

Chemical characterization of sphingophosphoglycolipids in rice leafy stems

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Abstract

Sphingophosphoglycolipids, phytoglycolipid-I (PGL-I) and phytoglycolipid-II (PGL-II), which show a single spot each on silicic acid plates, were separated from leafy stems in rice. The components and the chemical structures were then studied. The component fatty acids of PGL-I and PGL-II, consisted of hydroxy and normal acids, the former being predominant. The major component of PGL-I and PGL-II was mostly 2-hydroxylignoceric acid (40-46%). The component sphingoids of PGL-I and PGL-II consisted of trihydroxy and dihydroxy bases, the former being predominant, and the major one was mostly 4-hydroxysphinganine (82-93%). The molecular species of ceramide moieties in PGL-I and PGL-II were more similar to that of free ceramide than that of cerebroside. PGL-I contained the component sugars of mannose, N-acetylglucosamine, glucuronic acid and inositol in approximately equal ratios for sphingoid. According to analysis of components by GLC, we supposed PGL-II had at least two types of sugar chains because the proportion of arabinose, galactose, mannose, N-acetylglucosamine, glucuronic acid and inositol for sphingoid were 0.4 : 0.9 : 0.4 : 0.9 : 0.7 : 1.0.

Key words ; rice leafy stem, sphingolipid, phytoglycolipid

1. Introduction

Sphingolipides (neutral and acidic types) are known to be one component of cell membranes¹⁾. The neutral sphingolipids are reported to widely occur in higher plants²⁾, animals¹⁾ and fungi³⁾.

Previously, we established the chemical structures of neutral sphingolipids; ceramides, monoglycosyl-, diglycosyl-, triglycosyl- and tetraglycosylceramides, in rice and wheat grains^{4,5)}. However, only a few studies by Carter et al.^{6,7)} and Lester et al.^{8,9)} of the acidic sphingolipids (sphingo-

phosphoglycolipids, PGL) have been carried out on their structures, their metabolic pathway and their physiological role. They examined the structure of PGL from seeds and leaves, and suggested the complicated constitution of the oligosaccharide moieties in several types of molecules. Previously, we reported that we proved the chemical characterization of ceramides, a series of sphingoglycolipids and PGL of rice brans and the results were compared¹⁰⁾. Also, we proved the chemical characterization of ceramides and neutral sphingoglycolipids in leafy stems of rice¹¹⁾. This paper

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describes the separation and the chemical characterization of PGL in leafy stems of rice.

2. Experimental Methods

2.1 Preparation of sphingophosphoglycolipids from rice leafy stems.

Field-grown leaves of rice leafy stems (*Oryza sativa* L.) were harvested in the flowering season (in the middle of August). One Kg of leafy stems was homogenized in a polytoron with 750 ml of 100% (w/v) trichloroacetic acid and 2330 ml of 95% ethanol. This mixture was refluxed at 60°C for 60 min with continuous stirring. The homogenate was then added to an ethanol-diethyl ether-pyridine mixture (12:5:1, v/v) and brought up to pH 8.5 with conc. NH_4OH . The filter mixture was adjusted to pH 5.0 by adding acetic acid. The precipitate was most conveniently sedimented by storing the mixture at 5°C for 10 days and then centrifuged. The resulting precipitate was mixed with 1.5 times the volume of Hyflo Super Cel and then washed with acetone. The washed precipitate was poured into a glass column and eluted with a chloroform-methanol-water mixture (CHCl_3 -MeOH- H_2O , 16:16:5, v/v) containing 0.05 M $\text{CH}_3\text{COONH}_4$. This eluate was concentrated, and one-half volume of CH_3OH was added. The resulting precipitate was allowed to sediment for 16 days at 5°C. This precipitate slurry was centrifuged to remove the supernatant remaining after siphoning. The resulting precipitate was extracted with pyridine- H_2O (3:7, v/v) and then the extract solution was added to chelex 100 resin (Na^+) to change its ion form. The eluate was reprecipitated by adding acetone. The precipitate was then dissolved in a tetrahydrofuran (THF)- H_2O (3:1, v/v) solution. The THF- H_2O solution was separated on a Porasil column (4.5×43 cm) treated with base equilibrated with CHCl_3 , and solvent system of elution is shown as legend. The volume of each fraction was 500 ml. An aliquot was taken for phosphorus determination. Fractions were pooled as indicated in Fig. 1. The pooled fractions, A and

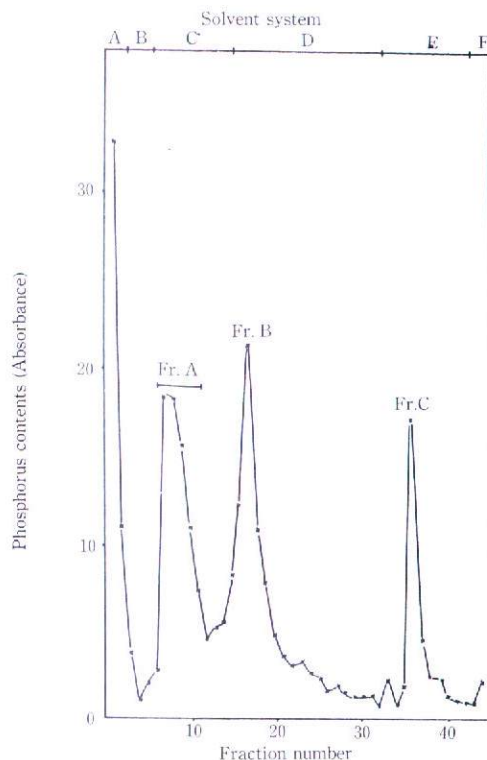


Fig. 1. Porasil column chromatography of crude Na^+ -phytoglycolipid in rice leafy stems. The solvent system used the following mixture, A: CHCl_3 -MeOH (65:40), B: CHCl_3 -MeOH- H_2O (65:40:3), C: CHCl_3 -MeOH- H_2O (56.4:38.4:5.8), D: CHCl_3 -MeOH- H_2O (54.5:39.4:7.3), E: CHCl_3 -MeOH- H_2O (43:43:14), F: CHCl_3 -MeOH- H_2O (3:48:47). The eluate volume of each fraction was 500 ml, and fr. 7-11 (Fr. A), fr. 15-19 (Fr. B) and fr. 36-37 (Fr. C) were collected.

B, were passed through DEAE-cellulose columns (acetate form). After the column was washed with 100 ml of CHCl_3 -MeOH- H_2O (16:16:5, v/v), the acidic lipids (mainly sphingophospholipids) were eluted with 40 ml of CHCl_3 -MeOH- H_2O (16:16:5, v/v) containing 2M $\text{CH}_3\text{COONH}_4$. The eluate was dialyzed for water and the inner solution was evaporated to get crude phytoglycolipid I (PGL-I from Fr. A), and crude phytoglycolipid-II (PGL-II from Fr. B). Each crude PGL was applied to re-

Porasil column chromatography by stepwise elution with CHCl_3 -MeOH- H_2O (from 65:40:3 to 65:44:7, v/v), and 10 ml of eluate was collected. Finally PGL-I was checked for purity by phosphorus determination and TLC¹⁰. Crude PGL-II applied to the re-porasil column by stepwise elution with CHCl_3 -MeOH- H_2O (from 65:42:5 to 65:47:9 v/v), and checked in the same way as PGL-I was.

2.2 Analytical methods

Phosphorus in each fraction and PGL-I and PGL-II were determined after perchloric acid digestion according to the Bartlett method¹². Component sphingoids, fatty acids in PGL-I and PGL-II were analyzed as reported¹⁰. Analyses of component sugar was as follows: PGL-I and PGL-II were heated under reflux with 5% methanolic HCl for 4hr. The reaction mixture was cooled and washed with hexane. The methanolic solution was evaporated after adding MeOH to remove HCl, and then after was acetylated with 1.2 ml of dry MeOH-dry acetic acid (3:1) and 7 mg of CH_3COOAg for 18hr at room temperature. After filtering, the eluate was evaporated several times to remove all the acetic acid. As for analyses of associated hexuronic acid, PGL-I and PGL-II were reduced using the Tayler method¹³. The sample dissolved in 2 ml of H_2O was added to 1 ml of aqueous 0.3M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The solution was stirred at room temperature for 2hr having a resulting pH of about 4.8 and then changed with conc. NH_4OH to pH 8.0. A drop of octyl alcohol followed by 5 mg of solid NaBH_4 were added and refluxed for 18hr at 55°C. The reaction mixture was dialyzed with H_2O and the resulting inner solution was evaporated to get carboxyl-reduced PGL-I and PGL-II. Carboxy-reduced samples were analyzed using the same methanolysis method. As for existence of a phosphorus inositol structure, the methanolic solution after extraction of methyl esters was evaporated, dissolved in an aliquot of MeOH, and then developed on the cellulose TLC with 1-propanol conc. NH_4OH - H_2O (6:3:1, v/v), with detection by the

Hanes-Ischerwood reagent¹⁴. These spots were extracted and resolved with 6N HCl for 40hr to get the inositol and the phosphoric acid. After the addition of a known amount of mannitol as an internal standard, inositol was analyzed by GLC using the SE-30 column at 180°C¹⁰.

3. Results and discussion

3.1 Identification of sphingophosphoglycolipids

PGL-I and PGL-II were separated from rice leafy stems. Yields of PGL I and PGL II from these separation methods were 11 mg and 16 mg, respectively. When PGL I and PGL II were chromatographed with CHCl_3 -MeOH-4NNH₄OH (9:7:2, v/v) on the silica gel TLC, PGL I and PGL-II each gave a single spot having R_f 0.45 and R_f 0.30, respectively. Color reactions on silica gel TLC were similar to those of standard sphingophosphoglycolipids from corn seeds with anthrone and Dittmer reagents. Moreover, purity of these lipids was confirmed by infrared spectrometric analyses, which showed the characteristic absorptions at 1650 cm^{-1} and 1550 cm^{-1} for the acid amide linkage, and a weak absorption at 1240 cm^{-1} for P=O. A broad band at 1100-1000 cm^{-1} was for C-O (alcohol) of the sugar moieties. Each absorption pattern was almost identical with those of corn and rice bran¹⁰, and was the characteristic absorption pattern of sphingophosphoglycolipid.

3.2 Fatty acid composition

The compositions and relative proportions of fatty acids associated with PGL-I and PGL-II are shown in Table 1. Normal, 2-hydroxy and 2, 3-dihydroxy fatty acids were observed in sphingophospholipids from rice leafy stems. The middle acids were predominant, as is generally the case in plant sphingolipids (ceramides, cerebrosides and oligoglycosylceramides)^{4,5} and in other plant sphingophospholipids⁶⁻⁹. Major normal fatty acids were commonly saturated C16, C24, C22 and C18, and major 2, 3 dihydroxy fatty acids were saturated C20 in PGL I, and C23, C20 and C26 in

Table 1. Composition of fatty acids in PGL-I and PGL-II from rice leafy stems (%)

Fatty acid	PGL-I			PGL-II		
	N*	2-H*	2,3-H*	N	2-H	2,3-H
16:0	4.0	—	—	2.4	—	—
18:0	1.7	—	—	0.4	—	—
20:0	0.9	2.8	5.2	0.4	1.8	0.6
21:0	tr.**	0.9	tr.	tr.	1.0	tr.
22:0	1.7	12.4	tr.	1.1	11.0	0.1
23:0	0.6	4.5	0.4	0.2	8.6	0.8
24:0	5.8	39.5	—	2.3	45.5	tr.
25:0	tr.	6.9	—	tr.	7.6	0.2
26:0	0.3	9.8	0.2	0.2	10.6	0.5
Others	—	2.2	0.2	—	3.9	0.8
Ratio of types***	15	: 79	: 6	7	: 90	: 3

*N, 2-H and 2, 3-H show types of normal, 2-hydroxy and 2, 3-dihydroxy fatty acid, respectively.

**Trace (tr.) show less than 0.1.

***Measured by quantitative TLC.

PGL-II. The relative amounts of these acids were slightly different from each other in the two PGLs. As component 2-hydroxy acids, at least seven species, from C20 to C26, were found. Especially, C24, C22 and C26 were major hydroxy acids in PGL-I and PGL-II, being the same components of ceramides, but not those of cerebrosides and oligo-

glycosylceramides in rice leafy stems¹¹⁾.

3.3 Sphingoid composition

Table 2 shows the sphingoid compositions, which were analyzed by GLC of fatty aldehydes derived from sphingoids by oxidation with NaIO₄. The rates of trihydroxy base and dihydroxy base were 90:10 in PGL-I and 98:2 in PGL-II. The major constituent of the two PGLs was 4-hydroxysphinganine (82% in PGL-I and 93% in PGL-II). We reported that the major component in ceramides was 4-hydroxysphinganine, but in the case of three sphingoglycolipids (monoglycosyl-, diglycosyl-, triglycosylceramide) the major bases were commonly 4-hydroxy-8-sphingenine and 4, 8-sphingadienine¹¹⁾. It is very interesting that the hydrophobic component of PGL-I and PGL-II is presumably closely related to free ceramides, but not to sphingoglycolipids.

3.4 Sugar composition and mole ratio

The methanolic solution after extraction of fatty acids of PGL-I and reduced PGL-I from the previously described method, which was reduced from uronic acid to neutral sugar by the carbodiimide method¹³⁾, were N-acetylated and trimethylsilylated to examine the component sugar (Fig. 2). We found the existence of about 1 mol of hexuronic acid as glucuronic acid (standard sugar) by the colorimetric method (Bitter and Muir method)¹⁵⁾, but the hexuronic acid peak can not be detected

Table 2. Composition of sphingoids in PGL-I and PGL-II from rice leafy stems (%)

Sphingoid	Aldehyde*	PGL-I	PGL-II	Ceramide**	Cerebroside**
4-Hydroxysphinganine	15:0	82.0	92.7	83.0	2.7
4-Hydroxy-8-sphingenine	15:1 ⁵	7.6	4.9	9.2	56.6
Sphinganine	16:0	6.3	2.1	1.8	<0.1
8-Sphingenine	16:1 ⁶	4.1	0.3	4.3	0.4
4-Sphingenine	16:1 ²	<0.1	<0.1	<0.1	1.4
4, 8-Sphingadienine	16:2 ^{2,6}	<0.1	<0.1	1.7	38.9
Trihydroxy sphingoids	C ₁₅ -Aldehydes	89.6	97.6	92.2	59.3
Dihydroxy sphingoids	C ₁₆ -Aldehydes	10.4	2.4	7.8	40.7

*Measured by GLC of fatty aldehydes derived from sphingoids by NaIO₄ oxidation.

**Information from ref. 11.

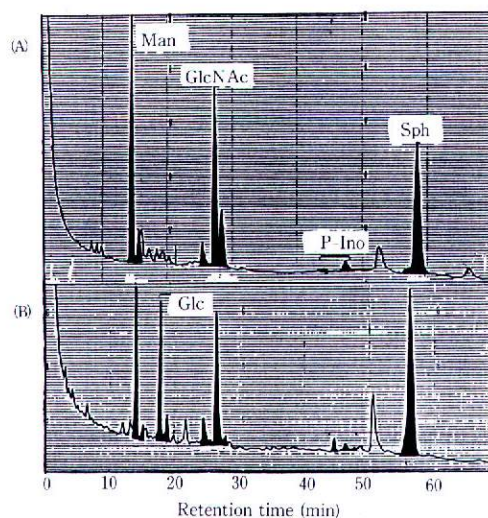


Fig. 2. Gas-liquid chromatogram of TMS derivative of methylglycoside from PGL-I. (A): PGL-I, (B): carboxyl reduced PGL-I.

Abbreviations; Man, mannose; Glc, glucose, GlcNAc, N-acetylglucosamine; P Ino, inositol phosphate; Sph, sphingoid.

from the methanolyzate of intact PGL I on the GLC chart. When it was reduced with carbodiimide before methanolysis, we found the existence of glucose which was derived from glucuronic acid. At the same time, we found only one peak of sphingoid and confirmed the existence of phosphorus inositol which did not show expected molar ratios. The composition of methylglycosides prepared from PGL-I is shown in Table 3 and Fig. 2. After PGL-I and PGL-II were reduced, mannose, glucose derived from glucuronic acid, N acetylglucosamine and sphingoid were found in a molar ratio of 0.9:1.0:1.0:1.0 in PGL-I. The composition in PGL-II contained arabinose and galactose in addition to those of PGL-I (Table 3). It then supported the form that PGL-II was a mixture of two molecular species. The sugar components of two species may consist of one group of arabinose, mannose, glucuronic acid and N-acetylglucosamine, the other having galactose, mannose, glucuronic acid and N-acetylglucosamine.

When PGL-I and PGL-II were resolved by meth-

Table 3. Sugar compositions and mol ratios in PGL I and PGL-II from rice leafy stems.

Sugar	PGL-I		PGL II	
	%	mol%	%	mol%
Arabinose	—	—	10.8	(0.4)
Mannose	31.6	(0.9)*	26.8	(0.9)
Galactose	—	—	13.4	(0.4)
Glucuronic acid	33.8	(1.0)	27.9	(0.9)
N-Acetylglucosamine	34.6	(1.0)	21.0	(0.7)
Inositol	+	1.0**	+	1.0**

*Numbers of parentheses show mol ratios for associated sphingoids.

**The mole ratio of inositol calculated for mannitol (internal standard).

analysis, they gave a single Hanes-Ischerwood reagent positive spot with 1-PrOH-conc. $\text{NH}_4\text{OH}-\text{H}_2\text{O}$ (6:3:1, v/v) on the cellulose TLC. After that these spots were extracted and resolved with 6N HCl for 40 hr, and only about 1 mol inositol was detected on GLC. Then it was found with colorimetric determination of phosphoric acid that PGL-I had about 1 mol phosphoric acid. We must analyze the binding form of the PGL-I and PGL-II, but because of the existence of only minor amounts we cannot examine in detail their sugar chain structures. These results as previously described together with the information on PGL in the literature⁶⁻¹⁰⁾ suggest that the possible structure of PGL-I isolated from leafy stems of rice is 1-O-[N-acetylglucosaminosyl-glucuronosyl-inositol]-phosphoryl-2-N'-hydroxylignoceroyl-4-hydroxysphinganine, being mannose combined somewhere on the sugar chain. PGL-II is the mixture of two molecular species, with the possible structure of one of them being 1-O-[N-acetylglucosaminosyl-glucuronosyl-inositol]-phosphoryl-2-N'-hydroxylignoceroyl-4-hydroxysphinganine with mannose and arabinose, while the other is 1-O-[N-acetylglucosaminosyl-glucuronosyl-inositol]-phosphoryl-2-N'-hydroxylignoceroyl-4-hydroxysphinganine with mannose and galactose.

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摘 要

スフィンゴホスホグリコリピド (フィトグリコリピド-I, PGL-I; フィトグリコリピド-II, PGL-II) をイネ茎葉より分別して, 構成成分および化学構造上の特徴を調査した。ケイ酸 TLC で単一スポットを示す PGL-I と PGL-II の構成脂肪酸には, ヒドロキシ酸と直鎖酸が存在し, 前者が主要であり 2-ヒドロキシシグノセリン酸 (40~46%) が主要成分であった。PGL-I と PGL-II の主要な構成スフィンゴイドはトリヒドロキシ塩基とジヒドロキシ塩基から成り, 前者が主要で 4-ヒドロキシスフィンガニンが 82~93% を占めていた。PGL-I および PGL-II とともにセラミド残基の分子種組成はイネ茎葉のセラブロシドのそれよりもセラミドのそれに類似していた。また, PGL-I の構成糖を GLC で調べたところ, スフィンゴイドに対してマンノース, N-アセチルグルコサミン, グルクロン酸およびイノシトールがほぼ等量認められた。また, PGL-II のそれはスフィンゴイドに対してアラビノース, ガラクトース, マンノース, N-アセチルグルコサミン, グルクロン酸, イノシトールが 0.4:0.9:0.4:0.9:0.7:1.0 の割合で認められることから, 少なくとも 2 種類の糖鎖から成る PGL の混合物であると推定した。

イネ茎葉のスフィンゴホスホ
グリコリピドの化学的特性

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