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STUDIES ON *GANODERMA LUCIDUM*[1]

1. Liquid Culture and Chemical Composition of Mycelium

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Abstract

Ganoderma lucidum (Fr.) Karst NTU G001, the wood rotting fungus and traditional Chinese medicine, was cultured in liquid media under various growth conditions. The growth of mycelia in complete media was found much better than those in Czapek solution alone or CM-cellulose supplemented Czapek solution at pH 4.25. CM-cellulose has significant effect on the growth of *G. lucidum* when supplemented to Czapek solution. Chemical composition of mycelia was also determined. Analysis of 14-day-old dried mycelia showed that total carbohydrates: 75.0% (reducing sugars: 29.8%), lipids: 4.6%, soluble proteins: 8.3%, and total ash: 3.62%. Further determination of fatty acid distributions in crude lipids by gas chromatography reveals that $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ are the predominant ones in saponifiable fractions. Considering free forms of fatty acids, the ratios of shorter chain ($C_{12:0}$, $C_{14:0}$) fatty acid were found slightly higher than those in esterified forms. Total sterol content was estimated to be 0.3% on dry weight base. This report reveals that the fatty acid patterns in *G. lucidum* will provide useful informations for the study of possible function in lipid metabolism.

Key words: *Ganoderma lucidum*; mycelium; carbohydrates; lipids; fatty acids.

Introduction

Ganoderma lucidum (Fr.) Karst, the wood rotting fungus, is a well known Chinese medicine with numerous controversial folklore beyond proper scientific evaluation. Primitive reports of the medicinal virtues of *G. lucidum* and related species scattered in both scientific and non-scientific literatures (Hour, 1960; Chen *et al.*, 1980). As a first part of our long term project on the medical fungus, we

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like to report here the liquid culture of *G. lucidum* collected in Taiwan and its chemical composition. This preliminary investigation is essential for the subsequent biological study, because liquid culture of *G. lucidum* can constantly provide raw materials in sizeable amounts. Difficulties like seasonal variations and aging of field collected fruiting bodies of *G. lucidum* inevitably limited their use for this purpose.

Materials and Methods

Materials

An isolate of *Ganoderma lucidum* (Fr.) Karst NTU G001 was kindly obtained from Dr. D. Cheng, Department of Plant Pathology, National Taiwan University (Chang, 1983). Growth media used in the liquid culture were purchased from Difco Co. (Michigan, USA) and E. Merck Co. (Darmstadt, W. Germany), LC garde organic solvents were purchased from E. Merck Co. or Alps Co. (Taipei, ROC). Methylated fatty acid standards and fat free bovine serum albumin (BSA) were obtained from Sigma Co. (Saint Louis, MO, USA). Diethyl ether used for continuous extraction of mycelial crude lipids was first peroxide freed and distilled before use. Fourteen-day-old mycelia of *G. lucidum* stationarily grown in potato-dextrose (PD) broth were dried at 50°C to constant weight and saved at 4°C for determination of chemical composition.

General Methods

Gas chromatography (GC) was carried out by a Hitachi model 163 instrument equipped with temperature programmer and FID detector. Identification and quantitation of fatty acid methyl esters were made in comparison with standards. Conditions of separation and results were reported in the text.

Liquid Culture of ***Ganoderma lucidum***

Ganoderma lucidum was maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at three-week intervals. For every pasteurized petri dish, 25 ml of MDA medium (malt extract: 3%, dextrose: 2%) was used. *G. lucidum* was inoculated at the center of petri dishes which were then incubated at 28°C±2°C for 10 days. The fine mycelia of *G. lucidum* on the surface of media were cut into pieces (approximately 0.7×0.7 cm) before transferring to 500 ml of potato dextrose broth in 1 l flask (Table 1). Flasks were maintained stationarily at 28°C ± 2°C for 14 days in a 24 hour-light incubation room. At the end of this growth period, yellowish brown mycelia were collected and stored at 4°C in a cold room.

Table 1a. *Compositions of synthetic media for the liquid culture of* ***Ganoderma lucidum***

	Media	Unit: gram per liter of broth
A	Czapek solution (pH 6.5)	$NaNO_3$: 3.0; $MgSO_4 \cdot 7H_2O$: 0.5; KCl: 0.5 K_2HPO_4: 1.0; $FeSO_4 \cdot 7H_2O$: 0.01; Sucrose: 30
B[1]	Czapek solution with pH adjusted to 4.25	$NaNO_3$: 3.0; $MgSO_4 \cdot 7H_2O$: 0.5; KCl: 0.5 K_2HPO_4: 1.0; $FeSO_4 \cdot 7H_2O$: 0.01; Sucrose: 30
C	Czapek solution with 1% dextran substitution (pH 6.5)	$NaNO_3$: 3.0; $MgSO_4 \cdot 7H_2O$: 0.5; KCl: 0.5; Dextran: 10 K_2HPO_4: 1.0; $FeSO_4 \cdot 7H_2O$: 0.01; Sucrose: 20
D	Czapek solution with 1% α-cellulose substitution (pH 6.5)	$NaNO_3$: 3.0; $MgSO_4 \cdot 7H_2O$: 0.5; KCl: 0.5; α-cellulose: 10 K_2HPO_4: 1.0; $FeSO_4 \cdot 7H_2O$: 0.5; Sucrose: 20
E[1]	Czapek solution (pH 4.25) with 0.5% α-cellulose substitution	$NaNO_3$: 3.0; $MgSO_4 \cdot 7H_2O$: 0.5; KCl: 0.5; α-cellulose: 5.0 K_2HPO_4: 1.0; $FeSO_4 \cdot 7H_2O$: 0.01; Sucrose: 25
F[1]	Czapek solution (pH 4.25) with 0.5% CM-cellulose substitution	$NaNO_3$: 3.0; $MgSO_4 \cdot 7H_2O$: 0.5; KCl: 0.5; CM-cellulose: 5.0 K_2HPO_4: 1.0; $FeSO_4 \cdot 7H_2O$: 0.01; Sucrose: 25

[1] The pH of medium was adjusted to 4.7 by 1 N HCl before sterilization, it will drop to 4.25 after autoclave.

Table 1b. *Compositions of complete media for* ***Ganoderma lucidum***

	Media	Unit: gram per liter of broth
G	10% MB	Malt extract: 100
H	5% MB	Malt extract: 50
I	3% MB	Malt extract: 30
J	3% MB & 2% D	Malt extract: 30; Dextrose: 20
K	3% MB, 2% D & 0.5% C	Malt extract: 30; Dextrose: 20; α-cellulose: 5.0
L	PDB	Potato: 250; Dextrose: 20
M	PCSB	Potato: 125; Corn: 125; Sucrose:20

Determination of Soluble Proteins

Homogenized mycelia of *G. lucidum* were extracted with sufficient distilled water at 4°C for 24 hours with gentle stirring. After filtration, the protein contents were determined by the Biuret method (Horwitz, 1980). Protein contents measured by the above procedure was found quite close to that obtained by a more careful extraction of protein with a polytron homogenized and removal of small molecules by dialysis.

Carbohydrate Contents

1. Soluble reducing sugars

Powdered dry mycelia of *G. lucidum* (0.5 g) in distilled water (25 ml) and stirred vigorously for 2 hours at room temperature. After filtration, the soluble reducing sugars was measured by the method of Nelson (Nelson, 1944).

2. Total carbohydrates

Dry mycelia (40 mg) were dissolved in sulfuric acid (0.4 ml, 72% concentration, w/w) and stood at room temperature for 24 hours. After dilution with 5 ml of distilled water, the contents were autoclaved at 121°C for 60 minutes. After neutralization with $BaCO_3$ powder and subsequent centrifugation to remove $BaSO_4$ precipitates, supernatant was collected. Following several washes of precipitates with distilled water, the total volume of the combined clear supernatant was adjusted to 250 ml with water and ready for carbohydrates determination (Yuan, 1974).

Determination of Total Ash

Mycelia of 14-day-old *G. lucidum* were dried to constant weight (2.003 g) and put into a crucible with suitable amount of 95% ethanol and ignited. After burning, the crucible was transferred to a muffle furnace and heated at 550±25°C for 2 hours. The contents were removed to desiccator containing fresh desiccant for cooling before weighing. The incineration step was repeated until the difference between two successive weighing was less than 2 mg. Totally six hours of heating was carried out at 550±25°C for complete incineration (I. S. O. Recommedation R928, 1969).

Determination of Lipids

1. Lipids

Ground powder of *G. lucidum* (2.0 g) was put into thimbles and continuously extracted with anhydrous ethyl ether (250 ml) for 12 hours. Etheral extract was carefully filtered through glass wool and evaporated to dryness by a rotary evaporated to dryness by a rotary evaporator. The residues were again put in a desiccator and further dried under high vacuum until the difference between two successive weighing was less than 2 mg.

2. Esterified fatty acid (Lowenstein, 1975)

Crude lipids were again taken up into suitable amount of ether and extracted with aqueous 1% KOH solution to remove free fatty acids. Again the etheral layer containing neutral and esterified lipids was collected and washed with water. After drying over anhydrous Na_2SO_4 powder, the organic layer was collected by evaporation of solvent. Saponification was carried out in aqueous 2 N KOH solution at

elevated temperature for 12 hours. After TLC analysis to secure complete hydrolysis with triglycerides run as control, the reaction was stopped and the mixture extracted with ethyl ether three times. Following acidification with 6 N HCl, free fatty acids were taken up into organic layer by extracting with *n*-hexane. Evaporation of solvent, methylation by excess anhydrous methanol/H_2SO_4, fatty acid methyl esters were again brought up to hexane layer by partition with aqueous alkaline solution to eliminate any possible existence of polar matters. Fatty acid methyl esters in *n*-hexane were concentrated under flux of N_2 for GC analysis.

Results and Discussion

Liquid Cultures of ***Ganoderma lucidum***

G. lucidum can be cultured in synthetic and complete media (Table 1). Great differences in mycelial biomasses showed that synthetic media tested were poor for maintaining vigorous growth (Table 2). Fourteen-day-old culture of *G. lucidum* in potato dextrose broth was chosen for the chemical analysis of compositions for

Table 2. *Dry mycelial weight of* ***Ganoderma lucidum*** *cultured at various media*

Some mycelia bound with cellulose powder and hard to be separated from broth such as media D and E. The actual weight of mycelia should be slightly higher. Mycelia in synthetic media A to F were 14-day old and in complete media G to M were grown for only 10 days under the same condition as described in text.

Media	Test A		Test B		Test C	
	weight	s. d.	weight	s. d.	weight	s. d.
	mg dry weight/100 ml broth					
A	16	2				
B	17	2				
C	11	2				
D	10	1				
E	28	2				
F	54	10				
G			735	76		
H			575	48		
I			244	26		
J			367	43		
K			359	18		
L					451	19
M					467	41

two reasons. Firstly, although it is not the best medium for the fungus growth but it is inexpensive to prepare a large scale of biomass. Secondarily, a preliminary time course study showed that the mycelial dry weight was still slightly increasing after 14 days growth (data not shown). Thus, in our search for the effective ingradients of *G. lucidum* on mammalian lipid metabolism, it is now possible for us to have constant supply of large amount of materials for biochemical studies.

Chemical Compositions of ***Ganoderma lucidum***

Contents of soluble proteins, carbohydrates, lipids and ash were all analyzed. Results presented in Table 3 were means of at least two duplicated assays. In comparison with similar analysis obtained from fruit body of *G. lucidum* (seasonal variation and growth condition were not specified), mycelia from liquid culture contained higher reducing sugars and total lipids. No further analysis of fatty acids of the cultured fruit bodies of the fungus was reported in the literatures. As specified in Table 4, crude lipids consist of 4.6% by weight of dry mycelia.

Table 3. *Chemical compositions of* ***Ganoderma lucidum***

The fungus was grown in potato-dextrose broth at 28° C ± 2°C for two weeks.

Composition	Percentage as total dry weight of mycelia				
Total carbohydrates	75.0	Reducing sugars Others	29.8 45.3		
Lipds	4.6	Saponified portion Unsaponified portion	1.4 2.9	Sterols	0.3
Soluble proteins	8.3				
Ash	3.62				

Table 4. *Fatty acid compositions of saponifiable crude lipids in* ***Ganoderma lucidum***

Determination was based on gas chromatographic method.

Fatty acid composition (% of total)											
$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	[1] $C_{18:1}$ $C_{18:2}$ $C_{18:3}$	$C_{19:0}$	$C_{20:0}$	$C_{21:0}$	$C_{22:0}$	$C_{14:0}$
trace	0.6	1.0	23.3	1.1	10.1	59.4	trace	1.5	trace	0.8	1.2

[1] Ratios of $C_{18:1}$ (12.8%), $C_{18:2}$ (38.3%) and $C_{18:3}$ (trace) determined by GC-MS.

Hexane extract of unsaponifiable portion was proved to contain many terpenoids. The major peak on the gas chromatogram (Fig. 1) has retention time similar to that of ergosterol and was later confirmed by GC/MS method. Gas chromatographic analysis of methyl esters of fatty acids obtained from saponification and methylation revealed that $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ fatty acids were the predominant species (Fig. 2). The presence of C_{15}, C_{17} fatty acids in low ratios was also confirmed. GC profiles of prepared methyl esters of free fatty acids in total lipids showed that the relative ratios of $C_{12:0}$ and $C_{14:0}$ fatty acids are higher than those in their esterified forms. Since there are few reports referring to the chemical composition of *G. lucidum*, particularly fatty acid patterns, thus it can provide useful informations for the study of possible function of the ingredients in *G. lucidum* on lipid metabolism.

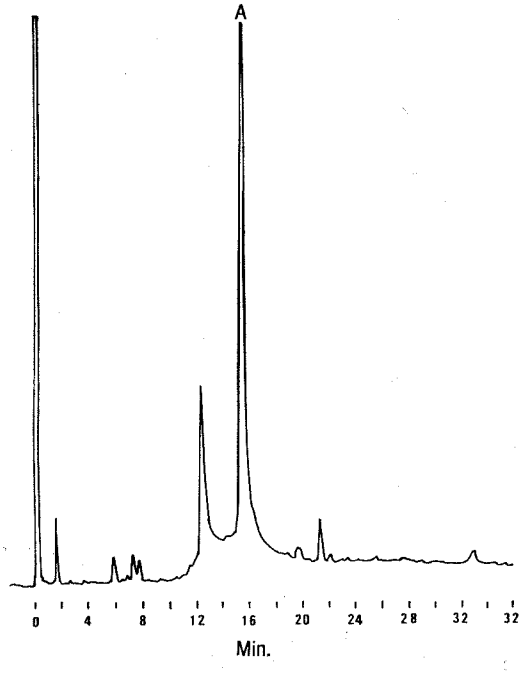

Fig. 1. Gas chromatogram of unsaponifiable non-polar matters in *Ganoderma lucidum*. A: Ergosterol (confirmed by GC/MS). Column: 10% SE-30 on 80/100 Chromosorb W, 1 meter, Temperature: 190-220°C at 5°C/min, Flow rate: 25 ml/min, N_2, Detector: FID, Sensitivity: Attenuation 64x, Sample size: 2μl.

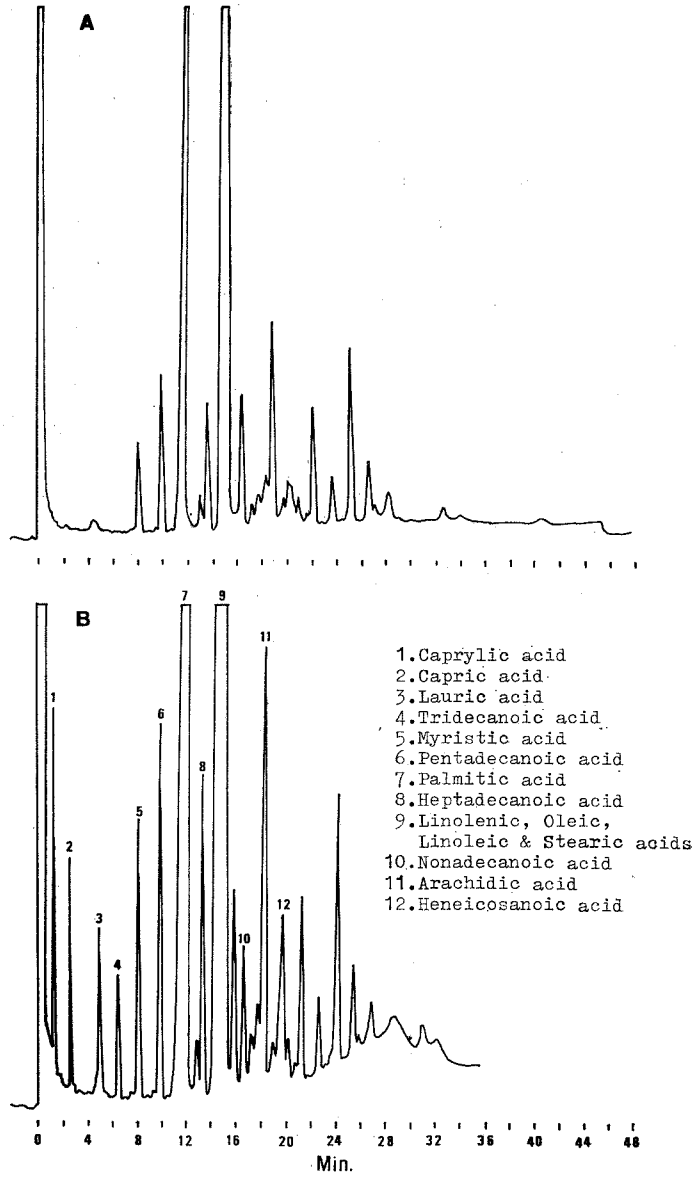

Fig. 2. Gas chromatograms of methyl esters of
(A): Saponifiable fatty acids of *Ganoderma lucidum*,
(B): (A) + fatty acid standards.
Conditions same as Fig. 1. except, Sample size:
(A) → 5 μl; (B) → 6 μl, Attenuation: (A) → 64x; (B) → 16x.

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Literature Cited

Chang, T. T. 1983. Studies on biology of several species of ***Ganoderma lucidum*** in Taiwan. MS thesis of National Taiwan University, 130 pp.

Chen, C. T., S. F. Chen, and H. C. Hsiu. 1980. A survey on a Chinese drug "Ling Chih". J. of Taiwan Sci. **34** (3-4): 145-147.

Horwitz, W. (ed.). 1980. Methods of Analysis, 13th edn. A. O. A. C., 1018 pp.

Hour, D. 1960. ***Ganoderma***, the first report. The ***Ganoderma*** research laboratory, Department of Botany, National Taiwan University, 25 pp.

I. S. O. Recommendation R928. 1969.

Lowenstein, J. W. (ed.). 1975. Methods in Enzymology, Vol. XXXV, Academic Press, New York, 654 pp.

Medico-Chemical Investigation Methods. 1970. Clinical laboratory, 11th edn. E. Merck, Darmstadt, Germany, 644 pp.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determation of glucose. J. Biol. Chem. **153**: 375-380.

Yokokawa, H. 1980. Fatty acid and sterol composition in mushroom of ten speciess of polyporaceae. Phytochemistry **19**: 2615-2618.

Yuan, H. F. 1974. A chemical study of the cell wall polysacchrides of Chinese celery. Bot. Bull. Academia Sinica **15**: 123-131.

靈 芝 之 研 究

一、液態培養及化學成份分析

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探討一種木材腐朽菌，同時也被用來當作傳統中藥之靈芝菌株，*Ganoderma lucidum* (Fr.) Karst NTU Gool，在各種液態培養基生長結果，發現該菌在完全培養基 (Complete medium) 生長，總比在 Czapek solution (pH 4.25) 或者 Czapek solution 加上 Carboxymethyl cellulose (CMC) 爲佳，CM-cellulose 顯然會促進該菌絲生長。分析培養14天之乾菌絲其化學成份：總碳水化合物佔75%，其中還原醣佔 29.8%；粗脂肪佔 4.6%；水溶性蛋白質佔8.3%；灰份佔3.62%。在粗脂肪中可被皂化的脂肪酸以 $C_{16:0}$，$C_{1:18}$ 和 $C_{18:2}$ 爲主，若觀察其自由態部份之脂肪酸則 $C_{12:0}$ 和 $C_{14:0}$ 比例略升；總固醇量爲0.3%。本研究結果顯示，就靈芝菌絲體脂肪酸之分佈模式，將可作爲未來研究靈芝脂質代謝作用之重要參考依據。