Technical

Glycolipids Isolated from Ginkgo Nuts *(Ginkgo biloba)* and Their Fatty Acid Compositions

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ABSTRACT

The total amount of glycolipids isolated from *Ginkgo biloba* nut was 3.12% of crude lipids, and the largest fraction of the glycolipids was digalactosyldiglyceride 64.1%, followed by monogalactosyldiglyceride 31.2% and cerebroside 4.7%. Chief fatty acids present in the former two were oleic and linoleic acids, while the main component amounting to 85% of total fatty acids in the last fraction was α-hydroxypalmitic acid. The sugar component in cerebroside was glucose, and its long chain base was found to be similar in structure with octadecasphingadiene that had been isolated from oyster glycolipids. The major compound in the cerebroside fraction, therefore, is considered to be N-α-hydroxypalmitoyl-glucosyloctadecasphingadiene.

INTRODUCTION

One of the authors has reported in a previous paper (1) that a small amount of glycolipids may be present in the phospholipid fraction isolated from ginkgo nuts. Detailed studies were, therefore, undertaken to elucidate the nature of sugar components and fatty acid compositions of glycoglycerolipids and cerebroside (Cer). The nature of a long chain base and a hydroxy fatty acid in Cer was also examined.

MATERIAL AND METHODS

Standard samples of fatty acids were purchased from Applied Science Laboratories, Inc. (State College, PA); 10% diethylene glycol succinate (DEGS on Shimalite W (60-80 mesh) and Wakogel B-5 for preparative thin layer chromatography (TLC) from Wako Pure Chemicals (Osaka, Japan); 1% silicone OV-1 on Chromosorb WAW HMDS (60-80 mesh), hexamethyldisilazane, trimethylchlorosilane, and TMS-PS (a special trimethyl silyl reagent for sugars) from Gaskuro Kogyo (Osaka, Japan); Sephadex G-25 from Pharmacia Fine Chemicals (Piscataway, NJ); 100 mesh silicic acid of the analytical grade from Mallinckrodt (St. Louis, MO); precoated silica gel TLC plates (0.25 mm in thickness) from E. Merck (Darmstadt, Germany); Amberlite IR-120 and IR-4B from Rohm and Haas Co. (Philadelphia, PA);Toyo No. 51 A for paper chromatography (PC) from Toyo Roshi Co., Ltd. (Tokyo, Japan); and analytical grades of reagents and solvents from Nakarai Chemicals, Ltd. (Kyoto, Japan).

TLC plates prepared with Wakogel B-5 (0.75 mm in thickness) were activated 1 hr. at 110 C prior to use.

Extraction and Purification of Glycolipids

A crude lipid fraction prepared from 450 g of shelled ginkgo nuts according to the method of Urakami et al. (1) was dissolved in a small volume of $CHCl_3$, the solution applied on a silicic acid column (30 g silicic acid mixed with 10 g Hyflo-Super-Cel), and eluted with $CHCl_3$-acetone, followed by acetone (refer to Table I) according to the method of Ito et al. (2), monitoring each fraction by TLC and anthrone reaction. The digalactosyldiglyceride (DGDG) fraction was further purified on a silicic acid column by eluting with $CHCl_3$ methanol 9:1. The monogalactosyldiglyceride (MGDG) and Cer fractions were purified by preparative TLC by developing with $CHCl_3$/methanol/acetic acid/water 170:30:20:7 and extracting each fraction from silica gel with $CHCl_3$/methanol 1:1. Each fraction thus obtained was purified on a small column of Sephadex G-25 to remove nonlipid components (3).

Hydrolysis of Lipids and Preparation of Fatty Acid Methyl Esters

Twenty mg each of DGDG and MGDG were treated with 15 ml of methanolic NaOH (0.3 N) and refluxed 1 hr. on a steam bath. After acidifying the reaction mixture with methanolic HCl, fatty acids (FA) were extracted with petroleum ether, the extract was washed with water, dried over anhydrous Na_2SO_4, and concentrated under vacuo and a nitrogen atmosphere. The methyl esters of FA were prepared as described previously (1).

A mixture of 11 mg of purified Cer in 5 ml of methanolic HCl (1N HCl containing 8.7 M water) (4) was refluxed 18 hr., the reaction mixture extracted with hexane, and the extract treated in the same manner as described above for the preparation of FA methyl esters.

Hydrolysis of Water-Soluble Fraction and Preparation of Derivatives

The methanolic phase obtained after hydrolysis of DGDG and MGDG was concentrated to a small volume, and the concentrate in 2N HCl was refluxed for 3 hr. The reaction mixture was diluted with water, and a portion of this was used for quantitative analysis of sugars and glycerol. The remainder was passed through an ion exchange column (Amberlite IR-120 H^+ and IR-4B OH^-). The concentrate of the effluent was used for PC and gas liquid chromatography (GLC).

The aqueous phase obtained by hydrolysis of Cer was cooled in an ice bath and treated, after diluting with distilled water, with 7 N NaOH solution to adjust its pH to 10, and a long chain base was extracted with diethyl ether. The reaction mixture was passed through the ion exchange column described above.

Trimethylsilyl derivatives of sugars and the long

chain base were prepared according to the methods of Bhatti (5) and Hayashi (6), respectively.

Analytical Methods

The purified lipids were analyzed by TLC by developing with $CHCl_3$/methanol/water 70:22:3 and Rf values of MGDG, DGDG, Cer, and other fractions were compared with those fractions prepared from spinach according to the method of Hirayama (7). The purity of each fraction was examined by the color reactions shown in Table I and also by quantitative analysis of sugars and glycerol by the method of Dubois et al. (8) and Sastry and Kates (9), respectively. Sugars and glycerol were separated by PC by developing with n-butanol/pyridine/water 6:4:3, and the spots were visualized by spraying a solution of $AgNO_3$-NaOH and periodate-benzidine.

GLC

Shimadzu GC 4 CM provided with a flame ionization detector was used. The conditions used for FA ester analysis were as follows: a glass column (3 mm. i.d. x 1.5 m.) packed with 10% DEGS, column temperature 180 C, and nitrogen 65 ml/min. Those used for sugar analysis: a glass column (3 mm. i.d. x 2 m.) packed with 1% OV-1, column temperature 140-200 C programmed at a rate of 0.5 C/min., and nitrogen 60 ml/min

GLC-Mass Spectrometry (MS)

Shimadzu LKB-9000 was employed. The analytical conditions used for the long chain base from Cer were as follows: a glass column (3 mm. i.d. x 2 m.) packed with 3% SE-30, column temperature 220 C, electron energy 70 eV, ion source and separator temperature 270 C and 235 C, respectively. The conditions used for the hydroxy acid methyl esters were similar to those given above, except that column, ion source, and separator tempeature were 200 C, 250 C, and 250 C, respectively.

RESULTS AND DISCUSSION

Composition of Glycolipids

Table I shows the results of column fractionation and qualitative analysis of each fraction by TLC and their purity by the color reactions. The trace amounts of Fraction I (Rf 0.90) and IV (Rf 0.63) are considered to be esterified sterol glycoside and sterol glycoside, respectively, in accordance with the report of Hirayama on spinach lipids (7). A total amount of purified glycolipids isolated from the crude lipid fraction was 3.12%, and the composition of glycolipids was 64.1% of DGDG, 31.2% of MGDG, and 4.7% of Cer. The IR spectrum of Cer showed the absorption bands characteristic to the N-acyl amide structure at 1640 cm^{-1} and 1540 cm^{-1} and no band at 1730 cm^{-1} for the carbonyl group.

Roughan and Batt (10) reported the presence of MGDG and DGDG in a proportion of 1.67:1 (μmole/g fresh tissue) in the leaves of *Ginkgo biloba* but no Cer.

Water-Soluble Components

The PC analysis of water-soluble hydrolysis products from MGDG and DGDG by developing with n-butanol/pyridine/water 6:4:3 showed the presence of glycerol at Rf 0.55 and galactose at Rf 0.29, corresponding to the Rf values of the standard samples. They were further confirmed by GLC analysis of their TMS derivatives on an OV-1 column; and quantitative analysis of glycerol and galactose showed mole ratios of 1:0.97 for MGDG and 1:2.01 for DGDG.

The sugar component of Cer, on the other hand, was found to be glucose, since cochromatography of the concentrate of the aqueous phase with the standard sample by TLC with the same solvent system mentioned above showed the same Rf at 0.42.

GLC-MS Analysis of Long Chain Base in Cer

The TLC analysis of the free base by developing with $CHCl_3$/methanol/ammonia water 80:20:2 showed a purple spot with ninhydrin reagent at Rf 0.45. The mass spectrum obtained for the TMS derivative of the long chain base was found to be identical with that for octadecasphingadiene from oyster glycolipids previously taken by Hayashi (6) with the same instrument as used in the present study. The characteristic fragment ions observed are shown below along with the mode of cleavage of the chemical structure reported by Hayashi (6). The position of the two double

Di-O-Tri-methyldisilyl ether of Octadecasphingadiene (6).

$$CH_3\text{-}(CH_2)_8\text{-}CH{=}CH\text{-}(CH_2)_2\text{-}CH{=}CH\text{-}$$

$$CH(OTMS)\}CH(NH_2)\}CH_2\text{ - }OTMS$$

m/e	309 } 132	338 } 103	m = 441
ion intensity	w s	w	

[a] w and s indicate weak and strong, respectively.

bonds in the present case, however, should be taken with some reservation since no GLC-MS analysis was performed on a derivative of its oxidation product. No phytosphingosine was detected in the aqueous phase.

It is interesting to find the octadecasphingadiene mentioned above in ginkgo nut but is not unusual since dehydrosphingosine and dihydrosphingosine have been found to be present in wheat four (11,12) and fungi (13).

Fatty Acid Composition and Identification of Hydroxy Acid

Oleic and linoleic acids are the major components in MGDG and DGDG, while they are minor ones in Cer as shown in Table II. The characteristic feature of MGDG is that it contains considerable numbers of short chain FA in the fraction indicated as U-1 amounting to 2.7%; it consists of nine peaks, and among them are decanoic acid and unsaturated dodecanoic acid. Dodecanoic acid has been reported to be present in both MGDG and DGDG isolated from *Euglena gracillis* (14). Myristic acid has been found to be present also in the neutral lipids of ginkgo nut (1) as well as in the leaves (15). Further study would be necessary for the identification of these short chain acids.

The most outstanding feature of the Cer fraction is the presence of a large amount of a hydroxy acid. The TLC analysis with hexane/diethyl ether 80:20 of FA from Cer showed a large spot at Rf 0.21; its Rt did not change after hydrogenation by GLC, and its TMS derivative on a DEGS column showed the same Rt as the TMS derivative of α-hydroxyhexadecanoic acid. The mass pattern of its ester-TMS derivative (M = 358) showed characteristic fragment ions at m/e 344 (M-15-1) and 300 (M-58) and that of the free hydroxy ester a medium intensity of M^+ at m/e 286

TABLE I

Characterization of Lipid Fractions from Ginkgo Nut Separated by Column Chromatography[a]

Column frn. no.	Solvent system C:A	Component	Yield[b] mg	Yield[b] %[e]	TLC[c] R_f	Color reactions[d] St	S	OH	Ch	NH
I	8:2				0.90	+				
II	7:3	MGDG[f]	73	0.97	0.81	-	+	+	-	-
III	4:6	Cer[f]	11	0.15	0.69	-	+	+	-	+
IV	2:8				0.63	+				
V	0:10	DGDG[g]	150	2.0	0.54	-	+	+	-	-

[a]Abbreviations: C:A = $CHCl_3$:acetone, St = steroids, S = sugars, OH = the vicinal hydroxyl group, Ch = choline, NH = the N-acyl amide group, MGDG = monogalactosyldiglyceride, DGDG = digalactosyldiglyceride, and Cer = cerebroside.

[b]7.5 g of crude lipids was applied.

[c]Developed with $CHCl_3$/methanol/water 70:22:3.

[d]All the components showed negative tests for the amino group and phosphorus; + represents positive and - negative test.

[e]Expressed as per cent in crude lipids.

[f]Purified by preparative TLC.

[g]Purified by column chromatography.

and a strong fragment ion at m/e 227 (M-59). The pattern of the latter was identical with that recorded for α-hydroxypalmitic acid in the laboratory of Hayashi. Since α-hydroxy acids in ceramides of plant origin are known to constitute the N-acyl amide moiety, the most probable structure for the major component in the cerebroside fraction from *Ginkgo biloba* nut may be expressed in the following manner;

$$CH_3\text{-}(CH_2)_x\text{-}CH{=}CH\text{-}(CH_2)_y\text{-}CH{=}CH\text{-}CH(OH)\text{-}CH\text{-}CH_2\text{-}O\text{-glucose}$$

$$\text{NH}$$

$$x + y = 10 \qquad O = C\text{-}CH(OH)\text{-}(CH_2)_{13}\text{-}CH_3$$

N-α-hydroxypalmitoylglucosyloctadecasphingadiene

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TABLE II

Fatty Acid Compositions[a] of Glycolipids from Ginkgo Nut[b]

FA	MGDG	DGDG	Cer
U-1	2.7		
14:0	tr		
14:1	0.1		
16:0	2.8	7.6	4.9
16:1	2.0	0.7	
16:2	0.5	1.1	
16:3		0.5	
18:0	0.5	1.3	1.9
18:1	26.3	36.0[c]	4.9
U-2	1.0		
18:2	56.3	48.8	2.9
18:3	6.9	4.2	tr
16:0H[d]			85.4
20:1	tr		
20:2	1.0		
22:0	tr		
20:4		tr	tr

[a]Calculated by area normalization of peaks. FA are expressed by carbon number: the number of unsaturated bonds.

[b]U and tr designate unknown and trace, respectively.

[c]Mixed with about 10% of an unknown FA.

[d]Characterized to be the α-hydroxy acid (see text).

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