Sphingolipids in Immature and Mature Soybeans

MASAO OHNISHI* and **YASUHIKO FUJINO**, *Department of Agricultural Chemistry, Obihiro, University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan*

ABSTRACT

Ceramides and cerebrosides were isolated from immature and mature soybeans, and structures of the constituents were investigated. As component fatty acids, normal, 2-hydroxy and 2,3-dihydroxy acids were found in ceramides, whereas only normal and 2-hydroxy acids were identified in cerebrosides. The principal fatty acid component was 2-hydroxylignoceric acid in ceramides, and 2-hydroxypalmitic acid in cerebrosides. Sphingoids in ceramides consisted mainly of trihydroxy bases, with 4-hydroxy-*trans*-8-sphingenine being predominant. In contrast, cerebrosides contained mainly dihydroxy bases, the principal constituent being *trans*-4,*trans*-8-sphingadienine. The only sugar in cerebrosides was glucose. The constituents of the two sphingolipids were similar to each other in immature and mature seeds. Possible metabolic relations of plant sphingolipids, based on composition, are discussed.

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INTRODUCTION

Sphingolipids are known to occur widely in organisms as components of the biomembranes. However, few studies have been carried out on the structure, the metabolic pathway and the physiological role of plant sphingolipids. Several analyses have been done concerning plant cerebrosides, a representative sphingolipid, but the detailed chemical composition, especially the component sphingoids, has not yet been completely elucidated. Previously, we examined cerebrosides isolated from rice, wheat grains and Azuki beans and found that typical sphingoids in seeds were 4,8-sphingadienine or 8-sphingenine, but not phytosphingosine (4-hydroxysphinganine) and dehydrophytosphingosine (4-hydroxy-*trans*-8-sphingenine) (1-3).

Free ceramide, a metabolic precursor of sphingolipids in animal tissues, was isolated for the first time in the plant kingdom from alfalfa leaves in this laboratory (4). Our recent papers reported that a comparatively large amount of ceramides was present in seeds such as rice, wheat and Azuki bean (1-3). As a preliminary survey of plant sphingolipids showed that ceramide was commonly distributed together with cerebroside in cereals, beans and leaves (3), ceramide seemed to be one of the typical sphingolipids in higher plants.

In this paper, we describe the chemical constituents and structures of ceramides and cerebrosides from immature and mature soybeans, and structural and possible metabolic relations of the two sphingolipids. Although cerebroside in immature soybeans has been isolated previously (5), individual components were not identified.

*Author to whom correspondence should be addressed.

EXPERIMENTAL METHOD

Isolation and Fractionation of Sphingolipids

Mature soybeans (Kitamusume variety, 2 kg), harvested at Hokkaido prefecture in 1978, were ground to powders, and extracted with hexane and then with chloroform/methanol (2:1, v/v) and water-saturated butanol. The latter two extracts were concentrated and washed with water to get pure lipids (1). Alternatively, soybeans of the same variety were cultured at the experimental farm in the university, and the immature plants were collected at 35 days after flowering. The beans (100 g) separated from the pods were extracted with hot isopropanol and chloroform/methanol to prepare total lipids (6). Both lipids (81 g and 40 g from mature and immature seeds, respectively) thus obtained were hydrolyzed with mild alkali to remove contaminating glycerolipids (1). Ceramides and cerebrosides were isolated from the alkali-stable lipid fractions by silicic column chromatography followed by acetylation, preparative thin layer chromatography (TLC) and subsequent deacetylation as described previously (1,2).

Purified ceramide was fractionated by silica gel TLC into three fractions according to degree of hydroxylation (7). On the other hand, cerebroside was subjected to silica gel/borax (98:2, w/w) TLC in order to separate into subfractions roughly according to the number of hydroxy groups on the ceramide moiety (8,9). These subfractions were analyzed directly by gas

chromatography-mass spectrometry (GC-MS) (7,10).

Analyses of Components

Each sphingolipid was degraded with methanolic 1 N HCl, aqueous methanolic 1 N HCl (11), aqueous dioxanic 10% $Ba(OH)_2$ (12) and methanolic 1 N KOH, respectively. Thus, methylglycosides, fatty acid methyl esters and sphingoids were obtained in every case, and analyzed as described previously (1-3).

The position of the double bonds in sphingoids was determined by periodate-permanganate oxidation (Von Rudloff degradation) and subsequent gas liquid chromatography (GLC) of the resultant monocarboxylic acid (13). The result was confirmed by GC-MS analyses of polyhydroxylated products of methyl ethers derived from sphingoids by oxidation with $NaIO_4$ followed by reduction with $NaBH_4$, methylation and subsequent oxidation with OsO_4 (1).

Each sphingolipid was dissolved in methanol and oxidized with $NaIO_4$ to determine fatty acid compositions of the two groups, which comprised the dihydroxy and trihydroxy bases (14). The products were subjected to silica gel TLC with chloroform/methanol (95:12, v/v) to be fractionated into the groups described above, and methanolyzed. 2-Hydroxy fatty acid methyl esters were isolated by silica gel TLC from the methyl ester fractions and analyzed by GLC.

For the determination of the anomeric configuration of the glycosidic linkages, acetyl cerebrosides were analyzed by a nuclear magnetic resonance spectrometer, operating in the Fourier transform mode at 200 MHz using deuterium chloroform as a solvent.

RESULTS

Confirmation of Sphingolipids

Purified ceramides were isolated in yields of 10 mg and 50 mg, and cerebrosides in yields of 33 mg and 130 mg, from immature and mature soybeans, respectively. These sphingolipids showed the same mobilities as those of authentic ones isolated from rice bran and Azuki beans on silica gel TLC (1,2). Moreover, infrared (IR) spectra of the lipids (not shown) exhibited the typical patterns of sphingolipids.

An oligoglycolipid, tentatively identified as diglycosylceramide, was also recognized in the fraction eluted from the silicic acid column by chloroform/methanol (80:20, v/v), but was not analyzed further in this work because of the insufficient amount.

Composition of Fatty Acids

The fatty acid composition, calculated from the relative ratio of each type and analyses by GLC, is shown in Table 1. The major acids in decreasing order were 2-hydroxylignoceric, 2-hydroxybehenic and palmitic acids in ceramides, whereas 2-hydroxypalmitic, 2-hydroxylignoceric and 2-hydroxybehenic acids, particularly the first one, were in cerebrosides.

Characterization of Component Sphingoids

Silica gel TLC, of the component sphingoids prepared from alkaline degradation of each sphingolipid, with chloroform/methanol/2 N ammonia (40:10:1, v/v) gave commonly three spots corresponding to *trans*-4-sphingenine (S_1), sphinganine (S_2) and 4-hydroxysphinganine (S_3). From GC-MS analyses of these spots as N-acetyl, O-trimethylsilyl ether derivatives (15), *trans*-4-sphingenine and sphingadienine were identified in S_1, sphinganine and x(not 4)-sphingenine in S_2 and 4-hydroxysphinganine and 4-hydroxysphingenine in S_3.

Von Rudloff oxidation of the acetyl sphingoids gave essentially a C_{10}-monocarboxylic acid, showing that the position of the double bond besides C-4 was exclusively C-8, which was confirmed by GC-MS analyses. The mass spectrum of the oxidized product originating from sphingadienine (Fig. 1(A)) showed ions at m/z 371 and 147 indicating the presence of a vicinal trimethylsiloxy group at C-2 and C-3 and ions at m/z 229, 257 and 289, which were the assignments for the other pair of trimethylsiloxy groups at C-6 and C-7. The mass spectrum of the polyhydroxylated compound derived from x(not 4)-sphingenine (Fig. 1(B)) exhibited ions at m/z 203 and 229, whereas that from unsaturated sphingoid S_3 (Fig. 1(C)) gave ions at m/z 189 and 229. These ions suggested that the presence of a vicinal trimethylsiloxy group were at C-6 and C-7 in the former, and at C-5 and C-6 in the latter, respectively. Thus, the positions of the double bonds in sphingoids turned out to be C-4 and/or C-8. When N-, O-acetyl derivatives of spots S_1 and S_3 in (mature) cerebroside were subjected to silica gel-$AgNO_3$ TLC (1), three spots (a, b and c) were found in both cases. Judging from their mobilities on TLC and results described above, S_1-a, -b and -c were characterized as *trans*-4-sphingenine, *trans*-4,*trans*-8-sphingadienine and *trans*-4,*cis*-8-sphingadienine, respectively. S_3-a, -b and -c were identified as 4-hydroxysphinganine, 4-hydroxy-*trans*-8-sphingenine and 4-hydroxy-*cis*-8-sphingenine, respectively (1,3, 16). Although spingoid S_2 was not analyzed in detail, 8-sphingenine was considered to be a

TABLE 1

Composition of Fatty Acids in Ceramides and Cerebrosides from Soybean Seeds (%)

Fatty acid	Immature seeds		Mature seeds	
	Ceramide	Cerebroside	Ceramide	Cerebroside
16:0	13	1	8	4
18:0	5	1	2	2
22:0	1	<1	2	<1
24:0	2	<1	1	<1
h_1 16:0[a]	3	73	2	79
h_1 22:0	14	10	26	5
h_1 23:0	4	<1	6	<1
h_1 24:0	42	13	41	9
h_1 25:0	5	<1	3	<1
h_1 26:0	2	1	<1	<1
h_2 22:0[b]	1	–	<1	–
h_2 24:0	3	–	3	–
h_2 25:0	1	–	2	–
h_2 26:0	<1	–	1	–
Others	4	1	3	1

[a] h_1 signifies 2-monohydroxy acid.

[b] h_2 signifies 2,3-dihydroxy acid.

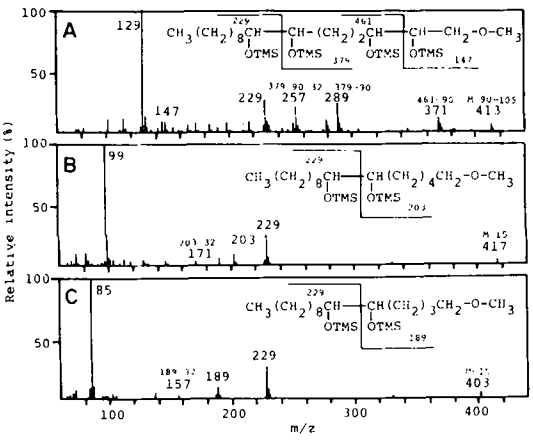

FIG. 1. Mass spectra of the trimethylsilyl ether derivatives of polyhydroxylated methyl ethers obtained from 4,8-sphingadienine (A), 8-sphingenine (B) and 4-hydroxy-8-sphingenine (C).

mixture of *cis*- and *trans*-unsaturated isomers, as in case of wheat sphingolipids (3,16).

Composition of Sphingoids

Table 2 shows the sphingoid composition analyzed and identified by GLC and GC-MS of aldehydes derived from sphingoids liberated by acid degradation (1). GLC of pentadecenals derived from the two geometric isomers of 4-hydroxy-8-sphingenine had the same retention time, so that the ratio of the isomers was determined by a combination of silica gel-$AgNO_3$ TLC and GLC of acetyl sphingoids. The principal constituents were 4-hydroxy-8-sphingenine and 4-hydroxysphinganine, the former being predominant in ceramides, whereas sphingadienines predominated in cerebrosides. Component sphingoids in soybeans generally possessed a *trans*-double bond rather than a *cis*-one at C-8, as in case of Azuki beans (2). This finding was supported by IR spectra of intact sphingolipids, in which the intensity of the absorption band at 970 cm^{-1} was significantly stronger than that of rice sphingolipid (1).

Identification of Component Sugars

Constituent sugars in cerebrosides isolated from immature and mature soybeans were found by GLC analyses to consist only of glucose. PMR spectra of the acetates (not shown) revealed the sharp doublet at 4.47 ppm ($J_{1,2}$= 7.8 Hz), indicating the β-glycosidic linkage (17,18). Therefore, the glucose linkage to the sphingoid moiety have the β-configuration.

Characterization of Molecular Species of Ceramide and Cerebroside

Table 3 shows hydroxy fatty acid composition based on sphingoid types of ceramide and cerebroside in mature soybeans. Constituent fatty acids in the dihydroxy base-containing species, which was minor in ceramide whereas major in cerebroside, were essentially only 2-hydroxypalmitic acid. On the other hand, the compositions in trihydroxy base-containing ceramide and cerebroside were similar to each other, the principal constituents being 2-hydroxylignoceric and 2-hydroxybehenic acids.

TABLE 2

Composition of Sphingoids in Ceramides and Cerebrosides from Soybean Seeds (%)

Aldehyde	Relative retention time[a]	Original base	Immature seeds		Mature seeds	
			Ceramide	Cerebroside	Ceramide	Cerebroside
15:0	0.66	4-Hydroxysphinganine	24	1	12	3
15:1 *trans*-5	0.75	4-Hydroxy-*trans*-8-sphingenine	73	16	83	10
15:1 *cis*-5	0.75	4-Hydroxy-*cis*-8-sphingenine		1	<1	7
16:0	1.00	Sphinganine	1	<1	<1	<1
16:1 *trans* & *cis*-6	1.15	*cis* & *trans*-8-Sphingenine	<1	1	<1	2
16:1 *trans*-2	1.92	*trans*-4-Sphingenine	<1	<1	–	<1
16:2 *trans*-2,*trans*-6	2.07	*trans*-4,*trans*-8-Sphingadienine	1	67	4	69
16:2 *trans*-2,*cis*-6	2.14	*trans*-4,*cis*-8-Sphingadienine	1	14	1	9
C_{15}-Aldehydes		Trihydroxy bases	97	18	95	20
C_{16}-Aldehydes		Dihydroxy bases	3	82	5	80

[a]Taking the retention time of 16:0 (10.4 min) as unity.

In order to confirm the results, subfraction I with Rf:0.4 and II with Rf:0.3 obtained by silica gel/borax TLC of immature soybeans were analyzed by GC-MS (7). The data are summarized in Table 4 (19). A single peak, comprising C_{16} hydroxy acid and sphingadienine, was found in fraction I, and five peaks in fraction II. The retention time and mass spectrum of peak 2 were identical with those of peak 1, so that peak 2 was assumed to be contaminant dihydroxy base-containing species, due to the incomplete resolution of fractions I and II. Peaks 3-6 were identified as trihydroxy base-containing species combined with hydroxy acids of C_{22-25}.

To confirm the major molecular species of ceramide, the principal ceramide components (Rf:0.6) were converted to trimethylsilyl ether derivatives and analyzed by GC-MS (Table 5) (7,19,20). Five peaks were found, the principal species being characterized as N-2′-hydroxylignoceroyl-4-hydroxysphingenine. The result was in good agreement with that deduced from the analyses of components described above. The other peaks, also identified as ceramides, were composed mainly of 4-hydroxysphingenine and 2-hydroxy acids of C_{22-26}.

DISCUSSION

In this study, the composition of ceramide and cerebroside in immature soybeans was substantially the same as that of sphingolipids in mature seeds. This suggests that the constituents in the two sphingolipids, unlike other lipid classes in soybeans (5), hardly changed during maturation.

The principal ceramide in soybean, identified as N-2′-hydroxy-lignoceroyl-4-hydroxy-*trans*-8-sphingenine, was identical with that of Azuki bean ceramide (2). Moreover, the major components in ceramide isolated from alfalfa leaves (4) and green bush bean leaves (21) were reported to be 4-hydroxysphingenine and 2-hydroxylignoceric acid. Regarding ceramide in cereals (rice and wheat) (1,3), we found that the principal fatty acid constituent was also 2-hydroxylignoceric acid, but the predominant base was the saturated homologue (4-hydroxysphinganine). Thus, in general, plant ceramide seems to consist mainly of saturated or 8-unsaturated trihydroxy bases of 18 carbons and 2-hydroxy fatty acids with longer carbon chains, particularly C_{24} acid, so that the structure of plant ceramides are not unique for plant species and organs but similar to one another. 2,3-Dihydroxy acids found in soybean ceramide have been recognized in rice, wheat and Azuki

TABLE 3

Composition of 2-Hydroxy Fatty Acids Based on Sphingoid Type in Ceramide and Cerebroside from Mature Soybean Seeds (%)

2-Hydroxy fatty acid	Ceramide		Cerebroside	
	Dihydroxy base-containing species	Trihydroxy base-containing species	Dihydroxy base-containing species	Trihydroxy base-containing species
16:0	92.4	0.4	100.0	2.9
22:0	0.3	29.9	<0.1	36.3
23:0	–	5.7	–	3.7
24:0	<0.1	58.2	<0.1	50.6
25:0	–	4.6	–	3.2
26:0	–	1.0	–	3.1
Others	7.3	0.2	–	0.2

bean seeds (1-3) and are, therefore, presumed to be widespread as the minor component of ceramide in higher plants. In any case, the stereoconfiguration of the hydroxy groups of 2,3-dihydroxy acids as well as monohydroxy ones in plant sphingolipids should be elucidated.

From the present findings, the major structure of cerebroside in soybean can be characterized as 1-O-β-glucosyl-N-2′-hydroxy-palmitoyl-*trans*-4,*trans*-8-sphingadienine, as in the case of Azuki bean (2). The main fatty acid (2-hydroxypalmitic acid) was ca. 40% in Azuki bean cerebroside, whereas ca. 70% in soybean cerebroside, though the compositions of constituent sphingoids in both cerebrosides were similar. Thus, it appears that soybean cerebroside is of the rather simple composition.

Biogenetically, ceramide has been proved to be the direct precursor of cerebroside in the animal tissues. However, overall compositions of constituents in ceramide and cerebroside isolated from soybean differed largely from each other, as in case of Azuki bean. This indicates a complex metabolic relationship between the two sphingolipids. However, the dihydroxy base- and trihydroxy base-containing ceramides were, respectively, highly similar to the ceramide moieties of dihydroxy base- and trihydroxy base-containing cerebrosides. It may suggest that the major dihydroxy base-containing cerebroside species is synthesized by the preferential glycosylation of the minor dihydroxy base-containing ceramide species. On the other hand, sphingoid and fatty acid components in the ceramide were nearly identical with those in phytoglycolipid obtained from soybeans (22). The constituents of the ceramide moiety in phytoglycolipid from green bush beans leaves were also different from those in their cerebroside, and significantly similar to those of their free ceramide (21). Thus, it seems to be general that plant ceramide is structurally related more to phytoglycolipid than cerebroside.

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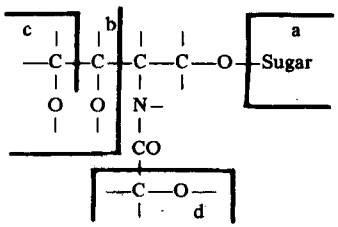
REFERENCES

1. Fujino, Y., and Ohnishi, M. (1976) Chem. Phys. Lipids 17, 275-289.
2. Ohnishi, M., and Fujino, Y. (1981) Agric. Biol. Chem. 45, 1283-1284.
3. Ohnishi, M., Ito, S., and Fujino, Y. (1980) Proc. Jpn. Conf. Biochem. Lipids 22, 379-382.
4. Fujino, Y., and Ito, S. (1971) Biochim. Biophys. Acta 231, 242-243.
5. Singh, H., and Privett, O.S. (1970) Lipids 5, 692-697.
6. Sastry, P.S., and Kates, M. (1964) Biochemistry 3, 1271-1280.
7. Ohnishi, M., and Fujino, Y. (1976) Agric. Biol. Chem. 40, 1419-1423.
8. Kean, E.L. (1966) J. Lipid Res. 7, 449-452.
9. Karlsson, K.-A., Samuelsson, B.E., and Steen, G.O. (1973) Biochim. Biophys. Acta 306, 317-328.
10. Ohnishi, M., Nakano, M., and Fujino, Y. (1975) Agric. Biol. Chem. 39, 743-744.
11. Gaver, R.C., and Sweeley, C.C. (1965) J. Am. Oil Chem. Soc. 42, 294-298.
12. Morrison, W.R. (1969) Biochim. Biophys. Acta 176, 537-546.
13. Hayashi, A., and Matsubara, T. (1971) Biochim. Biophys. Acta 248, 306-314.
14. MacMurray, T.A., and Morrison, W.R. (1970) J. Sci. Food Agric. 21, 520-528.

TABLE 4

Gas-Liquid Chromatographic and Mass Spectrometric Data for Trimethylsilyl Ether Derivatives of Soybean Cerebroside



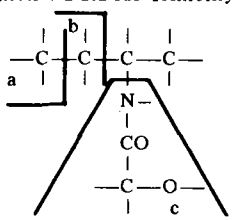
Peak number	Retention time (min)	Percentage of peak area	Component fatty acid			Component sphingoid			Molecule	
			M−a−b+73	d	Species[p]	b	c	Species[q]	M−15	Structure (sphingoid - acid - sugar)
Cerebroside I			m/z	m/z		m/z	m/z		m/z	
1	5.1	100	458	299	h_1 16:0	309	–	h_2 18:2	1130	h_2 18:2 - h_1 16:0 - Glc
Cerebroside II										
2	4.9	21	458	299	h_1 16:0	309	–	h_2 18:2	1130	h_2 18:2 - h_1 16:0 - Glc
3	16.0	25	542	383	h_1 22:0	–	297	h_3 18:1	1304	h_3 18:1 - h_1 22:0 - Glc
4	18.8	6	556	397	h_1 23:0	–	297	h_3 18:1	1318	h_3 18:1 - h_1 23:0 - Glc
5	22.5	43	570	411	h_1 24:0	–	297	h_3 18:1	1332	h_3 18:1 - h_1 24:0 - Glc
6	26.8	5	584	425	h_1 25:0	–	297	h_3 18:1	1346	h_3 18:1 - h_1 25:0 - Glc

[p] h_1 signifies monohydroxy acid.

[q] h_2 and h_3 signify dihydroxy and trihydroxy sphingoids, respectively.

TABLE 5

Gas-Liquid Chromatographic and Mass Spectrometric Data for Trimethylsilyl Ether Derivatives of Soybean Ceramide



Peak number	Retention time (min)	Percentage of peak area	Component fatty acid			Component sphingoid			Molecule	
			M−b	c+2	Species[p]	a	M−c−1−90	Species[q]	M−15	Structure (sphingoid - acid)
			m/z	m/z		m/z	m/z		m/z	
1	22.2	29	542	424	h_1 22:0	297	424	h_3 18:1	926	h_3 18:1 - h_1 22:0
2	26.9	11	556	442	h_1 23:0	297	424	h_3 18:1	940	h_3 18:1 - h_1 23:0
3	33.6	50	570	456	h_1 24:0	297	424	h_3 18:1	954	h_3 18:1 - h_1 24:0
4	39.9	8	584	470	h_1 25:0	297	424	h_3 18:1	968	h_3 18:1 - h_1 25:0
5	48.2	2	598	484	h_1 26:0	297	424	h_3 18:1	982	h_3 18:1 - h_1 26:0

[p,q]See Table 4.

15. Polito, A.J., Akita, T., and Sweeley, C.C. (1968) Biochemistry 7, 2609-2614.
16. Laine, R.A., and Renkonen, O. (1973) Biochemistry 12, 1106-1111.
17. Martin-Lomas, M., and Chapman, D. (1973) Chem. Phys. Lipids 10, 152-164.
18. Falk, K.E., Karlsson, K.-A., and Samuelsson, B.E. (1979) Arch. Biochem. Biophys. 192, 164-176.
19. Karlsson, K.-A., Pascher, I., Samuelsson, B.E., and Steen, G.O. (1972) Chem. Phys. Lipids 9, 230-246.
20. Hayashi, A., and Matsuura, F. (1973) Chem. Phys. Lipids 10, 51-65.
21. Carter, H.E., and Koob, J.L. (1969) J. Lipid Res. 10, 363-369.
22. Carter, H.E., Celmer, W.D., Galanos, D.S., Gigg, R.H., Lands, W.E.M., Law, J.H., Mueller, K.L., Nakayama, T., Tomizawa, H.H., and Weber, E. (1958) J. Am. Oil Chem. Soc. 35, 335-343.

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