products. (Periodate-Schiff, Solvent II)

with solvent II.

Figs. 9 (solvent I) and 10 (solvent II) show the results of the periodate-Schiff reaction. The main ninhydrin positive spots of PL2-Pk-Lp and PL2-Sv-Lp corresponding to GPE as well as the known GPE, gave a positive color reaction. In addition, these spots gave no color reaction with hydroxylamine which tests for the presence of esterified fatty acids. This means that the substances of these main ninhydrin positive, phosphate containing spots contain two adjacent free hydroxyl groups (positive periodate-Schiff reaction) and no esterified fatty acid (negative hydroxylamine test), and that these main spots are simply GPE.

The results of the above experiments are summarized in Table 5 and show that; 1) the main ninhydrin positive, phophate containing spot of PL2-Pk-Lp and PL2-Sv-Lp was glycerophosphorylethanolamine; 2) PL2-Pk and PL2-Sv were lysoderivatives of PL-2, and 3) the purified plakin preparation contained phospholipase A.

TABLE 5 *Color Reactions and Rf Values of Phospholipids and their Breakdown Products*

	Rf Values		Color Reaction				
	Solvent I	Solvent II	P^{32}	Mo	Nin	EFA	Schiff
PL2	0.46	0.84	+	+	+	+	−
PL2-Pk	0.26	0.67	+	+	+	+	−
PL2-Pk-Lp	0	0.27	+	+	+	−	+
PL2-Sv	0.25	0.68	+	+	+	+	−
PL2-Sv-Lp	0	0.27	+	+	+	−	+
PE	0.48	0.84		+	+	+	−
PE-Pk	0.25	0.68		+	+	+	−
PE-Sv	0.25	0.68		+	+	+	−
GPE	0	0.27		+	+	−	+
Ethanolamine	0.05	0.46		−	+	−	−
Glycerophosphate	0	0		+	−	−	+
Glycerol	0.62	0.26		−	−	−	+

TABLE 6 *Phospholipase A Activity of Plakin on Egg Yolk Lecithin*

Tube No.	1	2	3	4	5	6
	ml.	ml.	ml.	ml.	ml.	ml.
Lecithin (0.01 M)	0.2	0.2	0.2	0.2	0.2	0.2
Plakin	0.2	0.2	—	—	—	—
Snake venom	—	—	0.2	0.2	—	—
$CaCl_2$ (0.1 M)	0.1	—	0.1	—	0.1	—
Tris buffer (pH 8.0)	0.2	0.2	0.2	0.2	0.2	0.2
Water	0.3	0.4	0.3	0.4	0.5	0.6
↓						
Incubation for 2 hrs. at 37°C						
↓						
Addition of 4 ml Ethanol-Ether (=3 : 1)						
↓						
Measurement of Acyl Ester Content						
D_{520}	0.490	0.570	0.330	0.365	0.560	0.550
↓						
Measurement of Hemolytic Activity						
D_{540}	0.098	0	0.890	0.860	0	0

4. *Effect of plakin on egg yolk lecithin*

Because of its phospholipase A activity, plakin affected the phospholipids of sensitive bacteria. Therefore, further experiments were carried out to see whether phospholipids from other sources were susceptible to plakin; for this purpose, egg yolk lecithin was used as substrate. The constitution of each reaction mixture is shown in Table 6. After incubation at 37°C for 2 hours, 4 ml of ethanol-ether (3 : 1) were added to each reaction and the mixtures were centrifuged. Fatty acid ester was estimated using 3 ml of the supernatant. To 0.2 ml of the rest of each supernatant, 3ml of rabbit red blood cell suspension (6.2×10^7 cells per ml) were added and the mixtures were incubated at 37°C for 30 minutes. Then, after centrifugation, the hemoglobin content of the supernatants was estimated at 540 mμ in a Hitachi type EPU-2A spectrophotometer (Table 6). It can be seen that plakin, when activated by Ca^{2+}, exhibited a weak but definite activity on egg yolk lecithin; lysolecithin was detected by its hemolytic activity, and "Habu" venom heated at 100°C for 10 minutes showed very strong activity.

Another portion of the centrifuged supernatant from tubes 1, 3 and 5 was concentrated by evaporation and thin layer chromatography was carried out, using lysolecithin prepared according to the method of Hanahan (1952) as a control.

As can be seen in Fig. 11, a small but definite spot of lysolecithin was demonstrated in the

plakin digest.

FIGURE 11 TLC of the reaction products when plakin or snake venom was added to lecithin.

5. *Analyses of fatty acids liberated by plakin and heated "Habu" venom*

Since the purified preparation of plakin contained phospholipase A, an experiment was performed to investigate whether the enzyme exhibits substrate specificity for the fatty acids liberated. For this study, 9 mg of PL2 emulsified in 5.0 ml of M/10 borate buffer at pH 8.0 was incubated at 37°C for 2 hours with 5.0 ml of plakin. After incubation, 37.5 ml of chloroform-methanol (1 : 2) were added, and the mixture was centrifuged. The supernatant was dried *in vacuo* and applied to a column of silicic acid (Merck : 8 g) and chromatographed first with ether and then with methanol. The ether fraction (1.7 mg), containing free fatty acids, and the methanol fraction (7.2 mg), containing phospholipid and lysophospholipid, were separated. The fatty acids thus obtained were esterified by refluxing with methanol and sulfuric acid, and as a control, PL2 was treated with methanolic potassium hydroxide to yield the fatty acid methyl-ester. These esters were very kindly analyzed for us in a Hitachi-Perkin Elmer, model F6, gas chromatograph by Drs. J. YAMAKAWA and N. UEDA. Fig. 12 shows a chromatogram of the fatty acids of PL2, and that of the fatty acids liberated by plakin from PL2 is shown in Fig. 13. As can be seen from Fig. 12, many fatty acids were found. Although those with chains shorter than 13 carbon atoms were not identified, the most abundantly fatty acids detected were C-15 iso- and anteiso-acids as already reported by THORNE *et al.*, (1961) for the same *Bacillus megaterium* strain KM. As shown in Fig. 13 of the fatty acids liberated by plakin, C-13 and C-14 acids were most abundant and the amount at those with chains longer than C-15 was less than in Fig. 12.

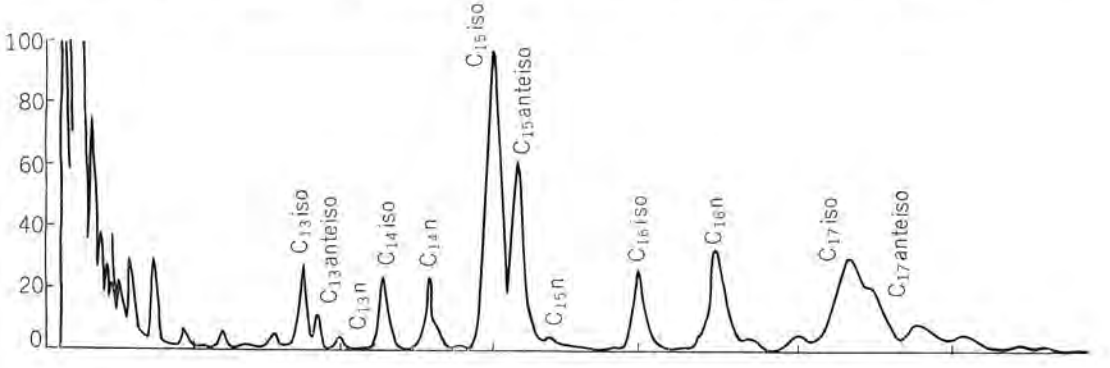

FIGURE 12 Gas chromatogram of the fatty acids of PL2.

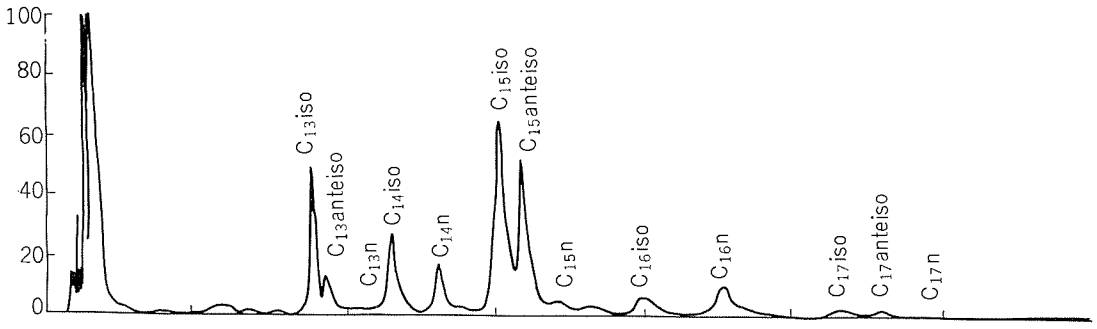

FIGURE 13. Gas chromatogram of the fatty acids liberated by plakin from PL2.

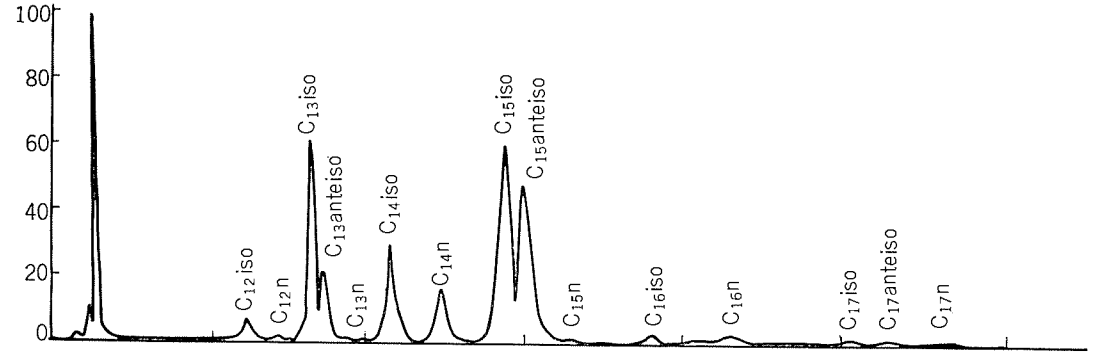

FIGURE 14 Gas chromatogram of the fatty acids liberated from PL2 by heated " Habu " venom.

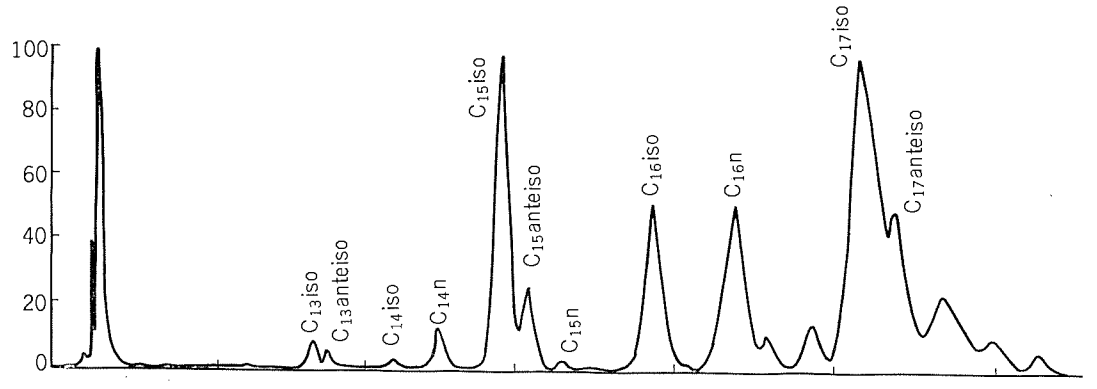

FIGURE 15 Gas chromatogram of the fatty acids of lysophosphatidylethanolamine.

A similar experiment was carried out with the fatty acids liberated from PL2 (phosphatidylethanolamine) by heated " Habu " venom and with the bound fatty acids of PL2-Sv (lysophosphatidylethanolamine). The latter fatty acids were esterified directly from PL2-Sv with methanolic potassium hydroxide, and the former ones were treated as above. Gas chromatography was performed and Figs. 14 and 15 present the chromatograms of the liberated and bound fatty acids, respectively. As can be seen from the two figures, there was a large amount of fatty acids shorter than C-14 acids in the liberated fraction and those longer than C-15 acids were abundant in the bound fraction. This contrast was very clear-cut.

DISCUSSION

Studies on plakin were begun about 15 years ago to clarify the host defense mechanism against infection of *B. anthracis*. For the first 10 years, the purification of plakin was not attempted because of the low yield of blood platelets. Without purified plakin, no progress could be made in studies on its nature. DEAE-cellulose chromatography improved the yield of plakin, as described in this paper; however, the purified preparation is still impure, because the plakin activity was eluted after the third protein peak. Further purification of this preparation was impossible because of the low yield of total protein, and the authors were forced to study the nature of plakin using this partially purified preparation.

Among the four phospholipids from *Bacillus megaterium* that named PL1 seems to be polyglycerophosphate by virtue of the high Rf value found for this substance by MACFARLANE (1961, 1962), who studied the phopholipids of *Staphylococcus aureus*, *Micrococcus lysodeikticus* and *Salmonella typhimurium*.

WAKUI *et al.* (1959) showed that: 1) heated " Habu " venom contains phospholipase A as the main enzyme; 2) it does not contain lysophospholipase; 3) phospholipase A lyses red blood cells only in the presence of added phospholipids. Therfore, we compared the phospholipase A of heated " Habu " venom with regard to their effects on bacterial phospholipids and the identify of the two enzymes was demonstrated. Here, another enzyme capable of hydrolyzing phospholipids, phospholipase C, must be considered. If it is present in both preparations, phosphorylethanolamine must be demonstrated in the reaction products. However, no trace of phosphorylethanolamine was found when ^{32}P-labelled phospholipids were used as substrates.

According to TATTRIE (1959), unsaturated fatty acids are combined at the β-position of egg yolk lecithin and according to Wakui (1961) unsaturated fatty acids are liberated by heated " Habu " venom. In the case of the phosphatidylethanolamine of *B. megaterium*, unsaturated fatty acids were scarcely detectable, and mainly fatty acids shorter than C-15 acids were liberated by treatment with heated " Habu " venom or plakin, although the activity of plakin was rather weak. From these observations it is suggested that fatty acids with shorter chains than C-15 acids are located mainly at the β-position while those with longer chains than C-15 acids are located at the α'-position of phosphatidylethanolamine in *B. megaterium*.

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