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A Study on the Lipid Components in Sporangiophores of *Phycomyces* sp.

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Phycomyces sp. 포자낭병의 脂質組成에 關한 硏究

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Abstract

Phycomyces sp. has been isolated from Meju and its lipid composition was investigated. The lipid content was 18.2 % of dry weight and composed of 69.2 % neutral lipids, 24.3 % glycolipids and 6.5 % phospholipids. It was possible to identify the presence of carotene, hydrocarbons, esterified sterols, fatty acid esters, triglycerides, free fatty acids and free sterols in the neutral lipid fraction. Major components in the neutral lipid fraction were free fatty acids and triglycerides. Twelve molecular species of triglycerides and 8 molecular species of diglycerides were fractionated by argentation TLC. The major fatty acids in total lipids were stearic, linoleic, arachidic and linolenic acids, and those in the free fatty acid fraction were stearic, linoleic and arachidic acids.

Introduction

Phycomyces has been found growing on the walls and timbers of an oil mill, on litter under beech trees, and on peeches in a refrigerator [1]. In Korea, it has been isolated from Meju [2]. It's sporangiophores are gigantic, elect, cylinderical and grow upward from mycelium, reaching 15~18 cm in height.

Phycomyces is capable of accumulating large amount of lipid. Lipids amount to 20~28% of the dry weight of mycelium, and glycerides, phospholipids and ergosterol are the principal constituents [3,4]

Biosynthesis of lipids, specifically of unsaturated fatty acids and carotenes, has received considerable attention from numerous investigators [5-10], but little work has been done on their metabolism and function. The main purpose of this study is to extend existing lipid data on the *Phycomyces* sporangiophores since almost all of the studies were done with mycelium, and hardly any with sporangiophores.

Materials and Methods

Microorganism and cultural condition

Phycomyces sp. used in this study was isolated from Meju (2), and was grown in a medium containing 10 g Bacto-soytone (Difco); 40 g glucose; 20 g agar per liter distilled water. Culturing was carried out in 500 ml Erlenmeyer flasks containing 100 ml of the inoculated culture medium and the cultures were incubated at 20 ± 1°.

Lipid extraction

Sporangiophores of *Phycomyces* were harvested 7 days after inoculation. The total lipids were extracted

from the sporangiophores according to the method Folch et al. [11] by stirring with chloroform-methanol (2 : 1, v/v) for 30~40 min. at room temperature. The supernatant was decanted and filtered through Whatman No. 42 filter paper. The residue was reextracted twice with chloroform-methanol(2 : 1, v/v); the extracts were combined and washed with 0.2 volumes Folch's salt solution [11]. The mixture was allowed to settle and the upper phase was discarded; the lower phase containing the lipid was washed twice more with Folch's mixture (chloroform-methanol-water, 3 : 48 : 47, v/v/v) containing 0.017 % $MgCl_2$, 0.02 % $CaCl_2$ and 0.29 % NaCl. After second washing, the lower chloroform phase was evaporated under nitrogen gas. The lipid was redissolved in chloroform for analysis.

Separation of lipid classes

Heat activated silicic acid (100 mesh, Mallinckrodt) was transferred in chloroform to a chromatography tube (2×10cm) [12] epuipped with Teflon stopcock, solvent reservoir and glass filter for retentation of adsorbent. Samples were applied in chloroform. The solvent systems described by Rouser et al. [13] were used, and 5 ml of fractions were collected. Solvent was removed by evaporation at low temperature and weighed for column chromatography. Neutral lipid fractions were dissolved in 2 ml of diethyl ether and examined by thin layer chromatography for identification of components.

Thin layer chromatography (TLC)

Thin layer chromatography was performed on standard 20×20 cm chromatoplates, coated with a 0.25 mm layer of silica gel G (E. Merck). The solvent system petroleum ether (30~70° fraction) —diethyl ether—acetic acid, 90 : 10 : 1 (v/v/v) was found to be the most suitable for the separation of neutral lipids. The lipids separated by TLC were identified by comparing Rf values with those reported previously [14] and with those pure compounds.

Lipid molecular species analysis was performed by argentation TLC. Three plates were coated at a time, 0.25 mm thick, with a slurry of 12 g of silica gel G and 2.9 g of silver nitrate dissolved in 24 ml of distilled water [15]. Freshly prepared plates were activated at 105° for 1.5 hour, and then stored before use in a desiccator in the dark. The $AgNO_3$-TLC solvent system used to separate glyceride molecular species was chloroform-acetic acid, 99.5 : 0.5 (v/v) [16].

Fatty acid analysis

A known amount of lipid classes were esterified according to the method of Metcalfe et al. [17]. The fatty acid methyl esters were analyzed using a Perkin-Elmer 900 Gas Chromatograph equipped with flame ionization detector. A stainless steel column (6″×1/8″) packed with 5 % DEGS and 1 % H_3PO_4 on Chromosorb G (80~100 mesh) was used at a temperature of 200°. The temperature of the injector and the detector were 250° and the flow rate of the nitrogen carrier gas was 26 ml/min.

The relative concentration of each fatty acid was calculated by triangulation of the peak areas on the chromatogram and was expressed as percentage of total peak area [18]. Each analysis was repeated 3~4 times.

Rusult and Discussion

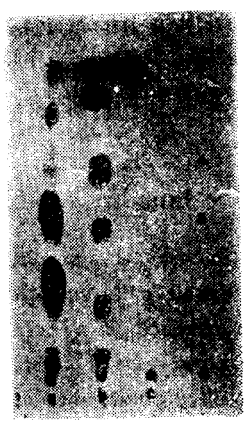


Fig. 1. **Thin layer chromatogram of total lipids of *Phycomyces* sp. sporangiophores developed in petroleum ether (bp. 30~70°) —diethyl ether—acetic acid, 90 : 10 : 1 (v/v/v) and visualized by charring with 25 % $NaHSO_4$ containing 3 % H_2SO_4**

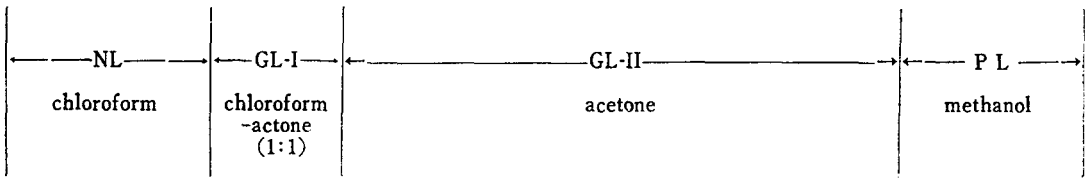
a, lipid extract of sporangiophores; b, standards; c, lipid extract of Bacto-soytone (media materials)

1, hydrocarbons; 3, esterified sterols; 4, fatty acid esters; 5, triglycerides; 6, free fatty acids; 7, free steroles; 8, polar lipids.

1. Total lipids of *Phycomyces* sp. sporangiophores

Total lipid content of *Phycomyces* was 18.2 % of dry weight of sporangiophores. Lipid content of mycelium had been reported previously [3,4] which were 20~28 %. These results indicate that *Phycomyces* synthesize a large amount of lipid which is worth studying.

Fig. 1. shows a thin layer chromatogram of total lipids developed in a solvent system of petroleum ether-diethyl ether-acetic acid, 90 : 10 : 1 (v/v/v). Nonpolar lipids were separated, and polar lipids remained at the origin. It was used that paraffin for hydrocarbons, β-carotene for carotenes, cholesteryl palmitate for esterified sterols, linoleic methyl esters for fatty acid esters, triolein for triglycerides, linoleic acid for free fatty acids, cholesterol for free

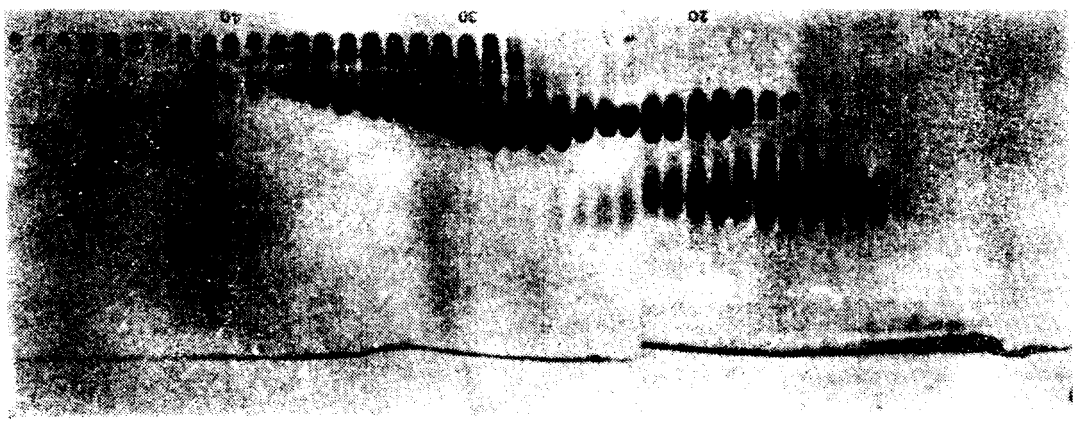


fraction number

Fig. 2. Silicic acid column chromatography of lipid of *Phycomyces* sp. sporangiophores

Column chromatography was accomplished as follows; sample, 180 mg; column size, 2×10 cm; elution with chloroform (240 ml), chloroform-acetone (1:1, v/v) (160 ml), acetone (600 ml) and methanol (200 ml) [13]; fraction volume, 5 ml.

NL, neutral lipids; GL-I, glycolipid-I; GL-II, glycolipid-II; PL, phospholipids.



fraction number

Fig. 3. Thin layer chromatographic pattern of a series obtained by silicic acid column chromatography

Each fraction was spotted on the 0.25 mm silica gel plate at 10 mm intervals. TLC conditions were the same as in Fig. 1.

sterols and phosphatidyl ethanolamine for phospholipid standard.

Comparison with standards indicates that there are esterified sterols and fatty acid esters in sporangiophore lipids which were not detected in mycelium[4].

The growth effect of lipid has been studied for mycelium grown in basal glucose-potassium acetate-ammonium sulfate medium. The addition of glycerol triolate to such a medium increases glucose utilization, growth and carotenogenesis. The added lipid seems to increase the rate of glucose uptake rather than forming a carbon and energy source [18]. Thus, Bacto-soytone which used in the medium was extracted by Folch's procedure.

Lipid content of Bacto-soytone was 0.6 % and the major lipid components did not affect lipid components in sporangiophores by incorporation.

2. Fractionation and identification of neutral lipid

Silicic acid column chromatography was used for initial separation of neutral lipids from phospholipids and glycolipids by stepwise elution.

The elution pattern is shown in Fig. 2. In glycolipids fraction, chloroform-acetone, 1:1 (v/v) and acetone effluents were named Glycolipid-I (GL-I) and Glycolipid-II (GL-II), respectively[13].

In neutral lipid fraction, each fraction was spotted on the silica sel G plate at 10 mm intervals (Fig. 3) standards (Fig. 4) and was identified by co-chromatography.

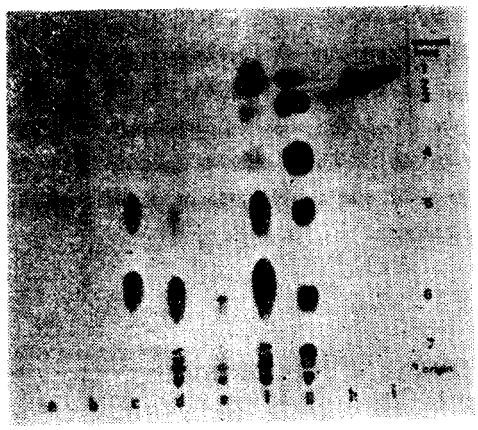


Fig. 4. Thin layer chromatogram of the fractions separated by silicic acid column chromatography

a, fraction number 6; b, fraction number 10; c, fraction number 20; d, fraction number 30; e, fraction number 40; f, spotted with a, b, c, d and e; g, standard; h, β-carotene standard; i, paraffin standard. 1, hydrocarbons; 2, carotene; 3, esterified sterols 4, fatty acid esters; 5, triglycerides; 6, free fatty acids; 7, free sterols

TLC conditions were the same as in Fig. 1.

Hydrocarbons eluted the first followed by carotenes esterified sterols, fatty acid esters, triglycerides, free fatty acids, free sterols, diglycerides and monoglycerides. Compared Fig. 4 with Fig. 1 most part of

fraction number

Fig. 5. Resolution of neutral lipid fractions by argentation TLC[17]. Developed in chloroform-acetic acid, 99.5:0.5 (v/v) [16] and visualized by charring with 25 % $NaHSO_4$ containing 3 % H_2SO_4.

Table. 1. Content of neutral lipids, glycolipids and phospholipids in *Phycomyces* sp. sporangiophores.*

	%
Neutral lipids	69.2
Glycolipids	24.3
Glycolipid-I	8.8
Glycolipid-II	15.5
Phospholipids	6.5

*Each lipid class was separated by silicic acid column chromatography and quantitated by gravimetric measurement.

neutral lipids were eluted during the fractionation procedure.

Each lipid class fraction was collected and quantitated by gravimetric measurement (Table 1). The total lipid was composed 69.2 % neutral lipids, 24.3 % glycolipids and 6.5 % phospholipids. This result is compatable with the conclusions of Watanabe et al.[20] that high lipid content microorganisms contained a high proportion of neutral lipids.

Fig. 5 shows a argentation thin layer chromatogram of neutral lipid fractions. Wilson et al.[15] fractionated 13 molecular species of triglycerides in soybean cotyledons according to degrees of unsaturation by argentation TLC and Suzuki et al.[21,22] reported 4 species of triglycerides and 3 species of phospholipids in *Lipomyces starkeyi* by gas chromatography-mass sepectrometry (GC-MS). In this study it was fractionated 12 spots in triglycerides and 8 spots in diglycerides.

Table 2. Quantity and composition of neutral lipids of *Phycomyces* sp. sporangiophores*

Lipid materials	%
Hydrocarbons	2.3
Esterified sterols	3.0
Fatty acid esters	1.4
Triglycerides	30.9
Free fatty acids	45.6
Free sterols	2.4
Diglycerides	6.1
Unknown	8.3

*Each lipid fraction was separated by TLC and quantitated by densitometric measurement.

From Fig. 5, chromatographic patterns are not varied with every lipid class.

The proportions of neutral lipid classes are shown in Table 2 and they are different from that reported for mycelium[4]. Hydrocarbons constitute 2.3 %, esterified sterols 3.0 %, fatty acid esters 1.4 %, triglycerides 30.9 %, free fatty acids 45.6 %, free sterols 2.4 % and diglycerides 6.1 % of the neutral lipid content.

3. Fatty acid composition

Free fatty acid content of *Phycomyces* sp. sporangiophores was 45.6 % of neutral lipids, and this value is strikingly larger compared with higher plants [23,24,25].

Tables 3 and 4 shows the fatty acid composition of the individual lipids. The total fatty acid compositions were quite different from that reported for mycelium[4,5]. The mycelium contains a large amount of palmitic, oleic, linoleic and linolenic acid while the sporangiophores were composed of a large amount stearic, linoleic, linolenic and arachidic acid, Fatty acid compositions of the mycelium reported in the literature is included in Table 3 for comparison with the present results.

Table 3. Fatty acid composition of total lipid, neutral lipids, glycolipids and phospholipids (%)

F.A.	Mycelium*	Total	NL	GL-I	GL-II	PL
14:0	1.49			tr.	0.83	
14:1	1.10			0.37	1.93	0.66
16:0	19.35	0.63	0.58	1.95	2.48	0.81
16:1	2.78			2.99		
18:0	7.68	36.53	43.12	5.38	31.50	37.50
18:1	27.91		0.33	1.74	4.84	0.45
u_1**		1.79	0.89	2.29	8.18	2.67
18:2	17.65	34.54	38.80	45.50	36.90	29.73
20:0		13.01	8.48	24.08	11.44	11.89
18:3	16.26	10.31	4.76	10.71	1.37	9.23
u_2**		3.61	2.84	4.12	1.02	7.58
20:4	1.68					

*Data reported by Chenouda[4].

**Unidentified fatty acids.

Total, total lipid; NL, neutral lipids; GL-I, glycolipid-I; GL-II, glycolipid-II; PL, phospholipids.

Table 4. Fatty acid composition of individual neutral lipids

F.A.	Total	FFM	TG	DG
14:1			0.45	
16:0	0.58	1.24	0.91	0.77
18.0	43.12	34.29	38.24	8.39
u_1	0.89	1.84	2.66	6.88
18:2	38.80	39.39	44.24	55.09
20:0	8.48	12.44	3.10	4.81
18:3	4.76	3.17	4.10	5.64
u_2	2.84	6.85	3.34	19.17

Total, total neutral lipids; FFA, free fatty acids; TG, triglycerides; DG, diglycerides.

The fatty acid composition of sporangiophores differed markedly from the aquatic *Phycomyces*[26], yeast[21,22], *Euglena*[27] and[28,29].

The fatty acid composition of individual lipid classes are similar, as shown in Tables 3 and 4, with the major components being stearic, linoleic, linolenic and arachidic acid. The notable differences among them are that the proportion of stearic acid in GL-I and DG are considerably smaller compared with the proportions observed in the other fractions.

γ-Linolenic acid is ostensively one of the most significant features of the fatty acids produced by lower fungi[5] but it was not possible to determine positional isomers here.

要 約

재래식 메주로 부터 分離한 *Phycomyces* 屬의 포자낭병의 脂質組成을 分析하여 다음과 같은 結果를 얻었다.

1. 乾體中의 脂質은 18.2%로서 그 組成은 中性脂質이 69.2%, 糖脂質이 24.3% 그리고 燐脂質이 6.5%였다.

2. 中性脂質의 構成成分은 carotene, 炭化水素, esterified sterol, 脂肪酸 ester, triglyceride, 遊離脂肪酸, 遊離 sterol등이고 主成分은 遊離脂肪酸과 triglyceride로서 각각 45.6%와 30.9%를 차지하였다.

3. $AgNO_3$-TLC에 의한 方法으로 triglyceride에서 12個, diglyceride에서 8個의 lipid molecular species를 分別하였다.

4. 總 脂質의 主脂肪酸은 stearic, linoleic, arachidic, linolenic acid이고 遊離脂肪酸의 主脂肪酸은 stearic, linoleic, arachidic acid 등이었다.

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