Structural analysis of novel bioactive acylated steryl glucosides in pre-germinated brown rice bran[1]

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Abstract Previous studies from our laboratory indicated that pre-germinated brown rice (PR) contained certain unknown bioactive lipids that activated two enzymes related to diabetes: Na^+/K^+ATPase and homocysteine-thiolactonase. In this paper, we report on the isolation and structural characterization of the activator lipids from PR bran as acylated steryl glucosides (ASGs). The activator lipid was isolated by silica gel column chromatography, and its chemical structure was determined by NMR, GC-MS, and tandem mass spectrometry. We demonstrated that the bioactive component consists of a mixture of acylated steryl β-glucosides. Δ8-cholesterol and 2-hydroxyl stearic acid were identified as constituents of ASGs. The steryl glucosides (SGs) subsequent to alkaline hydrolysis lost this enzyme activator activity. Soybean-derived ASGs were not active. This activity may be quite peculiar to PR-derived ASGs. Our findings suggest that the molecular species of ASG may play an important contributing role in the anti-diabetic properties of a PR diet.—Usuki, S., T. Ariga, S. Dasgupta, T. Kasama, K. Morikawa, S. Nonaka, Y. Okuhara, M. Kise, and R. K. Yu. **Structural analysis of novel bioactive acylated steryl glucosides in pre-germinated brown rice bran. *J. Lipid Res.* 2008.** 49: **2188–2196.**

Supplementary key words diabetes • steryl glucoside • homocysteine • glycolipids • ATPase

Pre-germinated brown rice (PR) is prepared by allowing brown rice (BR) to germinate slightly. PR is a new type of whole-grain foodstuff commercially available in Japan. The efficacy of the PR diet in the treatment of metabolic or lifestyle diseases such as hyperglycemia (1), depression (2), cognitive deficit (3), and hypercholesterolemia (4) has been explored by using appropriate animal models. In clinical studies, the PR diet has shown beneficial effects in lowering postprandial blood glucose levels in healthy subjects or prediabetic subjects with impaired fasting glucose (5, 6). This observation suggests that PR may be useful in treating and preventing diabetes and its complications (7). Furthermore, PR may enhance mental health and stimulate secretory immunoglobulin A in breast milk during lactation (8). The sprouting of brown rice causes the rice to generate γ-aminobutyric acid (GABA) (9). The level of GABA in PR is remarkably abundant, as compared with levels in white rice or BR, and is considered to be a nutrient that can prevent or improve certain metabolic diseases as mentioned above. Our previous report showed that PR-enriched diets improved hyperglycemia and peripheral neuropathy in streptozotocin (STZ)-induced diabetic rats (10). We found an unknown lipid fraction that enhanced activities of two enzymes in diabetic rats: homocysteine-thiolactonase (HTase) activity in HDL and Na^+/K^+ ATPase activity in sciatic nerve (10). Thus, our previous animal experiments suggested that the diabetes-improving component may be attributable to this lipid component. In this study, we investigated the structure of this bioactive lipid component in the total lipid extract of PR bran (TL_P).

MATERIALS AND METHODS

Preparation of lipoproteins and homocysteinylation of LDL

Lipoprotein fractions were prepared from rat serum using previously published procedures (11). Briefly, freshly collected serum (15 ml) obtained from normal male Wistar rats (n = 4) was pooled and adjusted to a density of 1.3 g/ml with solid potas-

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Abbreviations: ASG, acylated steryl glucoside; BR, brown rice; GABA, γ-aminobutyric acid; HPTLC, high-performance thin-layer chromatography; HT, homocysteine-thiolactone; HTase, homocysteine-thiolactonase; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; PR, pre-germinated brown rice; PVDF, polyvinylidene difluoride; SG, steryl glucoside; SIMS, secondary-ion mass spectrometry; S-AGS, soybean-derived acylated steryl glucoside; STZ, streptozotocin; TL_B, total lipid fraction from brown rice bran; TL_P, total lipid fraction from pre-germinated brown rice bran.

[1] Glycosphingolipid nomenclature is based on that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature.

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TABLE 1. Summary of the fractionation of HTase-activating lipid included in TLp

	Eluting Solvent	TL_B	TL_P	HTase activity
		mg/g[a]		%[b]
Total	Extraction C:M (1:1) 30 ml, C:M(2:1) 20 ml Silica gel column chromatography	240.96	213.04	164.0
Fr 1	C:H (1:1) 40 ml, C:H 9:1 70ml	161.89	171.71	73.4
Fr 2	C:M (9:1) 80 ml	37.89	19.6	135.4
Fr 3	C:M:W (7:3:0.1) 80 ml	21.07	3.73	91.6
Fr 4	C:M:W (3:6:0.8) 120 ml	18.56	11.71	101.1

HTase, homocysteine-thiolactonase; TL_B, total lipid fraction from brown rice bran; TL_P, total lipid fraction from pre-germinated brown rice bran.

[a] Values are means in individual experiments (n = 4).

[b] Values are means in individual experiments (n = 2) of HTase activity with 1.0 μg of additive (total TL_P or each fraction of Fr.1 to 4 of TL_P) and expressed as percent of control HTase activity without additive.

sium bromide. A discontinuous density gradient was formed in a centrifuge tube by layering normal saline (3.5 ml, 1.006 g/ml) over the adjusted serum (1.5 ml, 1.3 g/ml). Lipoproteins were separated by ultracentrifugation (370,000 *g*, 45 min, 4°C) in a TV865 rotor. Two major lipoprotein fractions (LDL and HDL) were collected and dialyzed overnight against PBS at 4°C. HDL was used for HTase activity assays.

In vitro homocysteinylation of LDL was done according to the procedure of Vignini et al. (12). Briefly, an aliquot of LDL (100 μg) was suspended in 100 μl of 10 mM PBS (pH 8.2) and incubated with 100 μl of homocysteine-thiolactone (HT) (100 μmol/l; Sigma, St. Louis, MO) and an indicated amount (0.01 to 10 μg) of various lipid fractions derived from brans with gentle stirring at 37°C for 2 h. After incubation, the mixture was passed through a Bio-gel P-2 column (30 × 1 cm ID; BioRad, Hercules, CA) equilibrated with 10 mM PBS (pH 8.2) to remove any unreacted HT and used for Na^+/K^+ ATPase activity assay as HT-modified LDL.

Na^+/K^+-ATPase assay

Na^+/K^+-ATPase activities were assayed as previously described (13). Briefly, a 0.2 ml assay medium containing 10 mM $MgCl_2$, 20 mM HEPES-Tris (pH 7.0), 120 mM NaCl, and 30 mM KCl, and 0.5 mg/ml of crude membrane preparations of sciatic nerves from normal male Wistar rats, 10 μg of HT-modified LDL, and 25 mM [γ-^{32}P]ATP (100,000 cpm) were incubated at 37°C for 15 min, followed by the addition of 0.1 ml of activated carbon (0.1 mg/ml). Parallel assays were also performed in which 1 mM ouabain was added to the assay medium. Following the addition of activated carbon, the samples were centrifuged at 1,500 *g* for 15 min at 4°C, the supernatants were collected, and the radioactivity (cpm) of the inorganic ^{32}P in the fractions was measured using a Beckman scintillation counter. Ouabain-sensitive Na^+/K^+ ATPase activity was calculated by subtracting the ouabain-sensitive activity from the Na^+/K^+-enhanced activity.

HTase activity assays

HTase activities in HDL fractions from normal male Wistar rats were measured with a commercial assay kit (Alfresa Auto

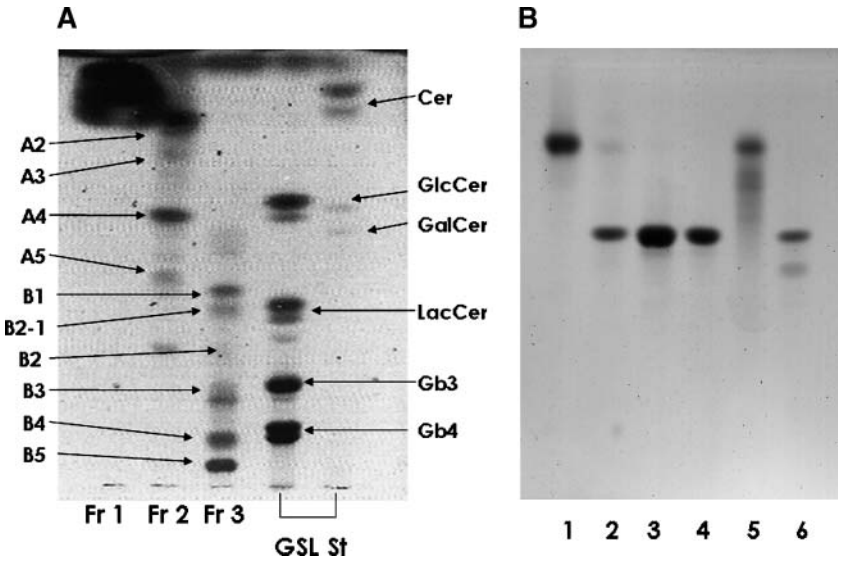

Fig. 1. A: High-performance thin-layer chromatography (HPTLC) of lipid fractions (Fr 1, 2, and 3) obtained by silica gel column chromatography of total lipid fraction from pre-germinated brown rice bran (TL_P) with the following solvents: 40 ml and 70 ml of chloroform-hexane (1:1 and 9:1; v/v); chloroform-hexane (9:1; v/v); and chloroform-methanol-water (7:3:0.1; v/v/v). Lane 1, Fr 1; lane 2, Fr 2; lane 3, Fr 3; lane 4, authentic glycolipids containing Cer, GlcCer, GalCer, LacCer, Gb3, and Gb4; lane 5, ceramide. The HPTLC plate was developed with the solvent system of chloroform-methanol-water (65:35:8; v/v/v) and subjected to visualization by orcinol reagent. B: Effect of alkaline hydrolysis on A2, A4, and soybean-derived acylated steryl glucoside (S-ASG). A2 and A4 were purified from pre-germinated brown rice (PR) bran by silica gel column chromatography. S-ASG was a commercial product from Matreya. A2, A4, and S-ASG were subjected to alkaline hydrolysis, and their motilities were compared on an HPTLC plate before and after alkaline hydrolysis. The HPTLC plate was developed with the solvent system of chloroform-methanol-water (65:35:8; v/v/v) and subjected to visualization by orcinol reagent. Lane 1, untreated A2; lane 2, alkaline-treated A2; lane 3, untreated A4; lane 4, alkaline-treated A4; lane 5, untreated S-ASG (authentic ASG derived from soybean); lane 6, alkaline-treated S-ASG.

HTLase; Alfresa Pharma Corp., Osaka, Japan) (14) that utilizes γ-thiobutyrolactone as the substrate. HTase hydrolyzes the lactone ring, generating free thiol, which reacts with 5, 5′-dithio-bis (2-nitrobenzoic acid). Generation of 5-thio-2-nitrobenzoic acid was monitored as a function of time by recording the absorbance at 450 nm using a spectrophotometer.

Lipid purification and analysis

The total lipid fraction was prepared by extracting 5 g of bran twice,with 30 ml and 20 ml of chloroform-methanol (1:1; v/v and 2:1; v/v, respectively) using the standard Folch procedure. The total lipid fraction from PR or BR bran (TL_P or TL_B, respectively) was further applied to silica gel column chromatography (15 × 1.0 cm ID, Iatrobeads, 6RS-8060; Iatron Laboratories, Inc., Tokyo, Japan), and eluted stepwise with the following solvents: *i*) 40 ml and 70 ml of chloroform-hexane (1:1 and 9:1; v/v); *ii*) 80 ml of chloroform-methanol (9:1; v/v); *iii*) 80 ml of chloroform-methanol-water (7:3:0.1; v/v/v); and *iv*) 120 ml each of chloroform-methanol-water (7:3:0.1 and 3:6:0.8; v/v/v). The fractions (Fr 1 to Fr 4) eluted with solvents *i*) to *iv*) were collected and evaporated to dryness. Each glycolipid fraction in an aliquot was developed on a high-performance thin-layer chromatographic (HPTLC) plate with the solvent system of chloroform-methanol-water (65:35:8; v/v/v) and visualized with an orcinol-sulfuric acid reagent (15). The mobility of each glycolipid on the plate was compared with that of authentic neutral sphingolipid samples (Cer, GlcCer, LacCer, Gb3, and Gb4). Each glycolipid band was quantitated by a TLC-densitometry GS-800 Calibrated Densitometer (BioRad). The carbohydrate content of Fr 1 to Fr 4 was further examined by the resorcinol-sulfuric acid method (16).

Each glycolipid in Fr 1 to Fr 4 visualized on the HPTLC plate was purified to homogeneity to yield a single spot by silical gel column chromatography (40 × 1.0 cm ID) as mentioned above by using a stepwise elution of 100 ml each of hexane-chloroform (1:1; v/v), chloroform, chloroform-methanol (4:1; v/v), chloroform-methanol-water (70:30:1; v/v/v), chloroform-methanol-water (60:40:2; v/v/v), and chloroform-methanol-water (50:50:2; v/v/v).

NMR analysis

All spectra were recorded in $CDCl_3$ at 25°C on Varian Inova 800 MHz and 900 MHz spectrometers. In addition to ^1H spectra, two-dimensional correlated spectroscopy and heteronuclear single quantum coherence (carbon-proton one-bond correlated data) and heteronuclear multiple bond correlation (carbon-proton multiple bond correlation data) spectra were collected using standard Varian pulse programs.

GC-MS analysis

FA and sterol compositions were analyzed by GC-MS (Hewlett-Packard 5980 gas chromatograph attached to a Hewlett-Packard 5972 mass spectrometer on a DB-5 column) with temperature programming from 50°C (initial) to 250°C (final) at 10°C/min increments for 15 min (17, 18). A2 or S-acylated steryl glucoside (S-ASG) (20 to 50 μg) was dissolved in 1 N anhydrous methanolic HCl in a screw-cap test tube under N_2, and subjected to methanolysis at 80°C for 16–24 h. After partitioning by addition of n-hexane, fatty acyl methyl esters in the upper hexane layer were removed, dried, and analyzed by GC-MS. The sterol fraction was separated by partitioning with 1.0 ml of petroleum ether and analyzed as described above.

TABLE 2. Summary of the fractionation of HTase-activating lipid included in TL_B and TL_P

	TL_B		TL_P	
	Composition[a]	HTase[b]	Composition[a]	HTase[b]
A2	0	ND	35.75	161.3
A4	trace	ND	22.47	93.0
B1	14.65	95.5	7.29	54.8
B2	7.5	101.0	3.29	86.9
B3	6.83	103.5	6.09	107.5
B4	15.23	94.0	9.24	91.0
B5	47.24	106.0	12.31	97.5
Others	8.55	ND	3.56	ND

ND, not detected.

[a] Values are means in individual experiments (n = 4) and expressed as percent of total glycolipids in TL_B or TL_P.

[b] Values are means in individual experiments (n = 2) of HTase activity with 0.1 μg of additive (each glycoconjugate; A2 to B5) and expressed as relative activity (%) of control HTactivity.

TABLE 3. ^1H and ^{13}C chemical shifts measured by two-dimensional NMR spectrometry of A2 in $CDCl_3$

		Shift for	
Position		^1H	^{13}C
Glucose			
	1	4.359 (7.7 Hz)	101.3
	2	3.339	73.68
	3	3.549	76
	4	3.352	70.1
	5	3.419	74.1
	6	4.483, 4.218	63.2
Sitosterol			
	C		
	5		n.o.
	10		n.o.
	13		42.8
	CH		
	3	3.525	79.63
	6	5.337	122.3
	8	1.425	32
	9	0.889	50.2
	14	0.958	56.81
	17	1.072	56.13
	20	1.323	36.2
	24	0.895	45.98
	25	1.633	29.24
	CH2		
	1	1.832, 1.031	37.4
	2	1.922, 1.590	29.8
	4	2.336, 2.245	39
	7	1.953, 1.494	32.05
	11	1.46, 1.42	21.18
	12	1.979, 1.132	39.8
	15	1.541, 1.043	24.4
	16	1.812, 1.240	28.35
	22	1.289, 0.979	34
	23	1.125	26.16
	28	1.243, 1.192	23.16
	CH3		
	18	0.665	12.2
	19	0.88 (?)	18.9 (?)
	21	0.88	18.9
	26	0.781	19.12
	27	0.803	19.94
	29	0.814	12.09
Acyl group			
	CH2		
		2.32	34.3
		1.59	25
		1.26	29.2
		0.86	14

TLC/secondary ion mass spectrometry and liquid chromatography-mass spectrometry analyses of individual lipid components

To determine the chemical structure of the glycolipid components in the A2 fraction, A2 was chromatographed on an HPTLC plate as described above. Lipid components on the plate were transferred to a polyvinylidene difluoride (PVDF) membrane. The lipid components were then excised from the membrane and analyzed by TLC/secondary-ion mass spectrometry (TLC/SIMS) (19, 20). Positive-ion mass spectra of A2 were recorded on a TSQ 700 quadrupole mass spectrometer (Finnigan Corp., San Jose, CA) equipped with a DEC Alpha Station data system. The A2 band on the PVDF membrane was excised (1.5 mm in diameter) and placed on a SIMS sample tip of the mass spectrometer, and a few microliters of 3-nitrobenzylalcohol were added as the SIMS matrix. The matrix with the sample was bombarded with a primary ion beam of Cs^+ at 20 keV. The ion multiplier was kept at 1.2 kV and the conversion dynode at 20 kV.

A2 was further characterized by reverse-phase liquid chromatography-mass spectrometry (LC-MS) using an Inertsil ODS-3 column (2.1 mm ID × 50 mm, particle size 2 μm; GL Sciences, Japan) equipped with Agilent 1100 series LC (Agilent Technologies, Inc., Santa Clara, CA) and an Esquire 3000 Plus quadrupole ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The analysis was performed under the following conditions: the solvent, 10 mM of ammonium acetate containing ethanol; flow rate, 0.05 ml/min; and column temperature, 30°C. Positive electrospray ionization was performed. Precursor ion scans were carried out at a collisional amplitude of 0.8 V.

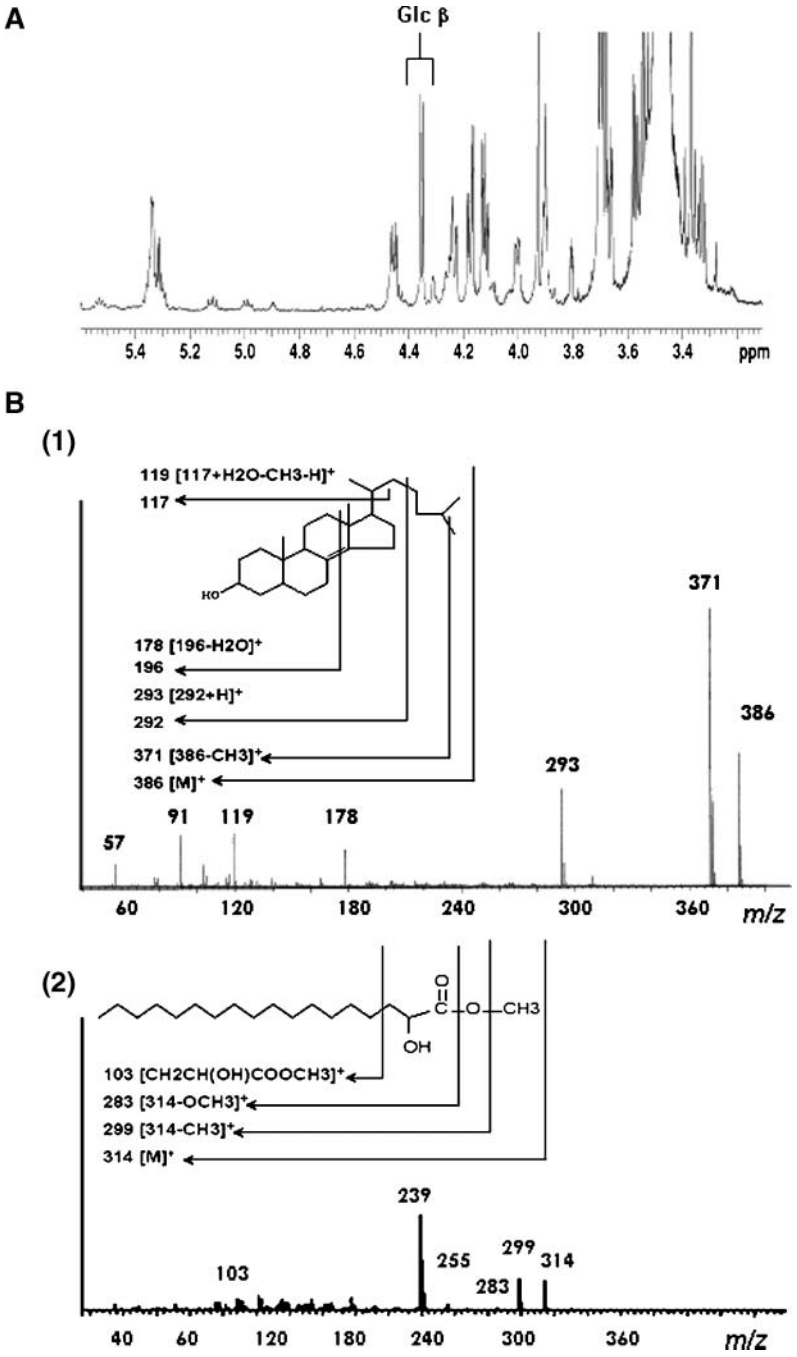

Fig. 2. A: ^{1}H-NMR spectrum of A2. B: Mass spectra of GC peaks at 25.554 min of retention time (corresponded to Δ8-cholesterol) (1) and at 23.579 min (corresponded to methyl ester of 2-hydroxyl stearic acid) (2) obtained by methanolysis of A2. Mass spectrometry fragmentation pattern of marked peaks shows the structure of the product.

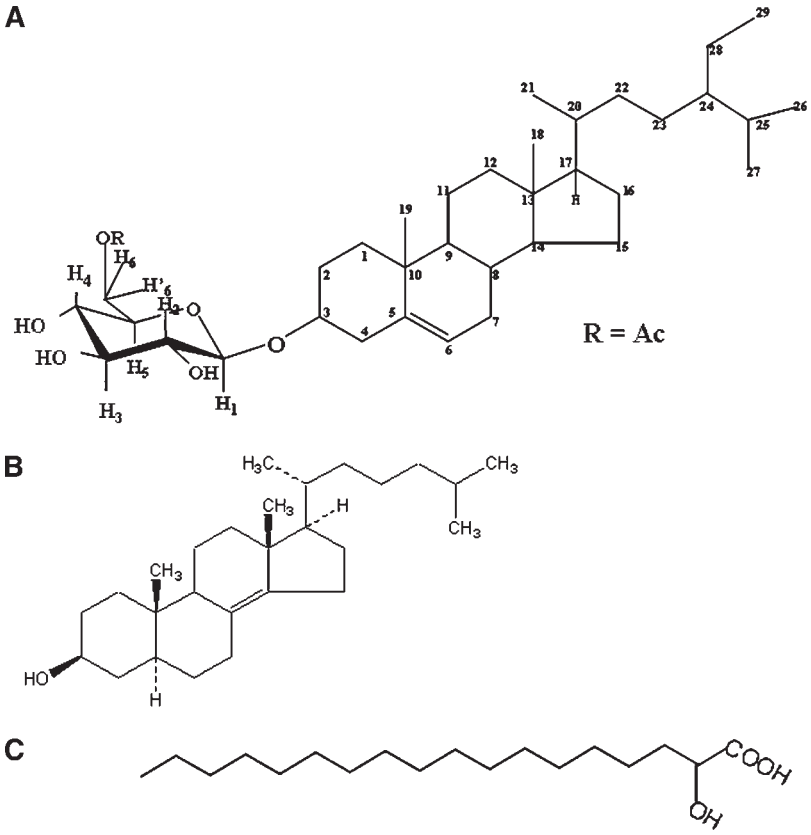

Fig. 3. A: Chemical structure of stigma-5-en-3-*O*-β-glucopyranoside (ASG characterized by β-sitosterol glucoside). The numbering is shown in Table 3 and corresponds to ^{1}H and ^{13}C chemical shifts of NMR spectrum. Ac is the acyl group. B: Structure of Δ8-cholesterol (5α-cholest-8(14)-en-3β-ol). C: Structure of 2-hydroxyl stearic acid ($18:0_{(2\ h)}$).

RESULTS

Lipid purification

The total lipid extract and Fr 1 to Fr 4 in TL_P and TL_B were estimated by weighing (**Table 1**). Most of the lipids recovered in Fr 1 did not contain any carbohydrate. The carbohydrate content of Fr 2 to Fr 4 (μg per g weight) was determined by the resorcinol-sulfuric acid method and expressed as mean ± SE (n = 3): Fr 2 was 214.6 ± 4.7 (TL_B) and 235.4 ± 41.5 (TL_P); Fr 3 was 4,155.7 ± 70.5 (TL_B) and 531.5 ± 108.6 (TL_P); Fr 4 was 912.7 ± 91.6 (TL_B) and 1,584.6 ± 141.6 (TL_P). Fr 4 of TL_P was most abundant in the carbohydrate content of all of these fractions, indicating that most of the glycolipids were present, but there was no activator activity for HTase (Table 1). Subsequently, Fr 1 to Fr 3 were analyzed by TLC with the developing solvent system of chloroform-methanol-water (65:35:8; v/v/v). A strong band was detected in Fr 1, shown by TLC as visualized by spraying with the orcinol-sulfuric acid reagent. However, the stain was not characteristic of sugar-containing lipids expected of orcinol staining. There was no glycolipid in Fr 1. In contrast, Fr 2 and Fr 3 contained many glycolipid components, which were classified into A- and B-numbered glycolipids (**Fig. 1**). In light of the relative mobility (R_f) values on TLC, Fr 4 probably did not contain any glycolipids, but only carbohydrate components. For this reason, Fr 4 was not subjected to further analysis by TLC. Each glycolipid component on the TLC plate was quantitated using the authentic standard of monogalactosyldiglyceride (Sigma) by densitometry and estimated as a percentage of all compo-

TABLE 4. Composition of sterol and FA from A2

	Sterol %				FA %						
Glycolipid	Campesterol	Stigmasterol	β-Sitosterol	≅Δ8-Cholesterol	16:0	18:0	$18:0_{(2\ h)}$	18:1	18:2	22:0	24:0
A2	8.9	4.7	66.6	19.7	27.1	7.4	7.1	29.3	21.6	0.0	7.5
S-AGS	17.3	15.6	67.1	0.0	41.6	29.2	0.0	12.0	2.6	10.5	4.1

S-AGS, S-acylated steryl glucoside. 2 h, 2-hydroxylation of carbon atom; FAs are expressed as number of carbon atoms:number of double bonds.

nents developed on the TLC plate (**Table 2**). TL_B included none or little of A-numbered glycolipids. On the other hand, there was a similarity of glycolipid-compositional patterns between TL_P and TL_B. TL_P was more abundant in lipids than was TL_B (Table 1). Subsequently, each of the A- and B-numbered glycolipids was purified by silica gel column chromatography as mentioned in the Materials and Methods section. Each of the purifed glycolipids was more than 99% pure based on TLC analysis. A2, A4, and B1 to B5 were tested for their stimulatory activity for HTase at the amount of 0.1 μg each (Table 2). Only A2 had stimulatory activity, showing 161.3% of relative activity as compared with the control.

Alkaline hydrolysis of A2

TLC analysis showed that A2 was alkali-labile, migrating to the same location (R_f of TLC plate) as did A4; on the other hand, A4 was alkali-stable (Fig. 1B). ASG, a commercially available lipid derived from soybean (S-ASG; Matreya, Pleasant Gap, PA), was used as an authentic standard for TLC analysis. S-ASG gave the same R_f as A2; alkaline-hydrolysis resulted in a compound with the same R_f value as that of A4. Chemical characterization based on the mobility on the TLC plate suggests that A2 may have the same structure as S-ASG or a structure similar to that of S-ASG, consisting of similar molecular components or stereo-isomeric linkages.

A2 and S-ASG were subjected to alkaline hydrolysis and converted to steryl glucoside (SG). Each SG was purified by C18 Sep-Pak cartridge (Waters, Milford, MA) and used for HTase activity assay.

^{1}H and ^{13}C NMR analysis

NMR data indicate that sample A2 was a mixture of compounds, in which the major component had the structure of β-sitosterol glucoside, stigma-5-en-3-*O*-β-glucopyranoside (21). The complete assignment of the major components of A2 is listed in **Table 3**.

Figure 2A shows the ^{1}H spectrum of the carbohydrate region for A2 that includes seven resonances due to β-glucose and additional signals due to glycerol and other minor components in the mixture. The chemical shift of H_1 (4.36 ppm) and the scalar coupling to H_2 (7.7 Hz) is diagnostic for the β-anomer. The chemical shift positions of H_6 and H_6' indicate that the 6-hydroxyl group is acylated. This acylation was confirmed by the HMBC data (not shown), which contain a cross peak connecting H_6 to a carbonyl carbon. The acyl chain that is connected to the glucose 6-hydroxyl is not identified, although there are signals due to CH2s at 2.32 and 1.59 ppm, which also show connections to the same carbonyl group, suggesting that the acyl group is a long-chain FA.

The peaks assigned as glycerol protons integrate to about 40% to 50% higher concentrations than does glucose. Although the HMBC shows a connection between the glycerol CH2 at 4.126 and 4.179 ppm and a carbonyl carbon, indicating that the glycerol may be esterified, there is no definitive connection to A2. The chemical structure of acylated β-sitosteroyl glucoside is shown in **Fig. 3A**.

GC-MS analysis

NMR analysis of A2 did not identify all of the components, such as sterol and FA. Sterol and FA compositions of A2 and S-ASG were determined by GC-MS analysis. Analysis of FAs (as methyl esters) showed that A2 contained 16:0, 18:0, $18:0_{(2\ h)}$, 18:1, 18:2, and 24:0; S-ASG contained 16:0, 18:0, 18:0, 18:1, 18:2, 22:0, and 24:0 (**Table 4**). There was a trace of 14:0 in A2 and S-ASG. A2 was characteristic of involvement of 2-hydroxylated stearic acid ($18:0_{(2\ h)}$) with a lesser amount of 16:0, as compared with that of S-ASG. Mass spectrometric analysis of the GC peak at 27.579 min of retention time is shown in Fig. 2B (2) and confirmed the presence of 2-hydroxyl stearic acid with the signals at *m/z* 314, *m/z* 299, *m/z* 283, and *m/z* 103, which were in agreement with

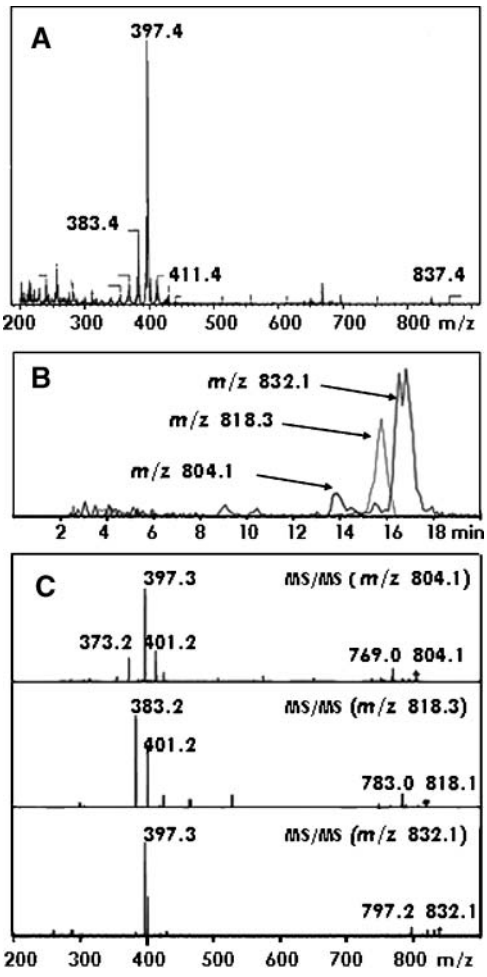

Fig. 4. TLC/secondary-ion mass spectrometry (SIMS) and liquid chromatography-mass spectrometry (LC-MS) of A2. A: Secondary-ion mass spectrum to demonstrate the ASG structure in A2. A2 was separated in an HPTLC plate and visualized with primulin. The plate was blotted on a polyvinylidene difluoride membrane to which A2 was transferred, and SIMS was performed as described in Materials and Methods. B: Three extracted ion chromatograms from separation of A2 on a reverse-phase column were traced by LC-MS scanning of *m/z* 804.1, *m/z* 818.1, and *m/z* 832.1. C: Tandem mass spectrometry (MS/MS) spectra from precursor ion of *m/z* 804.1 (top panel), *m/z* 818.1 (middle panel), and *m/z* 832.1 (bottom panel).

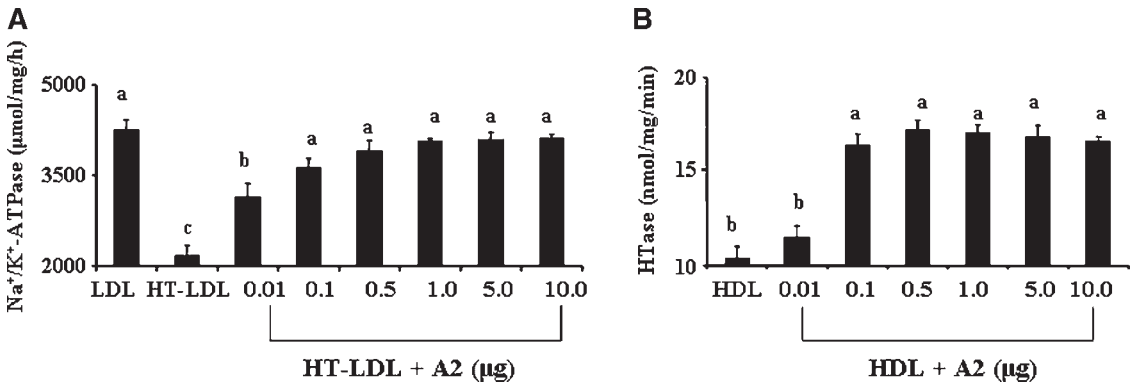

Fig. 5. A: Effects of A2 on Na^+/K^+ ATPase activity. Activity was assayed by incubation with the following amounts, as seen in columns (from left to right) 1 to 8: 1, no additive (normal LDL); 2, HT-treated campesterol; 3, A2 (0.01 μg)/HT-treated LDL; 4, A2 (0.1 μg)/HT-treated LDL; 5, A2 (0.5 μg)/HT-treated LDL; 6, A2 (1.0 μg)/HT-treated LDL; 7, A2 (5.0 μg)/HT-treated LDL; 8, A2 (10.0 μg)/HT-treated LDL. B: Effects of A2 on homocysteine-thiolactonase activity. Activity was assayed by incubation with the following amounts as seen in columns (from left to right) 1 to 7: 1, no additive (Non); 2, A2 (0.01 μg); 3, A2 (0.1 μg); 4, A2 (0.5 μg); 5, A2 (1.0 μg); 6, A2 (5.0 μg); 7, A2 (10.0 μg). Values are means ± SEM, n = 6 individual experiments. The values were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Values with different letters differ significantly among the columns. $P < 0.01$.

ions $[M]^+$, $[M\text{-}CH_3]^+$, $[M\text{-}OCH_3]^+$, and $[CH_2CH(OH)COOCH_3]^+$, respectively.

Analysis of sterols (as the free form) showed that S-ASG contained campesterol, stigmasterol, and β-sitosterol; A2 contained campesterol, stigmasterol, β-sitosterol, and Δ8-cholesterol (5α-cholest-8(14)-en-3β-ol). β-sitosterol is the major component of A2 as well as of S-ASG. However, A2 was characteristic of the involvement of novel sterols, such as Δ8-cholesterol, in a relatively high concentration. Δ8-cholesterol was confirmed from the mass spectra of the GC peak at 25.554 min of retention time with the signals at *m/z* 386, *m/z* 371, *m/z* 293, *m/z* *m/z* 178, and *m/z* 119, which were in agreement with ions $[M]^+$, $[M\text{-}CH_3]^+$, $[292+H]^+$, $[196+H_2O]^+$, and $[117+H_2O\text{-}CH_3\text{-}H]^+$, respectively. The chemical structures of Δ8-cholesterol and $18:0_{(2h)}$ are shown in Fig. 3B and C.

TLC/SIMS and LC-MS analyses

The structure of A2 was analyzed by TLC/SIMS. The TLC/SIMS spectrum obtained from A2 is shown in **Fig. 4A**. Characteristic ions were detected at *m/z* 837.4, 397.4, and 383.4, which were assigned as [palmitoylated β-sitosteryl β-glucoside $+Na]^+$, $[\beta\text{-sitosterol-}H_2O+H]^+$, and $[\text{campesterol-}H_2O+H]^+$, respectively.

Separation of the molecular species of ASG could be achieved on a reverse-phase column. Three extracted ion chromatograms from separation of A2 on a reverse-phase column were traced by LC-MS scanning of *m/z* 804.1, *m/z* 818.1, and *m/z* 832.1 (Fig. 4B). In Fig. 4C, the top panel shows the tandem mass spectrometry (MS/MS) spectrum of *m/z* 804.1 at 14 min of HPLC elution. The precursor ion (*m/z* 804.1) was consistent with [myristylated β-sitosteryl β-glucoside $+NH_4]^+$. The ion of *m/z* 397.3 was assigned as $[\beta\text{-sitosterol-}H_2O+H]^+$. The ion of *m/z* 769.0 was [myristylated β-sitosteryl β-glucoside $\text{-}H_2O+H]^+$. The ion of *m/z* 373.2 was [myristylated β-glucose $-H_2O+H]^+$. The middle panel was the MS/MS spectrum of *m/z* 818.3 from 15.7 to 16.0 min of HPLC elution. The precursor ion (*m/z* 818.1) was [palmitoylated campesteryl β-glucoside $+NH_4]^+$. The *m/z* 401.2 and 383.2 were assigned as [palmitoylated β-glucose $-H_2O+H]^+$ and [campesterol $-H_2O+H]^+$, respectively. The *m/z* 783.0 was assigned as [palmitoylated campesteryl β-glucoside $-H_2O+H]^+$. The bottom panel was the MS/MS spectrum of *m/z* 832.1 from 14 to 17.2 min of HPLC elution. The precursor ion (*m/z* 832.1) was consistent with [palmitoylated β-sitosteryl β-glucoside $+NH_4]^+$. From the MS/MS fragmentation, the ion of *m/z* 397.3 was assigned as $[\beta\text{-sitosterol} -H_2O+H]^+$, the ion of *m/z* 401.2 was assigned as [palmitoylated β-glucose $-H_2O+H]^+$, and the ion of *m/z* 797.2 was assigned as [palmitoylated β-sitosteryl β-glucoside $-H_2O+H]^+$.

Effect of A2 on Na^+/K^+-ATPase and HDL HTase activity

The purified A2 was tested for dose dependency in the ability to stimulate Na^+/K^+-ATPase and HTase activity. A2 showed a clear stimulatory effect on both enzymes at a range of 0.01 to 0.5 μg; however, higher doses of more than 0.5 μg resulted in saturation of the stimulatory effect (**Fig. 5**). In contrast to A2, S-ASG showed no stimulatory effect on Na^+/K^+-ATPase and HTase at the same dosage as A2 (data not shown).

A2 and S-ASG were subjected to alkaline hydrolysis. For each material, HTase activator activity was compared before and after alkaline hydrolysis (**Table 5**). Alkaline

TABLE 5. Comparison of HTase activation

	Alkaline Hydrolysis	
	Before	After
	(%)	
A2	157.9 ± 5.5	96.9 ± 4.0
S-ASG	95.7 ± 2.7	105.3 ± 5.3

Values are means in individual experiments (n = 4) of HTase activity with 0.1 μg of additive (A2 or S-ASG) before and after alkaline hydrolysis, respectively, and expressed as percent of control HTase activity without additive.

hydrolysis resulted in loss of activity for A2. S-ASG did not show any activation after alkaline hydrolysis.

DISCUSSION

Previously, we reported that STZ-diabetic rats fed with a PR diet showed a decrease in blood glucose levels and attenuation of peripheral nerve damage associated with neuropathy (10). PR bran contains some unknown lipid factors that stimulate Na^+/K^+ ATPase and HTase activity in in vitro assay systems. PR has an endosperm and other components (comprising bran) consisting of germ, combined aleurone and pericarp, etc. Our present study confirmed that the active ingredient is involved in the lipid fractions of bran, but not in the endosperm. We do not know whether this active fraction might be associated with the role of GABA in the PR. However, this activity seems to be related to the regulatory system involved in diabetic complications such as peripheral neuropathy rather than blood glucose control.

To characterize the bioactive ingredients, TL_P and TL_B were subjected to lipid fractionation and purification by silica gel column chromatography. The fraction containing the stimulatory effect for HTase was present in Fr 2 of the stepwise-solvent elution. Fr 1, Fr 2, and Fr 3 were analyzed by TLC. The remarkable compositional difference was that TL_P included A2 and A4, but TL_B did not.

Further analysis revealed that A2 had activator activities for HTase and Na^+/K^+ ATPase (Fig. 5). There are, however, no activities of HTase and Na^+/K^+ ATPase in S-ASG (data not shown). Structural analysis of A2 established that it consists of a molecular species of ASG, characterized by β-glucoside, sterols (campesterol, stigmasterol, β-sitosterol, and Δ8-cholesterol), and FAs (16:0, 18:0, $18:0_{(2\ h)}$, 18:1, 18:2, and 24:0). The presence of Δ8-cholesterol and $18:0_{(2\ h)}$ as components of ASG had been expected. Such a species of ASG containing Δ8-cholesterol and $18:0_{(2\ h)}$ was not detected by LC-MS. ASG containing myristic acid (14:0) was detected by LC-MS, although 14:0 was less than 1% of the total FA composition as revealed by GC-MS. Such an ASG might have been detected using an alternative eluting condition of LC. With the exception of the new molecular species ASG, the stimulatory activity of A2 was abolished by removal of the acyl group after alkaline treatment (Table 5). This finding suggests that the intact structure of A2 is needed for full stimulatory activity on HTase and Na^+/K^+ ATPase.

Several biological effects of SG and ASG are known, including anti-inflammatory (22), anti-ulcerogenic activity (23, 24), anti-diabetic (25) effects. In particular, the reported anti-hyperglycemic and insulin-releasing effects of SG by oral administration in hyperglycemic rats are relevant to our findings (25). However, our finding goes beyond the previous observation that ASG might be metabolized and converted to SG, subsequently, contributing to the anti-hyperglycemic effect. We emphasize that a certain novel ASG is probably involved in PR bran and functions as an enzyme activator. This function appears only in ASG of PR bran origin, but not in S-ASG. This observation may imply that the enzyme activator requires the special structure of sterol and FA components in the molecular species of ASG.

Previously, Fujino and Ohnishi (26) reported that rice bran contained steryl β-glucoside and that the sterol component consisted of sitosterol, campesterol, and stigmasterol. These investigators attempted to pretreat the combined glycolipid fractions with alkaline hydrolysis followed by purification of each of the glycolipid components. Clearly, ASG would have been destroyed under those conditions. In our own study presented here, we detected only a trace amount of steryl β-glucoside and no ASG in rice bran. The discrepancy between Fujino's and our studies may be due to the presence or absence of pre-alkaline hydrolysis.

Various molecular species of ASG have been isolated from plant seeds (27, 28). Some ASGs are also found in sprout-derived sterols of plants (29, 30) and play an important role as sprout-growing biochemical factors that are newly generated during the process of germination. Na^+/K^+ ATPase activity decreases in diabetic neuropathy because of injury on the peripheral nerve cell membrane. The germination-related ASG of the PR bran may be different from other ASGs in the binding affinity to HTase. Thus, the ASG of PR bran may be produced as sprout-derived sterols and may offer clinical efficacy for metabolic syndromes and lifestyle diseases.

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ERRATA

The authors of the article "Structural analysis of novel bioactive acylated steryl glucosides in pre-germinated brown rice bran" (*J. Lipid Res.* 2008. **49:** 2188–2196) have advised the *Journal* that the notation "Δ8-cholesterol" used in the article is incorrect and may lead to confusion. The correct notation should be "Δ8(14)-cholesterol" with the structure of "5α-cholest-8(14)-en-3β-ol" based on "IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature of Steroids" (*Pure Appl. Chem.* 1989. **61:** 1783–1822).

Please note that the correct structure is shown in Figure 3B as published in the original article.

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ERRATA

A previously published errata in the September 2009 issue of the *Journal of Lipid Research* was incorrect. The authors of the article "Structural analysis of novel bioactive acylated steryl glucosides in pre-germinated brown rice bran" (*J. Lipid Res.* 49: 2188–2196) have advised the *Journal* that the notation Δ8-cholesterol used in the original article is incorrect and the correct notation should be **Δ8(14)-cholestenol** with the following structure: 5α-cholest-8(14)-en-3β-ol based on "IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature of Steroids" (Pure & Appl. Chem. 61: 1783–1822, 1989). This misspelling appeared online in the September 2009 issue but has since been corrected. The *Journal* sincerely regrets this error.

Please note that the correct structure is shown in Figure 3B as published in the original article.

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The authors of the article "Quantitative analysis of phospholipids containing arachidonate and docosahexaenoate chains in microdissected regions of mouse brain" (*J. Lipid Res.* 51: 660–671) have advised the *Journal* that "docosahexaenoate" had been misspelled as "docosahexenoate". This misspelling appeared initially online but has since been corrected.

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