Chem. Pharm. Bull. 26(2) 343—349 (1978)

UDC 547.458.02.05 : 581.192

Lipopolysaccharide-like Substance isolated from Mycelia of *Cochliobolus miyabeanus*

Miyoko Kiuchi and Hisatora Kuroda

Kobe Women's College of Pharmacy[1]

(Received April 2, 1977)

The mycelia of *Cochliobolus miyabeanus* were treated with hot aqueous phenol and the lipopolysaccharide like substance (LPS-LS) was isolated from aqueous layer. By gel filtration, the LPS-LS was separated to 2 fractions (peak I, peak II) and further investigation was carried out about the peak II fraction and following results were obtained. 1) The molecular weight was about 42000. 2) It consisted of protein (2.0—2.2%), carbohydrate (83—88%), phosphorus (4.5—4.7%), and lipid (6.0—7.5%). 3) Arabinose, mannose, glucose, galactose glucosamine, fatty acids with $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ were detected in the carbohydrate and lipid moieties, respectively. 4) It indicated low degree of pyrogenicity and antigenicity to rabbits.

Keywords——fungi; mycelia; lipopolysaccharide; pyrogenicity; antigenicity

Introduction

Although many reports have been presented on the lipopolysaccharides in Gram negative bacterial cell walls,[2] little is known on the lipopolysaccharides in fungal mycelia. Iwata reported on the presence of toxic substances which have polysaccharide moieties in their molecules and indicated biological activities like as bacterial endotoxin in some pathogenic fungi[3] (*Aspergillus fumigatus*, *Candida albicans*, *Histoplasma capsulatum*). However chemical characters of these toxic substances remain to be established.

The present paper deals with the isolation of a lipopolysaccharide-like substance (LPS–LS) from mycelia of a plant pathogenic fungus *Cochliobolus miyabeanus* and the characterization of its chemical structure and biological functions.

Materials and Methods

Organisms——The strain of fungus used in this experiment was *Cochliobolus miyabeanus* as described in previous paper.[4]

Extraction of LPS-LS——Mycelial LPS-LS was extracted with hot aqueous phenol according to the method of Westphal and Jann.[5] Mycelia grown in potato-sucrose media at 27° for 2—3 days with shaking were harvested, washed with tap water, distilled water successively and lyophilized. Hot 90% (w/v) phenol (350 ml) was added to a suspension of 20 g of the lyophilized mycelia in 350 ml of distilled water at 65° and the mixture was stirred for 15 min at 65°. After cooling with ice bath, the mixture was separated to three layers (aqueous layer, phenol layer, and insoluble matter floating at boundary) by centrifugation. The phenol layer and the insoluble matter were reextracted with an additional 350 ml of H_2O under the same conditions. The combined aqueous layer was dialyzed against tap water, distilled water and centrifuged for removal of insoluble materials. The supernatant was lyophilized and the resultant white fluffy powder was termed the crude LPS-LS (Fig. 1).

1) Location: *Motoyama-kita-machi, Higashinada-ku, Kobe.*
2) G.A. Adams, C. Quadling, and M.B. Perry, *Can. J. Microbiol.*, **13**, 1605 (1967): P. Mühlradt, *Eur. J. Biochem.*, **11**, 241 (1969): G. Schmidt, I. Fromme, and H. Mayer, *Eur. J. Biochem.*, **14**, 357 (1970).
3) K. Iwata, *Tokyo Igaku Zasshi*, **72**, 85 (1964).
4) A. Kumano, S. Okamoto, and H. Kuroda, *Eisei Kagaku*, **10**, 165 (1964).
5) O. Westphal and K. Jann, "Methods in Carbohydrate Chemistry," Vol. 5, ed. by R.L. Whistler, Academic Press, New York, 1965, pp. 83—91.

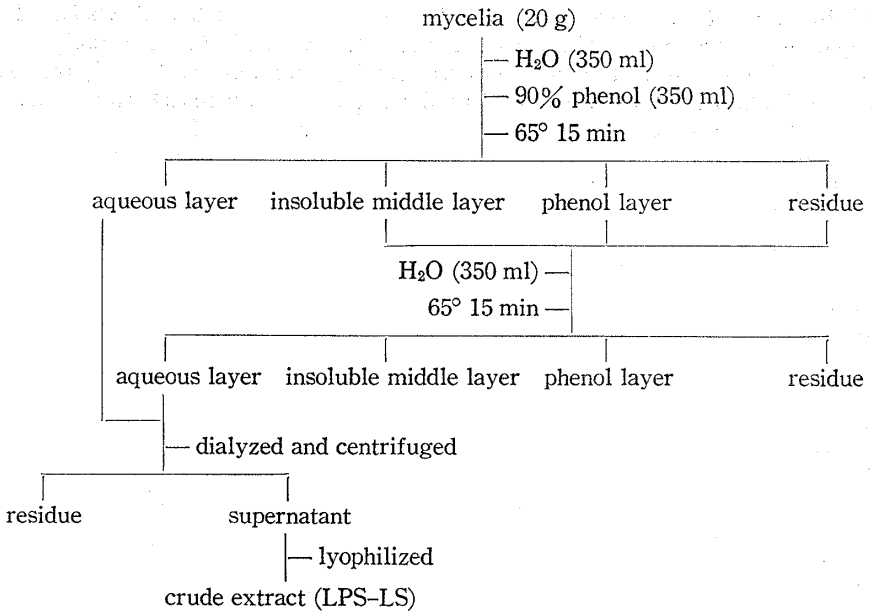

Fig. 1. Extraction of Lipopolysaccharide-like Substance (LPS-LS)

Removal of Nucleic Acid from Crude LPS-LS——Since crude LPS-LS seems to be contaminated with a lot of nucleic acid, the crude LPS-LS was treated with cationic detergent "cetavlon" to remove the impurities (Fig. 2). A 2% cetavlon solution in 0.5 M NaCl was added to the crude LPS-LS solution (0.5—1.0%) in 0.5 M NaCl so that the ratio of crude LPS-LS to cetavlon in the mixture was 1: 1.5. When the mixture was gradually diluted with H_2O, almost all of RNA-cetavlon salt precipitated at a NaCl concentration of about 0.3 M. After removal of the precipitates by centrifugation, the supernatant was lyophilized, dissolved in 0.5 M NaCl solution, and poured into a ten-fold volume of EtOH. After centrifugation, the precipitates were dissolved in H_2O, dialyzed, and lyophilized. Resultant white powder was termed the LPS-LS.

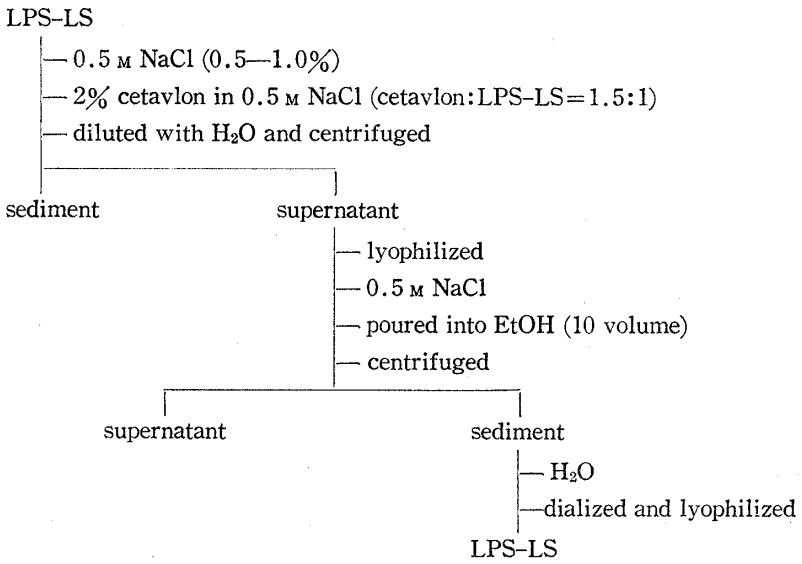

Fig. 2. Removal of Nucleic Acid from Crude LPS-LS

Identification of Fatty Acids and Sugars——Fatty acids and sugars were identified by gas–liquid chromatography (GLC). The fatty acid moieties in lipid fraction were methylated prior to GLC analyses. The lipid fraction was allowed to react with 1 ml of 5% HCl–MeOH in sealed tube at 100° for 4 hr, and resulting methylated fatty acids were extracted 3 times with 6 ml each of hexane and 3 times with 3 ml each of petroleum ether in succession. The combined extract was evaporated *in vacuo* and the concentrated residue was injected into a gas liquid chromatograph (Shimadzu Model GC-1C) under the condition described below. To probe the presence of fatty acids with –OH group a part of the concentrated residue was treated with the trimethylsilyl agent (TMS-HT, Tokyo Kasei Co., Ltd. Tokyo) prior to be submitted to GLC analysis.

After leading to alditol-trifluoroacetyl (TFA) derivatives or sugar-trimethylsilyl (TMS) derivatives, neutral or basic sugars were analyzed by GLC (Shimadzu Model GC-4BPTF). For analysis of neutral sugars, the sample was dissolved in 0.5 ml of H_2O and was reduced by 0.5 ml of 1% $NaBH_4$ solution at room temperature for 30 min. Amberlite 120A was added to the reaction mixture and the filtrate was evaporated to dryness *in vacuo*. Boric acid was removed as methyl borate by several distillations with methanol under reduced pressure. After drying over P_2O_5, the final residue was dissolved in 0.1 ml of AcOEt, followed by reaction with 0.1 ml of anhydrous trifluoroacetic acid for 10 min, and the reaction mixture was submitted to GLC analysis under the condition described below. For analysis of basic sugars, the sample was hydrolyzed with 6 N HCl at 100° for 5 hr. After removal of HCl by repeating of evaporation with H_2O, the residue was dried over P_2O_5, and treated with the trimethylsilyl agent (TMS-HT, Tokyo Kasei Co., Ltd. Tokyo). The reaction mixture was submitted to GLC analysis under the condition described below.

GLC Analyses——GLC analyses were carried out under the following conditions. a) Analysis of fatty acid methyl ester: column, stainless steel (3 mmϕ × 1.8 m); packing, 15% diethylene glycol succinate on 60—80 mesh Celite; injector and detector temperature, 250°; column temperature, 180°; nitrogen flow 40 ml/min; detector, FID. b) Analysis of TMS derivatives of sugars: column, glass (4 mmϕ × 1.5 m); packing, 5% SE-30 on 60—80 mesh Shimalite; injector and detector temperature, 250°; column temperature, 160°; nitrogen flow, 40 ml/min; detector, FID. c) Analysis of TFA derivatives of alditols: column, glass (4 mmϕ × 1.5 m); packing, 1.5% QF-1 on 60—80 mesh Chromosorb; injector and detector temperature, 200°; column temperature, 140°; nitrogen flow 40 ml/min; detector, FID.

Gel Column Chromatography——The LPS-LS (100 mg) was dissolved in a small amount of H_2O, and applied to a Sephadex G-75 column. Elution was done with H_2O. The fractions (6.5 ml) were collected and each fraction was assayed by Anthrone method.[6] A molecular weight of the fractionated material was determined by comparison with standard proteins (chymotripsinogen A, egg albumin).

Infrared Spectrophotometric Analysis——The infrared spectrum of the LPS-LS was analyzed by a Hitachi EPIG II Spectrophotometer using KBr pellets.

Other Analytical Methods——Total potein was determined by Lowry's method,[7] total carbohydrate by Anthrone's method,[6] total phosphorus by Bartlett's method,[8] heptose by the cystein-H_2SO_4 method,[9] and 2-keto-3-deoxyoctonate by the thiobarbituric acid method.[10]

Antigenicity of Mycelia to LPS-LS——To prepare antiserum to whole mycelial cells, white rabbits (4—5 kg) were given intravenous injections 3 doses in a week with 30 mg each of washed cells which were suspended in 0.9% NaCl solution. Animals were bled 7 days after the last injection. The effectiveness of the antisera was tested by agglutination of a suspension of LPS-LS. To the LPS-LS suspension (1 mg of the LPS-LS in 1.5 ml of 0.9% NaCl solution), 0.5 ml of antisera were added, and after standing still for 12 hr at 37°, the agglutination was tested.

Pyrogenicity Test——Three of rabbits were used. The body weight of the animals is shown in Table II. They had been previously tested in a trial with pyrogen-free saline. Each rabbit was injected intravenously with the suspension containing 260 μg of the LPS-LS. The body temperature was recorded for the next 3 hr at intervals of 60 min.

Results

The yield rate of the crude LPS–LS from mycelia of *Cochliobolus miyabeanus* by the method of Westphal and Jann with hot aqueous phenol was 4.5—7.0%, and the treatment with cetavlon to remove the contaminated nucleic acids yielded 1—2% LPS–LS of dried mycelia. Although the strong ultraviolet (UV) absorption in 260 nm was shown in the crude LPS–LS, this UV absorption was disappeared by treatment of the crude LPS–LS with cetavlon. The LPS–LS which was given by treatment with the detergent was fractionated by Sephadex G-75 column chromatography. As shown in Fig. 3, the LPS–LS was separated to 2 fractions (peak I and peak II in Fig. 3). Since almost all of the LPS–LS consisted of peak II, thereafter the investigation about the fraction of peak II was carried out. A molecular weight of this fraction was determined as about 42000 by comparison with standard proteins (chymotripsinogen A, 25000; egg albumin, 45000).

6) L.H. Koehler, *Anal. Chem.*, **24**, 1576 (1952), **26**, 1914 (1954).
7) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) G.R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).
9) B.G. Wright and P.A. Rebers, *Analytical Biochemical*, **49**, 307 (1972).
10) V.S. Waravdekar, L.D. Saslaw, *J. Biol. Chem.*, **234**, 1945 (1959).

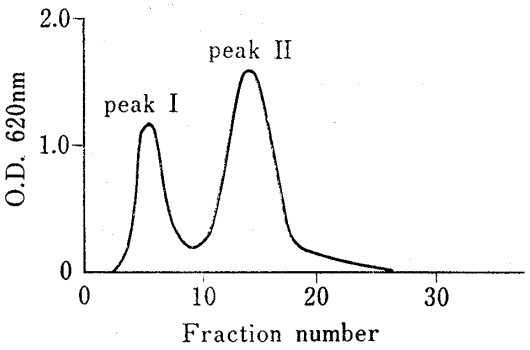

Fig. 3. Fractionation of LPS-LS by Sephadex G-75 Column Chromatography

The column (15×700 mm) was eluted with H_2O. Effluent was collected in 6.5 ml fraction and monitered for carbohydrate by O.D. at 620 nm after the anthrone reaction.

Fig. 4 is the infrared spectrum of the peak II. The absorption bands occurred at 3300—3400 cm^{-1}, 1630 cm^{-1} indicate the presence of –OH group and –CONH– group. The presence of –CO–, –COC– of glycosidic linkage is suggested by the broad absorption at 1000—1150 cm^{-1}. The chemical constitution of the peak II is given in Table I. As was expected, almost all of the peak II (83—88%) consisted of carbohydrates. As like the bacterial LPS which has been known to have some heptose molecules in its carbohydrate moiety, the presence of heptose in the peak II fraction was suggested by the cystein-H_2SO_4 method (Fig. 5). To identify the constituents of carbohydrate moiety in the peak II, the fraction of peak II was hydrolyzed with 1N H_2SO_4 at 100° for 60 min. After the removal of lipid moiety with $CHCl_3$, the residue was hydrolyzed for additional 120 min and neutralized with $Ba(OH)_2$ solution. The supernatant was passed through a 15×200 mm column of Amberlite 120A. The combined effluent was reduced with $NaBH_4$ followed by conversion to TFA-derivatives and it was submitted to GLC analysis. The results of GLC analyses clarified the presence of arabinose,

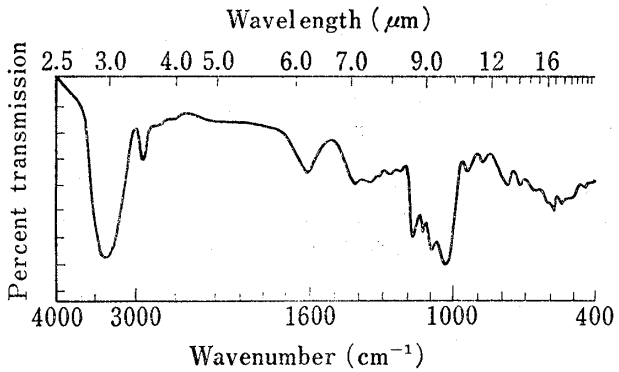

Fig. 4. Infrared Spectrum of Peak II Compound (KBr)

Table I. Chemical Composition of peak II Fraction

Composition[a)]	Percentage
Phosphorus	4.5— 4.7
Protein	2.0— 2.2
Sugar	83 —88
Lipid	6.0— 7.5

a) Methods of analyses were described in Materials and Methods in the text.

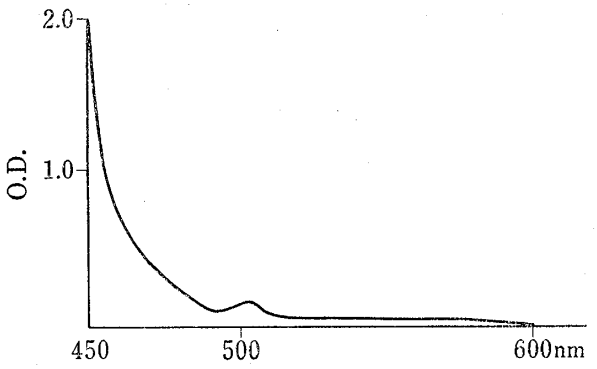

Fig. 5. Absorption Profile of Peak II Fraction by Method of Cystein-H_2SO_4

Sample 99 mg/ml.

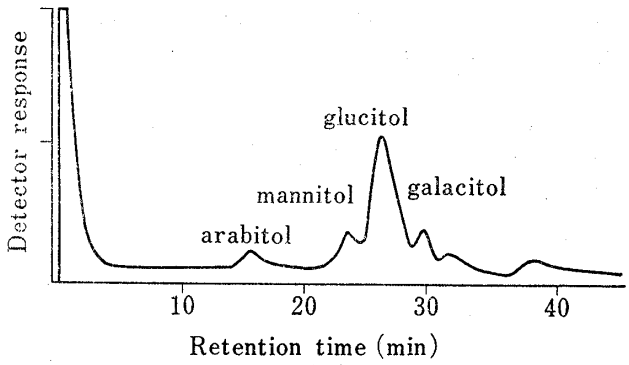

Fig. 6 Gas Liquid Chromatogram[a)] of TFA Derivatives of Alditols given from Hydrolysate of Peak II Fraction

a) Analytical condition was shown in the text.

mannose, glucose, galactose in addition to an unidentified peak which has long retention time (t_R=37 min) in the hydrolysate of peak II fraction (Fig. 6). Although, judging from the presence of absorption peak at 500 nm by cystein-H_2SO_4 method on peak II fraction, it is considered that this peak showing long retention time corresponds with heptose, the identification is not carried out. Fig. 7 indicates the results of GLC analyses on the TMS-derivatives given from the neutralized hydrolysate of peak II fraction without passing through the column of Amberlite 120A. As shown in Fig. 7, a peak corresponding to authentic glucosamine was detected in addition to the peaks corresponding to glucose, galactose, and mannose. The presence of 2-keto-3-deoxyoctonate is usually confirmed in the bacterial LPS. Although the

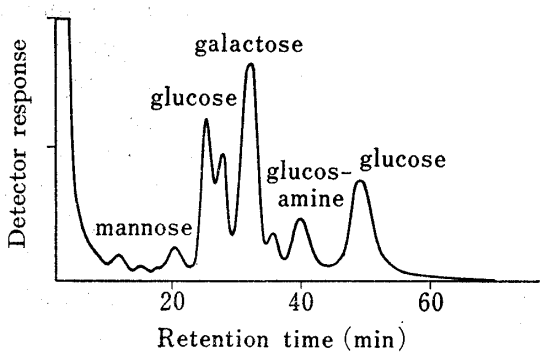

Fig. 7. Gas Liquid Chromatogram[a] of TMS Derivatives of Sugars given from Hydrolysate of Peak II Fraction

a) Analytical condition was shown in the text.

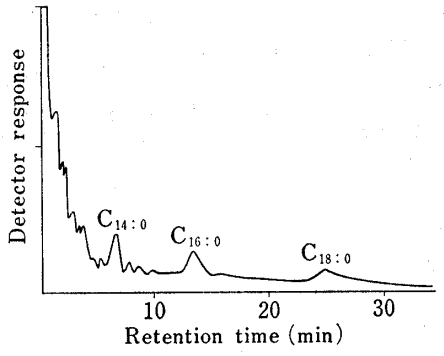

Fig. 8. Gas-Liquid Chromatogram[a] of Fatty Acid Methyl Esters in $CHCl_3$ Soluble Fraction from Hydrolysate of Peak II Fraction

a) Analytical condition was shown in the text.

TABLE II. Biological Activities of Peak II Fraction

a) Pyrogenicity Test

Time		Rabbit 1	Rabbit 2	Rabbit 3	Temperature	Humidity
	Weight	4.9 kg	5.3 kg	4.0 kg		
Before	3.5 hr	39.0°	39.5°	39.5°	22°	37%
	2.0	39.3°	39.4°	39.5°	22°	37%
	1.0	39.2°	39.4°	39.3°	22°	37%
Amount injected		260 μg	269 μg	260 μg		
After	1.0 hr	39.8°	39.9°	39.9°	22°	37%
	2.0	39.7°	39.8°	39.7°	22°	37%
	3.0	39.5°	39.7°	39.5°	22°	37%
Δ		0.6°	0.5°	0.6°		
Pyrogenicity		+	−	+		

b) Agglutination Test of Peak II Fraction by Antisera to Whole Cells

Amount injected	Agglutination titre for antisera
3×30 mg	1: 200 +
	1: 400 +
	1: 800 ±
	1:1600 −

hydrolysate of peak II fraction by 0.25 N H_2SO_4 was allowed to react with thiobarbituric acid, no spectrum of the chromophore corresponding to 2-keto-3-deoxyoctonate (549 nm) was recognized. This result indicates that any keto-saccharic acid is not contained in the LPS–LS. By hydrolysis, 6.0—7.5% of dried weight of the peak II fraction was transferred to $CHCl_3$ layer. The composition of fatty acids being in this $CHCl_3$ extractable fraction was clarified by GLC analysis (Fig. 8). The acids with $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ were detected as main components in addition to some minor unidentified acids. Even if the fatty acid methyl esters in $CHCl_3$ soluble fraction were treated with trimethylsilyl agent, the almost same gas liquid chromatogram as to Fig. 8 was given GLC analysis and no new peaks were detected, while in the case of bacterial LPS, the presence of β-hydroxy myristic acid was confirmed by GLC analysis of fatty acid methyl esters treated with the trimethylsilyl agent.[11] These results suggest that the acids with $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ are main components of this LPS–LS and acid with -OH group as β-hydroxy myristic acid is not contained. Table II indicates the results of biological tests. The peak II fraction was barely pyrogenic, a dose of 50—65 μg/kg producing temperature rises of 0.5°, 0.6°, and 0.6° in each of 3 rabbits tested. As shown in Table II, repeated injections of 30 mg each of whole mycelial cells to rabbits gave the antisera with low antibody titre against the peak II fraction. The agglutination titre was 1 : 400.

Discussion

The LPS–LS was isolated from mycelia of *Cochliobolus miyabeanus* which is a typical plant pathogenic fungus in Japan. Although this LPS–LS can be isolated by the same procedure as case of the bacterial LPS from Gram negative bacterial cell walls, some different characters are recognized between the fungus LPS–LS and the bacterial LPS. For instance, no 2-keto-3-deoxyoctonate and β-hydroxymyristic acid are detected in the fungus LPS–LS, despite of, these compounds are usual components of the bacterial LPS. Moreover, the fungus LPS–LS has very lower biological activities with regard to the pyrogenicity and antigenicity than those of the bacterial LPS as shown in Table II. From the facts described above, the lipopolysaccharide obtained from fungus mycelia was termed the lipopolysaccharide like substance (LPS–LS). It is suggested that no molecules of the fungus LPS–LS usually aggregate in aqueous solution, while molecules of the bacterial LPS aggregate with each other by 10^6—10^7 molecular size,[11] because the molecular size of this fungus LPS–LS indicated no change with the treatment of the detergents (Data are not shown). Since the formation of hydrophobic bonds between some fatty acid molecules in lipid moieties participates in the LPS aggregation, as the reason why the aggregation of the fungus LPS–LS is not taken place, it is thinkable that this LPS–LS consists of very smaller lipid moiety (6.0—7.5%) than those of the bacterial LPS (*Salmonella abortus* equi 26%,[12] *Escherichia coli* 28%,[13] *Vibrio marinus* PS-207 61%,[14] *Salmonella minnesota* R-595 70%[15]). It was confirmed by GLC analysis that the carbohydrate moiety of the LPS–LS consisted of glucose as a main component and arabinose, mannose, galactose glucosamine as minor components. Although, the presence of a small amount of heptose was suggested with color reaction by cystein-H_2SO_4 method, the identification is not carried out yet.

As to fatty acid composition of lipid moiety, it is characteristic of the fungus LPS–LS thet it has no β-hydroxymyristic acid, which is a usual component of the bacterial LPS. The fatty acids of the LPS–LS consists of only saturated acids with $C_{14:0}$, $C_{16:0}$, $C_{18:0}$.

11) N. Kasai and A. Nowotny, *J. Bacteriol.*, **94**, 1824 (1967).
12) O. Westphal and O. Lüderitz, *Angew. Chem.*, **66**, 407 (1954).
13) A. Taylor, K. Knox, and E. Work, *Biochem. J.*, **99**, 53 (1966).
14) C.F. Deneke and R.R. Colwell, *Can. J. Microbiol.*, **19**, 1211 (1973).
15) N. Kasai and A. Nowotny, *J. Bacteriol.*, **94**, 1824 (1967).

The authors are interested by that, so far as quality, the fungus LPS–LS and the bacterial LPS indicate some similar biological activities to rabbits in spite of the presence of the differences between their chemical constituents. Iwata[3] reported that by injection of whole cells of *Candida albicans* to rabbit, the neck of the animal began to bent either to the right or the left, and the same phenomenon occurred by injection of this fungus LPS–LS. Further studies are proceeding to clarify the mechanism of those biological phenomena caused by the fungus LPS–LS.