Composition and Molecular Species of Ceramide and Cerebroside in Scarlet Runner Beans (*Phaseolus coccineus* L.) and Kidney Beans (*Phaseolus vulgaris* L.)

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Ceramide and cerebroside were isolated from scarlet runner bean (*Phaseolus coccineus* L.) and kidney bean (*Phaseolus vulgaris* L.), and their components and principal molecular species were studied. In the ceramide of the two beans, three types of normal, 2-hydroxy, and 2,3-dihydroxy fatty acids were observed and showed a ratio of about 25:74:1. The major component of the fatty acid was 2-hydroxylignoceric acid, while the major component in the sphingoid was 4-hydroxy-*trans*-8-sphingenine. In cerebroside of the two beans, normal and 2-hydroxy fatty acids were observed, the latter showing more than about 90%. The major fatty acid of cerebroside was 2-hydroxypalmitic acid, while the major sphingoids were *trans*-4,*trans*-8-sphingadienine and *trans*-4,*cis*-8-sphingadienine. The sugars of both cerebrosides were almost glucose. From GC–MS and reversed-phase HPLC data, the major ceramide species in the two beans was found to be *N*-2′-hydroxylignoceroyl-4-hydroxy-*trans*-8-sphingenine, and the major cerebroside was found to be 1-*O*-β-glucosyl-*N*-(2′-hydroxypalmitoyl)-*trans*-4,*trans*-8-sphingadienine. The major ceramide residues in the cerebroside from the two *Phaseolus* beans were similar to those of soybean and Adzuki bean but different from that of pea bean, which were mainly *N*-2′-hydroxypalmitoyl-*trans*-8-sphingenine residues.

INTRODUCTION

It is well-known that glycolipids in animals, mostly sphingolipids, are membrane components and have important physiological activities (Karlsson, 1982). In higher plants, three series of glycolipids, namely glyceroglycolipids, sphingoglycolipids, and steryl glycosides, are widely known (Fujino, 1983), and we found not only the well-known mono- and diglycosyl types but also the oligoglycosyl types with up to five sugar chains, from rice (Fujino, 1983; Fujino et al., 1985a), wheat (Ohnishi et al., 1985; Fujino et al., 1985b), maize (Tanaka et al., 1984), and Adzuki bean (Kojima et al., 1989, 1990). Recently, plant glycolipids were also reported to have physiological effects (Okuyama and Yamazaki, 1983; Sakata and Ina, 1983), and we reported that certain molecular species of cerebrosides from wheat grain were active upon the fruiting of *Schizophyllum commune* (Kawai et al., 1986). The most remarkable characteristic in plant cerebroside was reported to be the structural variety of the ceramide residues according to plant species (Ohnishi and Fujino, 1981, 1982; Ito et al., 1985a). But the properties of ceramide and cerebroside species in the similar family have not yet been characterized. In this paper we describe the components and the molecular species of ceramides and cerebrosides in two near species of the *Phaseolus* genus, scarlet runner bean (*Phaseolus coccineus* L.) and kidney bean (*Phaseolus vulgaris* L.), in which phosphatidylcholine and phosphatidylethanolamine species resembled but triacylglycerol species differed from mutually (Sasaki et al., 1989). Moreover, the molecular species of cerebrosides were compared with those among several bean seeds.

MATERIALS AND METHODS

Isolation of Ceramides and Cerebrosides. Commercially available scarlet runner bean (*P. coccineus* L., 200 g) and kidney bean (*P. vulgaris* L., 100 g) seeds were ground to powders, immediately steamed to deactivate the enzymes under boiled water, and extracted three times each with 600 mL of chloroform–methanol (2:1 v/v) and water-saturated butanol for 2 h of severe shaking, respectively. After the combined extracts were evaporated to dryness by use of a rotary evaporator, the residue was dissolved in 180 mL of a chloroform–methanol solution (2:1 v/v) and partitioned according to the method of Folch (Folch et al., 1957) to get total lipids. The total lipids were treated with 0.4 N KOH in methanol and then sonicated for 4 h to remove contaminating glycerolipids. The alkaline-stable lipids, which were prepared for a Folch's partition, were then applied by silicic acid column chromatography using the chloroform and methanol system. The crude ceramide and cerebroside fractions were eluted with chloroform–methanol from 98:2 and 95:5 (v/v) and chloroform–methanol from 90:10 to 80:20 (v/v), respectively. The individual sphingolipid fractions were further purified by silicic acid column chromatography and by acetylation followed by preparative thin-layer chromatography (TLC) with subsequent deacetylation (Fujino et al., 1985a; Ohnishi et al., 1985).

Analyses of Fatty Acids. Ceramide (3 mg) and cerebroside (3 mg) were heated at 100 °C under reflux with 1.5 mL of 5% methanolic HCl for 4 h. The solution was then cooled and extracted with hexane. The hexane phase was washed with an equivalent amount of water, evaporated to dryness, and then subjected to TLC on silica gel G in hexane–diethyl ether–acetic acid (80:30:1 v/v) to separate normal and hydroxy fatty acid methyl esters. They were then analyzed by gas–liquid chromatography (GLC) as reported (Tanaka et al., 1984; Fujino et al., 1985b).

Analyses of Sugars. The rest of the methanolic solution which extracted the fatty acid methyl ester as previously described was made alkaline (pH 9–10) with 7 N NaOH solution and washed with diethyl ether to remove the sphingoids. The solution was deionized by passage through ion-exchange resin (Dowex 50, H^+ type; Amberlite IR-C, OH^- type) columns. The eluate solutions were evaporated to dryness to yield the constituent sugar as methyl glycosides. Moreover, half of the methyl glycosides was hydrolyzed in 1 mL of 1 N HCl for 3 h at 100 °C. After the solution was cooled, it was deionized through an ion-exchange resin (Amberlite IR-C, OH^- type) column. After the eluate solution was evaporated to dryness, $NaBH_4$ (10 mg) and 1 mL of water were added for reduction and left for 1 h at room temperature. To finish the reduction, the excess material ($NaBH_4$) was dissolved by using a few drops of acetic acid and then

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evaporated with methanol to remove the acetic acid. The residue (alditols) was heated at 100 °C under reflux with 1 mL of acetic anhydride for 2 h to be changed to alditol acetate. The methyl glycosides and alditol acetates were analyzed by GLC as reported (Fujino et al., 1985b; Ohnishi et al., 1985).

Analyses of Sphingoids. Cerebrosides (5 mg) were hydrolyzed with 5 mL of 1 N aqueous HCl in methanol for 18 h at 70 °C. The ceramides (3 mg) were heated at 100 °C under reflux with 3 mL of 1 N KOH in methanol for 20 h. Each reaction solution was cooled and washed with hexane four times. The methanol phase of the remainder was made alkaline (pH 9–10) with 6 N KOH solution, and then the sphingoids were extracted with an equivalent volume of 3 mL of diethyl ether three times, washed, and evaporated to dryness.

The sphingoids were solubilized in 1 mL of methanol and then added to 0.1 mL of 0.2 M $NaIO_4$ solution. The mixture was then oxidized for 1 h at room temperature. The fatty aldehyde induced from the sphingoids was extracted with hexane, washed, and evaporated to dryness. Moreover, half of the aldehyde was dissolved with 2 mL of chloroform–methanol (1:3 v/v) and then added to 2 mL of 0.1 M NaOH solution which included 1 mg of $NaBH_4$. The mixture was then left for 2 h. After the reaction, Folch's partition (Folch et al., 1957) was done and the lower phase was evaporated to dryness to yield the fatty alcohol.

Analyses of Molecular Species of Ceramides and Cerebrosides. To examine the molecular species of the ceramides, the ceramides and cerebrosides were changed to trimethylsilyl ether derivatives, which was performed on a gas chromatograph–mass spectrometer (GC–MS) (Hitachi RMU-6MG instrument) (Ohnishi and Fujino, 1982; Fujino et al., 1985b). Moreover, the underived cerebrosides were analyzed by reversed-phase high-performance liquid chromatography to separate the geometric isomer.

Infrared Spectrometer. Infrared spectra were measured on an infrared spectrometer (IR-3A type, Nippon Bunko-Kogyo Co., Ltd., Tokyo) using KBr pellets with 1 mg of lipid to 100 mg of KBr.

Gas–Liquid Chromatography (GLC). GLC analyses were performed on Hitachi 063 and 163 gas chromatographs (Hitachi Seisakusho Co., Ltd., Tokyo), fitted with flame ionization detectors, and the carrier gas was high-purity nitrogen used at a flow rate of 30–40 mL/min. The normal fatty acid methyl esters and fatty aldehydes were analyzed on a 3 mm × 2 m glass column of 5% DEGS on 80–100-mesh Chromosorb W-AW-DMCS (Gaskuro Kogyo Inc., Tokyo) at 180 and 140 °C, respectively. The hydroxy fatty acid methyl esters (from 180 to 290 °C at 2 °C/min), methyl glycosides (at 160 °C), sphingoids (at 190 °C), and fatty alcohols (at 175 °C) were analyzed as trimethylsilyl ether derivatives on a 3 mm × 2 m glass column of 3% silicone SE-30 on 80–100-mesh Chromosorb W-AW-DMCS (Gaskuro Kogyo). The hydroxy fatty acid methyl esters were identified by comparison with authentic esters. The alditol acetates were then analyzed on a 3 mm × 2 m glass column of 3% ECNSS-M on 80–100 mesh Chromosorb W-AW-DMCS (Nihonchromato Inc., Tokyo) at 190 °C. Trimethylsilyl ether derivatives of ceramides and cerebrosides were analyzed on a 3 mm × 1 m glass column of Diasolid ZT (180–100 mesh, Nihonchromato). Column temperatures were from 200 to 260 °C at 3 °C/min for the ceramides and from 250 to 300 °C at 5 °C/min for the cerebrosides.

Gas–Liquid Chromatography–Mass Spectrometry (GC–MS). GC–MS analyses were performed on the Hitachi RMU-6MG instrument and interfaced with the Hitachi 0002B-8DK computer data system. The trimethylsilyl ether derivatives of the ceramides and cerebrosides were analyzed on a Diasolid ZT column (3 mm × 1 m) at 300 and 330 °C, respectively. All mass spectra were taken under the same conditions as described in a previous paper (Ohnishi and Fujino, 1982).

High-Performance Liquid Chromatography (HPLC). HPLC analyses were done with a Shimadzu Model 6A instrument (Shimadzu Co., Kyoto). Reversed-phase HPLC was performed by using an Inertsil ODS-2 column (250 × 4.6 mm, Gaskuro Kogyo). A variable-wavelength spectromonitor Model SPD-6A was used at 220 nm for the underivatized cerebroside at 40 °C. Methanol–water (25:1 v/v) was used as eluent at a flow rate of 1 mL/min.

Table I. Composition (Percent) of Fatty Acids of Ceramides and Cerebrosides in Scarlet Runner Beans and Kidney Bean Seeds

	ceramide		cerebroside	
fatty acid	scarlet runner bean	kidney bean	scarlet runner bean	kidney bean
16:0	2.7	8.6	3.6	4.6
16:1	0.2	0.3	0.1	0.7
18:0	0.6	3.0	0.3	0.7
18:1	0.6	1.4	0.2	0.4
18:2	0.7	0.4	0.3	0.2
20:0	0.5	0.9	0.2	<0.1
22:0	2.0	1.2	0.2	0.3
23:0	1.8	1.3		
24:0	11.1	5.4	0.4	0.3
25:0	2.2	1.6		
26:0	2.4	1.1		
$14h_2$:0[a]			0.8	0.8
$15h_2$:0			0.9	0.6
$16h_2$:0	6.4	2.4	58.0	58.2
$18h_2$:0			0.2	0.3
$20h_2$:0		0.7	0.4	0.5
$22h_2$:0	8.7	11.4	7.2	5.6
$23h_2$:0	5.1	8.2	1.7	1.3
$24h_2$:0	43.0	41.7	23.3	23.3
$25h_2$:0	5.8	5.9	1.1	0.9
$26h_2$:0	4.7	3.9	1.2	1.2
$22h_{2,3}$:0[b]	<0.1	0.1		
$23h_{2,3}$:0	<0.1	0.2		
$24h_{2,3}$:0	0.5	0.3		
$25h_{2,3}$:0	0.3	<0.1		
$26h_{2,3}$:0	0.1	<0.1		
normal	24.8	25.0	5.3	7.2
hydroxy	75.2[c]	75.0[c]	94.7	92.8

[a] h_2, 2-hydroxy fatty acid. [b] $h_{2,3}$, 2,3-dihydroxy fatty acid. [c] Hydroxy showed 2-hydroxy fatty acid plus 2,3-dihydroxy fatty acid.

RESULTS

Identification of Ceramides and Cerebrosides. Ceramides (10 and 5 mg) and cerebrosides (32 and 17 mg) were isolated from the lipids of scarlet runner bean and kidney bean, respectively. When these pure ceramides and cerebrosides were chromatographed on silicic acid plates, both lipids showed the same pattern: ceramides of both beans gave four spots (R_f 0.81, 0.72, 0.56, and 0.34) with chloroform–methanol (9:1 v/v), while cerebrosides gave only one broad anthrone positive spot (R_f 0.20–0.35) with chloroform–methanol (95:12 v/v) on silica gel TLC. But on high-performance thin-layer chromatography (HPTLC, Merck Co. Ltd.) with the same solvent, cerebrosides were separated into at least seven spots. The major spot is shown at R_f 0.20–0.30 on account of the four bands, and the R_f 0.1 band of the cerebroside from two beans showed the same mobility as a standard cerebroside contained in trihydroxy-based and 2-hydroxy fatty acids. The infrared spectra of each ceramide and cerebroside showed absorption at 1650 and 1550 cm^{-1} for the acid amide linkage and at 970 cm^{-1} for the *trans* double bound. However, there was not one at 1220 cm^{-1} for P=O or at 1730 cm^{-1} for the ester carbonyl group. Cerebrosides showed near 1050 cm^{-1} a broad peak for the C–O of the sugar moiety. This spectra pattern was in agreement with those of the reference ceramide and cerebroside (Fujino et al., 1985a).

Composition Analysis of Ceramides and Cerebrosides. Ceramides of both beans contained three types of fatty acids (normal, 2-hydroxy, and 2,3-dihydroxy fatty acids), their ratio being 25:74:1, respectively. The 2-hydroxy fatty acids showed two spots, R_f 0.24 and 0.30, for a difference in chain length on the silica gel TLC with hexane–diethyl ether–acetic acid (85:15:1 v/v). Fatty acid

Table II. Composition (Percent) of Sphingoids of Ceramides and Cerebrosides in Scarlet Runner Bean Seeds and Kidney Bean Seeds

sphingoids		ceramide		cerebroside	
		scarlet runner bean	kidney bean	scarlet runner bean	kidney bean
4-hydroxysphinganine	(t18:0)	14.9	21.2	0.3	0.3
4-hydroxy-*trans*-8-sphingenine	(t18:1^{8t})	61.8	61.7	12.6	11.0
4-hydroxy-*cis*-8-sphingenine	(t18:1^{8c})	6.9	6.9	9.7	8.5
sphinganine	(d18:0)	2.5	0.7	<0.1	0.2
trans-8- and *cis*-8-sphingenine	(d18:1^{8t} and d18:1^{8C})	0.3	2.2	1.0	2.7
trans-4-sphingenine	(d18:1^{4t})	3.7	1.2	<0.1	<0.1
trans-4,*trans*-8-sphingadienine	(d18:2^{4t,8t})	7.0	5.0	45.1	60.1
trans-4,*cis*-8-sphingadienine	(d18:2^{4t,8c})	2.9	1.0	31.2	17.3
dihydroxy sphingoids		16.4	10.2	77.4	80.2
trihydroxy sphingoids		83.6	89.9	22.6	19.8

compositions of the ceramides and cerebrosides in both beans are shown in Table I. The ratio of normal to 2-hydroxy fatty acids differed for ceramides and cerebrosides; the latter consisted of almost all 2-hydroxy acids, and the former had a ratio of about 1:3. Major fatty acid components of cerebrosides were 2-hydroxypalmitic acid and 2-hydroxylignoceric acid (about 5:2) in both bean cerebrosides, which were more than 80% of the cerebrosides. The major fatty acid of the ceramides was 2-hydroxylignoceric acid, which was more than 40% of the two beans. The major normal fatty acids of ceramides in several plants contained palmitic acid; however, the two *Phaseolus* seeds also contained about 5–11% lignoceric acid as normal acid.

Sphingoids and fatty alcohols from sphingoids were trimethylsilyl ether derivatives, and fatty aldehydes from sphingoids were intact, which were analyzed with GLC. As for the results of spinach leaves (Ohnishi et al., 1983), seven peaks were obtained for both lipids. The quantitative ratio of sphingoids differed for ceramides and cerebrosides, but ceramides of the two beans and their cerebrosides were alike. After these results previously described were taken into considerations, the composition of the sphingoids in the two beans is shown in Table II. The major sphingoid of both ceramides is composed of 4-hydroxy-*trans*-8-sphingenine and 4-hydroxysphinganine, in a ratio of 4:1 (scarlet runner bean) and 3:1 (kidney bean). The major sphingoid of both cerebrosides is composed of *trans*-4,*trans*-8-sphingadienine, *trans*-4,*cis*-8-sphingadienine, and 4-hydroxy-*trans*-8-sphingenine, formed in a ratio of 1:1.5:2 (scarlet runner bean) and 1:1:2 (kidney bean). These three sphingoids in the two beans occupied more than 90% of the total sphingoids.

The sugar was prepared from cerebrosides in the two beans, which was converted to the trimethylsilyl derivatives of methyl glycosides and alditol acetates, and then analyzed with GLC. These data agreed with the relative retention time of standard methyl α- and β-glucosides and glucitol acetate. These results showed that the sugar of each cerebroside consisted of only glucose.

Molecular Species of Ceramide and Cerebroside. Trimethylsilyl ether derivatives of ceramide were analyzed by GLC (Figure 1) and GC–MS (Figure 2) and showed at least five peaks. The chromatogram patterns of the two bean ceramides were alike and showed a similarity between the two ceramide species. The mass spectrum of peak 1 in Figure 1 is shown in Figure 2. Molecular weight ions at m/z 941 (M^+), 926 (M – 15), 831 (M – 90), and 748 (M – 103 – 90), fragment ions of fatty acid at m/z 500 (b + 1 + 73), 426 (b + 2), 542 (M – a), and 616 [M – (a – 73)], and fragment ions of sphingoid at m/z 297 (c), 644 (M – c), and 218 [M – (b + c)] were found to come from the trimethylsilyl ether of *N*-(2′-hydroxybehenyl)-4-hydroxy-*trans*-8-sphingenine. In the same manner, peaks 2–5 in Figure 1 were analyzed. The mass spectrum of each peak

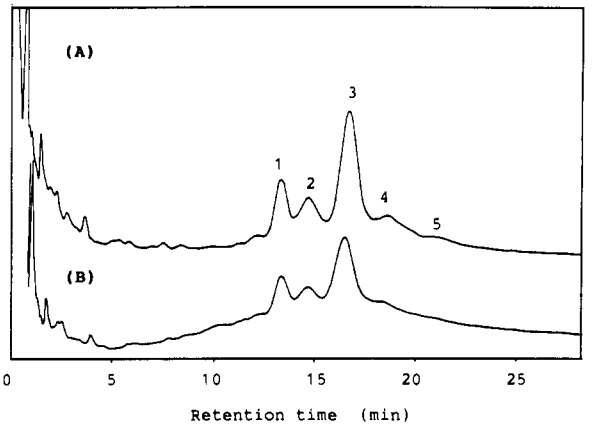

Figure 1. Gas–liquid chromatograms of ceramides from scarlet runner beans (A) and from kidney beans (B). Trimethylsilyl ether derivatives of ceramides were analyzed by gas–liquid chromatography. A glass column, 3 mm × 1 m, was packed with Diasolid ZT and temperature programmed at 3 °C/min from 200 to 260 °C.

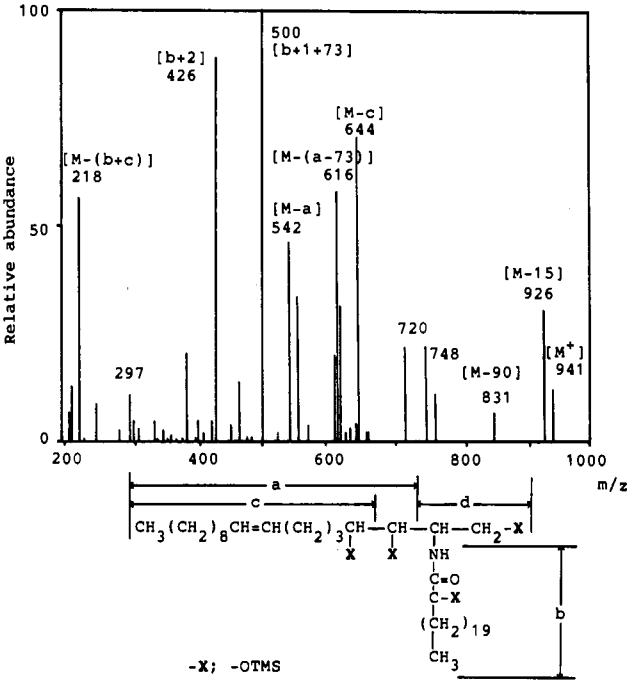

Figure 2. Mass spectrum of peak 1 in Figure 1. Trimethylsilyl ether derivatives of ceramides were analyzed by GC–MS. A glass column, 3 mm × 1 m, was packed with Diasolid ZT. Column temperature was 300 °C. The energy level of the ion source was 20 eV, and the ionizing current was 80 μA.

showed the molecular weight ion (M – 15) at m/z 940 (peak 2), 954 (peak 3), 968 (peak 4), and 982 (peak 5), fragment ion of fatty acid (b + 1 + 73) at m/z 514 (peak 2), 528 (peak 3), 542 (peak 4), and 556 (peak 5), and a

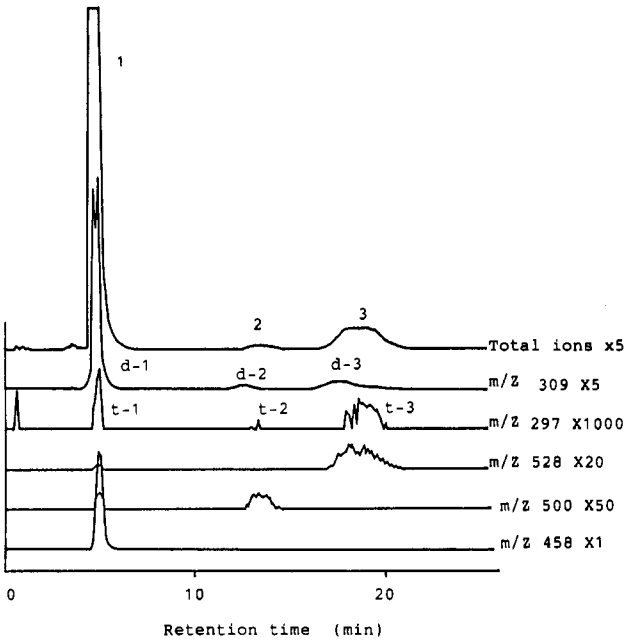

Figure 3. Mass chromatogram of cerebroside from kidney beans. Trimethylsilyl ether derivatives of cerebrosides were analyzed by GC–MS with a computer data system. A glass column, 3 mm × 1 m, was packed with Diasolid ZT and temperature set at 330 °C.

fragment ion of sphingoid (c) at m/z 297 (peaks 2–5). On the basis of analyses of components and GC–MS data, the major ceramide species of the two beans were 2-hydroxy C_{22-26} fatty acids—4-hydroxy-*trans*-8-sphingenine. A minor ceramide could not be elucidated because the fatty acid and sphingoid fragment ions in the mass spectra were not clear.

GLC of the trimethylsilyl ether derivatives of the cerebroside in two beans gave mainly three peaks, which formed in a ratio of about 11:3:1 for the two bean cerebrosides. To confirm the three species of the kidney bean cerebrosides, trimethylsilyl ether derivatives of the cerebroside were assayed by the mass chromatographic technique (Ohnishi et al., 1983) (Figure 3). A total ion chromatogram gave three peaks, the mass chromatogram of m/z 309 due to 4,8-sphingadienine revealed three peaks (1-d, 2-d, 3-d), and that of m/z 297 due to 4-hydroxy-8-sphingenine revealed three peaks (1-t, 2-t, 3-t). These peaks agreed with those in the mass chromatograms of m/z 458 due to 2-hydroxypalmitic acid, m/z 500 due to 2-hydroxybehenic acid, and m/z 528 due to 2-hydroxylignoceric acid. The results indicated that the species of peak 1 were 1-*O*-β-glucosyl-*N*-(2′-hydroxypalmitoyl)-4,8-sphingadienine (main) and 1-*O*-β-glucosyl-*N*-(2′-hydroxypalmitoyl)-4-hydroxy-8-sphingenine (minor), of peak 2 were 1-*O*-β-glucosyl-*N*-(2′-hydroxybehenyl)-4-hydroxy-8-sphingenine (main) and 1-*O*-β-glucosyl-*N*-(2′-hydroxybehenyl)-4,8-sphingadienine (minor), and of peak 3 were 1-*O*-β-glucosyl-*N*-2′-hydroxylignoceroyl-4-hydroxy-8-sphingenine (main) and 1-*O*-β-glucosyl-*N*-2′-hydroxylignoceroyl-4,8-sphingadienine (minor). In this GLC and GC–MS technique, the isomers having cis and trans configurations of 8-position unsaturation in the sphingoid moiety cannot be separated. To confirm the geometric isomer of cerebroside species, underived cerebrosides in bean seeds were analyzed by reversed-phase HPLC. Underived cerebrosides in kidney bean and pea seeds gave at least 10 major peaks (5 groups) (Figure 4), which turned out to have combinations of 2-hydroxy fatty acids (C_{14-25}) and sphingoids 8-sphingenine (d18:1[8]), 4-hydroxy-8-sphingenine (t18:1[8]) and 4,8-sphingadienine (d18:2[4,8]). The geometrical isomers of the B and C groups were completely separated by reversed-phase HPLC. Namely, peak B_1 was the 4-trans,8-cis isomer and peak B_2 the 4-trans,8-trans isomer of the sphingoid moiety in kidney bean cerebroside, and peak C_1 was the 8-cis isomer and peak C_2 was the 8-trans isomer of that in pea cerebroside (Table III). Other groups were not confirmed cis and trans isomers, because of the mixture of at least four species having geometrical isomers on each peak group (Table III).

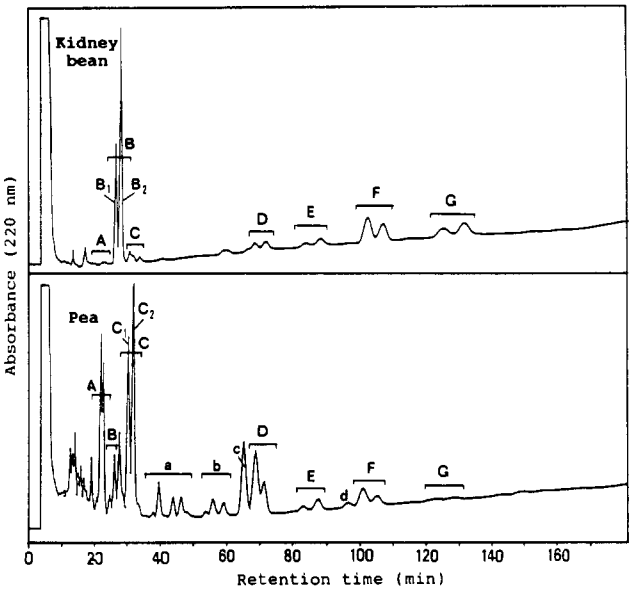

Figure 4. Reversed-phase HPLC chromatograms of cerebrosides in kidney bean and pea seeds. The Inertsil ODS-2 column, 250 × 4.6 mm, was used, and the column temperature was set at 40 °C. About 1 mg of the cerebroside was injected, and the detector range was set at the level of 0.05. Group numbers correspond to Table III. The molecular species of ceramide residues of pea cerebrosides, group a was $18h_2$:0-t18:1[8], $18h_2$:0-d18:2[4,8], and $18h_2$:0-d18:1[8], group b was $20h_2$:0-t18:1[8] and $20h_2$:0-d18:2[4,8], peak c was $20h_2$:0-d18:1[8], and peak d was $22h_2$:0-d18:1[8]. Abbreviations are the same as indicated in Tables I and II.

Table III. Molecular Species of Cerebroside from Several Bean Seeds (Percent)

group	ceramide residue[a] (fatty acid–sphingoid)	kidney bean	scarlet runner bean	Adzuki bean	soybean	pea
A	$14h_2$:0–d18:1[8]	<1	<1	<1	<1	16
	$16h_2$:0–t18:1[8]					
(B_1)	(4t,8c)	(18)	(23)	(9)	(14)	
B	$16h_2$:0–d18:2	55	53	59	74	5
(B_2)	(4t,8t)	(37)	(30)	(50)	(60)	
(C_1)	(8c)					(13)
C	$16h_2$:0–d18:1	<1	<1	<1	<1	31
(C_2)	(8t)					(18)
D	$21h_2$:0–d18:2[4,8]	6	9	7	9	14
	$22h_2$:0–t18:1[8]					
E	$22h_2$:0–d18:2[4,8]	5	4	5	6	3
	$23h_2$:0–t18:1[8]					
F	$23h_2$:0–d18:2[4,8]	19	17	17	6	6
	$24h_2$:0–t18:1[8]					
G	$24h_2$:0–d18:2[4,8]	13	15	10	6	2
	$25h_2$:0–t18:1[8]					
	others	2	2	2	2	23[b]

[a] Abbreviations are the same as indicated in Tables I and II. [b] $20h_2$:0–d18:1[8] (peak C in Figure 4) species include about 10%.

DISCUSSION

It has been assumed that plant cerebrosides participate in important physiological actions of the tonoplast and plasma membrane, because this lipid is located in these membranes as a major lipid constituent (Yoshida and Uemura, 1986; Rochester et al., 1987) and has high phase

transition temperatures (Yoshida et al., 1988). The phase transition temperatures of cerebrosides in tonoplast and plasma membrane differed between mung beans (chilling sensitive plant) and peas (chilling resistant plant), being 7–10 °C higher in mung bean cerebrosides (Yoshida and Washio, 1987). The difference may be occurrence due to the structural variety of the ceramide residue according to plant species. The structures of ceramide residues in cerebrosides are quite complicated compared with those of animal sphingoids, due to the presence of the nine species of plant sphingoids and more than seven species of 2-hydroxy fatty acids (Ohnishi and Fujino, 1981, 1982; Ito et al., 1985a). The molecular species of cerebroside in kidney bean seeds resembled those of scarlet runner beans. Although the phase behavior of the cerebroside is significantly affected by the presence of other lipid molecules such as phospholipids, substantial difference was not noted in the molecular species of cerebrosides and phospholipids in two similar family beans. By reversed-phase HPLC analysis, we concluded that the species patterns of cerebrosides in the two similar family beans are the same as those of Adzuki bean and soybean cerebrosides but different from those of pea cerebrosides. The major cerebroside species, except for peas (1-*O*-β-glucosyl-*N*-(2′-hydroxypalmitoyl)-*trans*-8-sphingenine), was 1-*O*-β-glucosyl-*N*-(2′-hydroxypalmitoyl)-*trans*-4,*trans*-8-sphingadienine, while the major ceramide species of five beans was *N*-2′-hydroxylignoceroyl-4-hydroxy-*trans*-8-sphingenine. We found that phase transition temperatures of the cerebroside species varied with the configuration at the 8-position unsaturation in sphingoid, and generally the *trans*-8-unsaturated species revealed higher phase transition temperatures (45–65 °C) than the cis-8-unsaturated ones (18–38 °C) (Ohnishi et al., 1989). Those results may show that the physiological characteristics of both bean membranes are similar to each other.

The clarification of the biosynthesis pathways of cerebrosides will also serve to elucidate the role of the physiological function of cerebroside species in plants. Since the major molecular species of cerebrosides and ceramide residues of cerebroside were different, we theorized that special species of ceramide were often connected with glucose and the cerebrosides were synthesized. Another species of ceramide was composed of phosphorus oligosaccharide and were synthesized to phytoglycolipid, which is a class of sphingolipids. Because the phytoglycolipid-like compounds showed that sphingoids contained 4-hydroxy-8-sphingenine and 4-hydroxysphinganine (about 2:1) and the major fatty acid was 2-hydroxylignoceric acid as reported (Ito et al., 1985b; Kondo and Nakano, 1987), components of the phytoglycolipid-like compounds were quite alike, not so much in cerebroside components as in ceramide components. It would be of interest to extend the research to the level of biosynthetic mechanism of the many sphingolipids, that is, unsaturation in associated sphingoids, hydroxylation in associated sphingoids, and glycosylation in ceramides.

Recently, it was reported that the sphingoid derivatives, which are the most potent inhibitors of protein kinase C in neutrophiles (Wilson et al., 1986), also inhibit transplasmalemma electron transport in oat mesophyll cells in the dark (Dharmawardhane et al., 1989). However, these model experiments were carried out with sphingoids found in animals, namely sphinganine, *trans*-4-sphingenine, and 4-hydroxysphinganine. It will be necessary to examine whether the plant sphingoids, especially with the configuration at the 8-position unsaturation in sphingoid, are effective or not and to study breakdown products of cellular sphingolipids and mechanism of sphingolipid turnover in plants.

LITERATURE CITED

Dharmawardhane, S.; Rubistein, B.; Stern, A. I. Regulation of transplasmalemma electron transport in oat mesophyll cells by sphingoid bases and blue light. *Plant Physiol.* **1989**, *89*, 1345–1350.

Fujino, Y. Lipids in cereals. *Jpn. Oil Chem. Soc. (Yukagaku)* **1983**, *32*, 67–81 (in Japanese).

Fujino, Y.; Ohnishi, M.; Ito, S. Molecular species of ceramide and mono-, di-, tri- and tetraglycosylceramide in bran and endosperm of rice grains. *Agric. Biol. Chem.* **1985a**, *49*, 2753–2762.

Fujino, Y.; Ohnishi, M.; Ito, S. Further studies on sphingolipids in wheat grain. *Lipids* **1985b**, *20*, 337–342.

Ito, S.; Ohnishi, M.; Fujino, Y. Investigation of sphingolipids in pea seeds. *Agric. Biol. Chem.* **1985a**, *49*, 539–540.

Ito, S.; Kojima, M.; Fujino, Y. Occurrence of phytoglycolipid in rice bran. *Agric. Biol. Chem.* **1985b**, *49*, 1873–1875.

Karlsson, K. A. Glycosphingolipids and surface membranes. In *Biological Membranes*; Chapman, D., Ed.; Academic Press: New York, 1982; Vol. 4, pp 1–74.

Kawai, G.; Ohnishi, M.; Fujino, Y.; Ikeda, Y. Stimulatory effect of certain plant sphingolipids on fruiting of *Schizophyllum commune*. *J. Biol. Chem.* **1986**, *261*, 779–784.

Kojima, M.; Ohnishi, M.; Ito, S.; Fujino, Y. Characterization of acylmono-, mono-, di-, tri and tetraglycosylsterol and saponin in Adzuki bean (*Vigna angularis*) seeds. *Lipids* **1989**, *24*, 849–853.

Kojima, M.; Seki, K.; Ohnishi, M.; Ito, S.; Fujino, Y. Structure of novel glyceroglycolipids in Adzuki bean (*Vigna angularis*) seeds. *Biochem. Cell Biol.* **1990**, *68*, 59–64.

Kondo, Y.; Nakano, M. Sphingolipid-saccharide complex from black gram sprouts. *Biochim. Biophys. Acta* **1987**, *919*, 156–163.

Ohnishi, M.; Fujino, Y. Chemical composition of ceramide and cerebroside in Adzuki bean seeds. *Agric. Biol. Chem.* **1981**, *45*, 1283–1284.

Ohnishi, M.; Fujino, Y. Sphingolipids in immature and mature soybeans. *Lipids* **1982**, *17*, 803–810.

Ohnishi, M.; Ito, S.; Fujino, Y. Characterization of sphingolipids in spinach leaves. *Biochim. Biophys. Acta* **1983**, *752*, 416–422.

Ohnishi, M.; Ito, S.; Fujino, Y. Sphingolipid class and their molecular species in wheat flour. *Agric. Biol. Chem.* **1985**, *49*, 3609–3611.

Ohnishi, M.; Imai, H.; Kojima, M.; Yoshida, S.; Murata, N.; Fujino, Y.; Ito, S. Separation of cerebroside in plants by reversed-phase HPLC and their phase transition temperature. Proceedings of ISF-JOCS World Congress 1988. *Jpn. Oil Chem. Soc.* **1989**, 930–935.

Okuyama, E.; Yamazaki, M. The principles of *Tetragonia tetragonoides* having anti-ulcerogenic activity II. Isolation and structure of cerebrosides. *Chem. Pharm. Bull.* **1983**, *31*, 2209–2219.

Rochester, C. D.; Kjellbona, P.; Andersson, B.; Larsson, C. Lipid composition of plasma membranes isolated from light-grown barley (*Hordeum vulgare*) leaves: Identification of cerebroside as a major component. *Arch. Biochem. Biophys.* **1987**, *255*, 385–391.

Sakata, K.; Ina, K. Digalactosyldiacylglycerols isolated from a brown alga as effective phagostimulants for a young abalone. *Agric. Biol. Chem.* **1983**, *47*, 2957–2958.

Sasaki, S.; Ohnishi, M.; Mano, Y.; Kojima, M.; Ito, S.; Fujino, Y. Characterization of triacylglycerol species in oil-poor beans. *Agric. Biol. Chem.* **1989**, *53*, 2021–2023.

Tanaka, H.; Ohnishi, M.; Fujino, Y. On glycolipids in corn seeds. *Nippon Nogeikagaku Kaishi* **1984**, *58*, 17–24 (in Japanese).

Wilson, E.; Olcott, M. C.; Bell, R. M.; Merrill, A. H., Jr.; Lambeth, J. D. Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. *J. Biol. Chem.* **1986**, *261*, 12616–12623.

Yoshida, S.; Uemura, M. Lipid composition of plasma membranes and tonoplasts isolated from etiolated seedling of mung bean (*Vigna radiata*). *Plant Physiol.* **1986**, *82*, 807–812.

Yoshida, S.; Washio, K. Possible relation between chilling injury and phase transition of tonoplast glucocerebroside in *Leguminous* plants. *Annu. Rep. Interdiscip. Res. Inst. Environ. Sci.* **1987**, *7*, 45–53 (in Japanese).

Yoshida, S.; Washio, K.; Kenrick, J. R.; Orr, G. Thermotropic properties of lipids extracted from plasma membrane and tonoplast isolated from chilling-sensitive mung bean (*Vigna radiata* [L.] Wilczek). *Plant Cell Physiol.* **1988**, *29*, 1411–1416.

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Registry No. 16:0, 57-10-3; 16:1, 373-49-9; 18:0, 57-11-4; 18:1, 112-80-1; 18:2, 60-33-3; 20:0, 506-30-9; 22:0, 112-85-6; 23:0, 2433-96-7; 24:0, 557-59-5; 25:0, 506-38-7; 26:0, 506-46-7; $14h_2$:0, 2507-55-3; $15h_2$:0, 2507-54-2; $16h_2$:0, 764-67-0; $18h_2$:0, 629-22-1; $20h_2$:0, 16742-48-6; $22h_2$:0, 13980-14-8; $23h_2$:0, 2718-37-8; $24h_2$:0, 544-57-0; $25h_2$:0, 58394-60-8; $26h_2$:0, 14176-13-7; $22h_{2,3}$:0, 31888-40-1; $23h_{2,3}$:0, 136044-42-3; $24h_{2,3}$:0, 116028-49-0; $25h_{2,3}$:0, 136044-43-4; $26h_{2,3}$:0, 136044-44-5; 4-hydroxysphinganine, 13552-11-9; 4-hydroxy-8-sphingenine, 81520-97-0; sphinganine, 764-22-7; *trans*-8-sphingenine, 41679-34-9; *cis*-8-sphingenine, 41679-35-0; *trans*-4-sphingenine, 123-78-4; *trans*-4-*trans*-8-sphingadienine, 40878-79-3; *trans*-4-*cis*-8-sphingadienine, 41679-33-8; $14h_2$:0-d18:1^8 ceramide, 136044-38-7; $16h_2$:0-t18:1^8 ceramide, 136044-39-8; $16h_2$:0-d18:2 ceramide, 125074-05-7; $16h_2$:0-d18:1 ceramide, 136044-40-1; $21h_2$:0-d18:$2^{4,8}$ ceramide, 136044-41-2; $22h_2$:0-t18:1^8 ceramide, 124960-68-5; $22h_2$:0-d18:$2^{4,8}$ ceramide, 124960-67-4; $23h_2$:o–t18:1^8 ceramide, 124960-69-6; $23h_2$:0-d18:$2^{4,8}$ ceramide, 134719-88-3; $24h_2$:0-t18:N^8 ceramide, 87384-98-3; $24h_2$:0-d18:$2^{4,8}$ ceramide, 124960-70-9; $2sh_2$:0-t18:1^8 ceramide, 124960-71-0.