PHOSPHOLIPIDS IN THE PERITROPHIC MEMBRANE OF THE SILKWORM, *BOMBYX MORI* L.*

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INTRODUCTION

The silkworm, *Bombyx mori* L., possesses a soft colloidal thin membrane, called "Peritrophic membrane", in the alimentary canal. Examination of the peritrophic membrane shows that it consists of a single uniform circumference throughout its length, and under the electron microscope, of a fibrous network with a thin film between[4]. It is constantly renewed, the old one being discharged with feces or the solute mucus. In the formation of lamellae, this is regard as secretion of substance which condenses to membrane sometimes as transformation of a part of cell surface[12]; but there is no real distinction between these atternatives. The physiological function of this membrane is generally regarded as protection of the epidermis from abrasion by hard fragment in the food, and rapid removal of substances which are useless for the silkworm[14].

The peritrophic membrane has some components: a basis of chitin, with protein incorporated in it[13]. The remainder is made up of other mucopolysacchrides including, apparently, hyaluronic acid[7][9][14].

On the other hand, it is investigated that the peritrophic membrane of having flacheria, which is one of the fatal disease for silkworm, being liquefied or disappeared at all as the condition of the disease, and then dissolves in the presence of the insect pathogens[5][10][11]. The degradation of the peritrophic membrane of the insect in the pathogens[8], in views of the insect pathology, is one of the most interestings.

Bacillus thuringiensis group, which is pathogenicity to the larvae of certain insects such as the lepidoptera, produces the enzyme, phospholipase C (E. C. 3-1-4-3), which is one of the classification key for pathogens in these insects[6]. Authors, therefore, who have researched the infection mecha-

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nism of the disease of the silkworm caused by *B. thuringiensis* var. *thuringiensis* BERLINER, are expecting of the existence of phospholipids which is substrates of this enzyme, in the peritrophic membrane of the silkworm. However, meager work has been done with phospholipids in tissues of the insects, though it is believed that the phospholipids exist in invertebrate and have similar function in invertebrate as in vertebrate.

This paper provides additional informations on the chemical natures of phospholipids material in the peritrophic membrane of the silkworm.

MATERIALS AND METHODS

Material of the peritrophic membrane: After starving the silkworm (strain; Japanease 131 × Chinease 131) for six hours the peritrophic membrane were removed by dissection from approximately four thousand individuals at a stage of the 5th instar. They were thoroughly washed with cold saline to except the fragments of leaves and digestive fluid, and then treated with fresh acetone. All treatments were at low temperature unless otherwise noted.

Preparation of phospholipids sample: The procedure of FOLCH and SLONANE-STANLEY (1957) was mainly followed[3]. The washed sample were homogenized with chloroform-methanol mixture, 2 : 1 by volume in a high speed blender. After standing overnight in the darken with stirring, the emulsion was centrifuged at 3,000 rpm for 20 min. and filted with glass filter. The residue was again extracted with same technique. The extracts were washed by FOLCH's method[2], as follow. The put filtrate together was violently shaken for 2–3 min. with 0.2 volumes of 0.9% NaCl solution, and leaned this overnight at the temperature 0°C. The upper aqueous layer containing same solution phosphorous impurities was removed and discarded. After the low layer was rewashed similarly with "pure solvents upper layer", taken to dryness in vacuo, then dissolved in equal volume of chloroform and analysed for phosphorus.

Procedure for phospholipids analysis: The BARTLETT's method (1959)[1] was modified for estimation of total phospholipids. Up to 2.0 ml. of the sample to be analyzed and 0.5 ml. of 10N–H_2SO_4 were placed in a tube and heated on a sand-bath at 150–160°C for 3 hours. Four drops of 30%–H_2O_2 were added, and solution was returned to the bath for 1.5 hours more to complete the combustion. After cooling at room temperature, 4.6 ml. of 0.22%–ammoniummolybdate and 0.2 ml. of the Fiske-Sabbarow reagent were added, mixed throughly, and heated for 7 min. in a boiling water-bath. The optical density at 830 mμ was recorded with the used of colorphotometer (Shimazu Co.).

Column chromatography: Silicic acid chromatography was employed for

the separation of phospholipids. Silicagel (Kanto Chemical Co., Tokyo, for chromatography, 100 mesh) was suspended in methanol and repeated a washing to clarify. After drying the silicic acid, it was activated in an oven at 110°C for 12 hours and stored in a desiccator. A slurry of 75 g of activated silicic acid with methanol was packed into a chromatographic tube, which was 450 mm in length and 26.4 mm inner diameter, with a constriction at its lower. The whole silkworm lipids (slightly greenish yellow) were quantitatively transferred to the top of the column. Development was continued with chloroform, then with increasing percentages (by volume) of methanol in chloroform. The elution schedule was the following solvent system; 20 ml. of chloroform, 20 ml. of acetone, 30 ml. of chloroform-methanol (4 : 1) 30 ml. of chloroform-methanol (3 : 2), 40 ml. of chloroform-methanol (1 : 1), 40 ml. of chloroform-methanol (1 : 4), and 40 ml of methanol, successively. The speed of elusion was regulated at 1 ml/sec. and the effluent was collected 5 ml. Phospholipids were estimated in each fraction of the effluent from the column.

Thin layer chromatography (TLC): Kieselgel G (Merck) was used as absorbent. Development was performed by the usual ascending method using the solvent system of chloroform : methanol : water (70 : 30 : 5). Preparative TLC was carried out by using glass plates (20 × 20 cm.) coated with Kieselgel G layers 0.25 mm. in thickness. The chromatoplates were activated at 110°C for 30 min. After developing 13 or 15 cm., the individual compounds were detected by spraying 50% H_2SO_4, 5%-phosphomolybdic acid, Dragendorff reagent, Hans reagent and iodine vapor etc. over the plate. Standerd phospholipids for identification were used from the liver (procedure of Sapporo Medical College).

RESULTS AND DISCUSSION

Very many techniques have been described in literature for extraction of phospholipids from tissue, every method uses extracting solution 10 times the volume of tissue. A good technique should minimized formation of new covalent bounds and breaking of covalents in the native lipids molecules. In this experiment, when acetone is used in the first treating for extraction of phospholipids, though losses acid-phosphorous compounds, in the peritrophic membrane, no more 3 times the volumes of tissue are necessary. The extraction temperature being maintained, as far as possible at 0°C.

The extracts were purifyed following FOLCH's washing procedure to remove water-soluble substance. This method is seemed most attractive for this purpose and lipid extracts obtained was subjected to FOLCH's washings with brine, evaporated, and the chloroform-methanol soluble residue was

chromatographed on silicic acid. This method is also reproducible more than dialysis and cellurose-column technique for most chemical investigation of lipids. Slightly greenish-yellow extracts were obtained. The yield of the conjugated lipids were 1.42% of the extracts.

The standard curve following KH_2PO_4 to estimation of total phospholipids is shown in Fig 1. The plotting value of each concentration of KH_2PO_4 is the avaraga of 20 measurements to improve in appoximations, an error of 0.1% was involved in the measurements of density. The total phospholipids to be 25 times of the value on the standard curve.

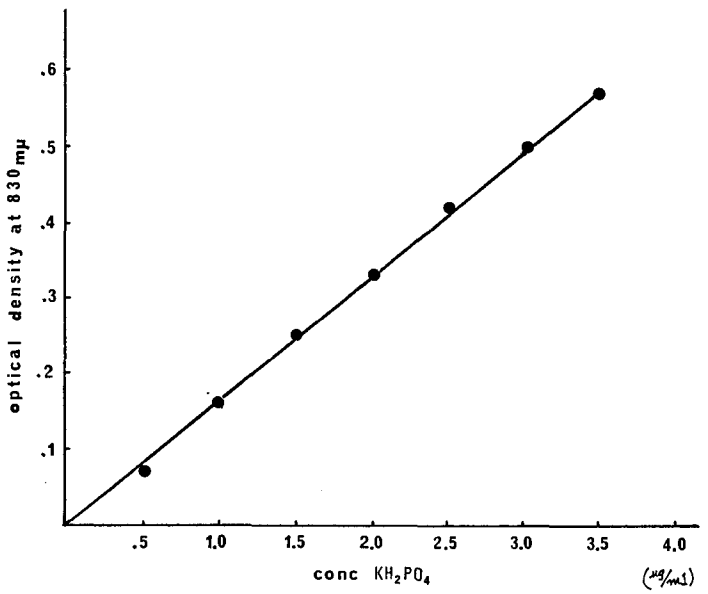

Fig. 1. Standard curve of phosphorus by the heating method
The reaction mixture was consisted 2 ml of standard solution of KH_2PO_4 after wet-combustion 4.6 ml of 0.22% ammoniummolybdate and 0.2 ml of the Fiske-Sabbarow reagent. The mixture was heated for 7 min. and the optical density was measured at 830 mμ.

The method for analysis of phosphorous is widely experimented gravimetric, volumetric, and colormetric. The colormetric is known for its simplicity, rapidity as microassay, BARTLETT's method has offered significant advantages in case of manipulation, accuracy, and sensitivity. Solutions with higher color values, however, could, by dilution with water, be brought into the most accurate range for colormetry without losing proportionality.

From this standard curve, 64.24 μg/ml. of phospholipids were culculated from the peritrophic membrane of four thousands, this was yielded 0.12%

a larva.

The separation and identification of this membrane by the onedimentional thin layer chromatography was carried out.

As shown in Fig. 2-b, a number of spots were detected by the TLC of whole lipids originated from extracts. It was characteristic that neutral lipids,

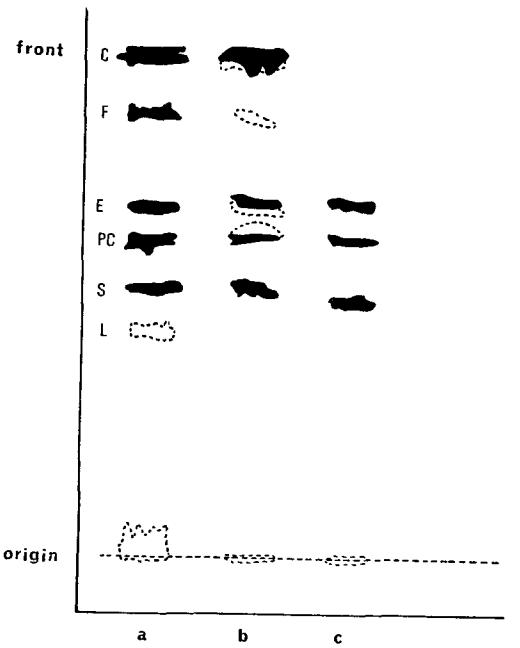

Fig. 2. Thin layer chromatography on silicic gel G plate
Developed by chloroform-methanol-water (70 : 30 : 5)
Detected by 50%-H_2SO_4, iodine, Dragendolff
a: standard sample with liver (C; neutral fat, F; free fatty acids E; phosphatidylethanolamine PC; phosphatidylcholine) S; sphymgomyerine L; lysolecithin)
b: whole extracted lipids c: effluent lipids from silicic acid column.

fatty acids and a relatively small amounts of phospholipids were present in the whole lipids, in contrast to general observation on the abundance of these lipids in liver (Fig. 2-a).

In order to examine the nature of lipid components, the whole phospholipids were first seperated by silicic acid column chromatography. The described elution schedule was used for effluates from column and separated 100 fractions were collected. The elution pattern of lipids isolated from the

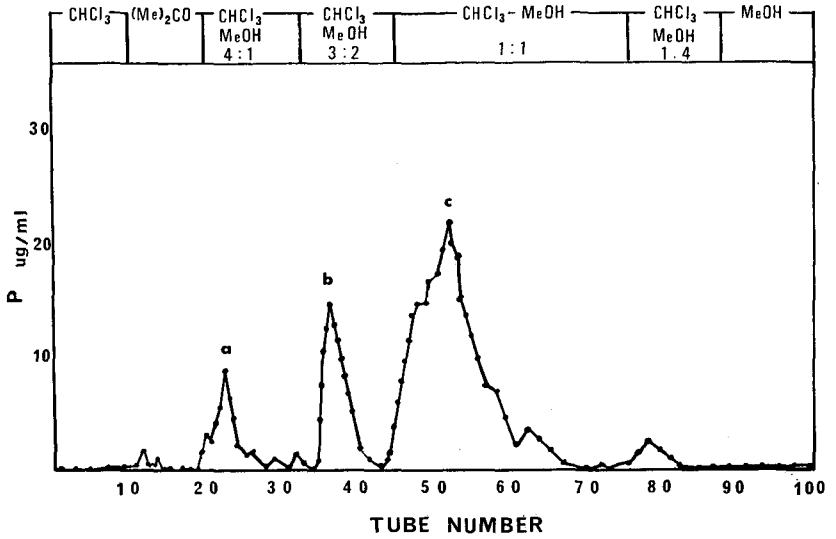

Fig. 3. Silicic acid column chromatogram of the phospholipids from the peritrophic membrane of the silkworm
a: phosphatidylethanolamine b: phosphotidylcholine c: sphingomyelin

whole lipids is typically presented in Fig. 3, showing the three major independent peaks and several minor peaks. For the purpose of detecting the lipid components of the individual peak fractions shown in Fig. 2-c, each of them was examined by the onedimentional TLC. The results of chromatogram by spraying some reagents are investigated in Table 1.

TABLE 1. Color reactions of the lipids separated by TLC.

reagents	spot No.*				
	1	2	3	4	5
phosphomolybdic acid	+	+	+	+	+
50% H_2SO_4	+	+	+	+	+
Iodine	+	+	+	+	+
$K_2Cr_2O_7$-H_2SO_4	+	+	+	+	+
Dragendorff	−	−	−	+	−
ninhydrin	−	−	+	−	−

* corresponds to those in Fig. 2

From the investigated results above, the major peaks will be discussed in order of aspearance.

Fraction a (Eluted with chloroform-methanol mixture (4 : 1)); the effluent

was identified as phosphatidylethanolamine. This spot on TLC showed the following results; phosphomolybdic acid was positive, Rhodamine 6G was blue, Dragendroff reagent was negative and Shiff reagent was positive.

Fraction b (Eluted with chloroform-methanol (3 : 2)); the major constituent in this fraction was identifies as Rhodamine 6G was yellow. This fraction was indicated to be phosphotidylchorine by these reagents.

Fraction c (Eluted with chloroform-methanol (1 : 1)); this fraction was presumed to be sphymgomyeline.

The yield of other several fraction of minor peaks were nonphospholipids components. For example, the first peak was eluted with chloroform alone from the silicic acid column, as shown high spots (Rf) in Fig. 2-b, sterol esters and large amounts of triglycerides were present, and free fatty acid were detected. Compounds of these fractions could be easily identified on TLC becaused of their faint reddish color by spraying many reagents.
The estimation of each of the separated phospholipid was culculated on the presented standard curve after harvesting from each spot of the chromatogram.

From tne investigated results above, the peritrophic membrane of the silkworm is found to have phospholipids in addition to protein, chitin and polysacchride. So far as authors aware, no detailed study of lipids from silkworm tissue has been published.

YAMAZAKI (1955) suggested the function of this membrane. But it is possible for bacteria to multiply in this membrane, as *Bacillus thuringiensis* produced the enzyme, which degrades the phospholipids.

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SUMMARY

The phospholipids were isolated from peritrophic membrane of the silkworm, *Bomcyx mori* L., in the following investigation with combination of successive chloroform-methanol mixture from silicic acid chromatographic separation and thin layer chromatographic analysis. Total lipids were extracted from the membrane with the solvents mixture of chloroform-methanol (2 : 1) at low temperature. The yield of the conjugated lipids were 1.42% of the peritrophic membrane.

By column chromatography and thin layer chromatography, 6 spots were

detected from the conjugated lipids and 3 of them were phospholipids. They were phosphatidylethanolamine, phosphatidylchorine, sphingomyeline, phosphatidic acids and free fatty acids.

Total phospholipids were approximately 64.24 g/ml. (0.12% of the conjugated lipid a larva) of the peritrophic membrane.

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