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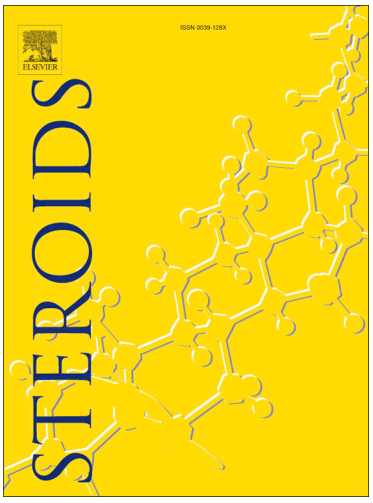

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Analysis of steryl glucosides in rice bran-based fermented food by LC/ESI-MS/MS

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Abstract

Steryl glucosides (SGs) and acylated steryl glucosides (ASGs) are phytochemicals found in plant-based foods and are known as bioactive compounds with potential health benefits. These include anti-inflammatory properties, anti-diabetic effects, and modulation of immunoregulatory functions as well as having cholesterol lowering effects. In this study, three major SGs, i.e., glucosides of β-sitosterol, stigmasterol, and campesterol, were synthesized and used as standards for measurement of their contents in rice bran (RB)-based fermented food (FBRA) utilizing *Aspergillus oryzae* and raw material (RM). The compounds were quantified using liquid chromatography/electrospray ionization-tandem mass spectrometry. It was found that β-sitosteryl glucoside was most abundant among the analyzed glucosides in both samples, and the contents of each SG in FBRA decreased about 35% from those of RM. In contrast to SGs, the contents of ASGs in FBRA increased 1.5-fold during the fermentation process as evidenced by an alkaline hydrolysis. The present results suggest that the FBRA might have greater beneficial effects than the RM, since ASGs have shown to have more potent cholesterol lowering effects and stronger anti-diabetic properties than SGs.

Key words steryl glucoside, acylated steryl glucoside, rice bran, fermentation, *Aspergillus oryzae*, LC/ESI-MS/MS

Introduction

Plant sterols (phytosterols) are one of the main constituents of plant membranes, playing important roles in cell membrane stability and in signal transduction [1]. Dietary phytosterols, on the other hand, can occur in plant-derived foods in multiple forms -- as free sterols, steryl esters (esterified to fatty acids or hydroxycinnamic acids, such as ferulic acid or *p*-coumaric acid), and as glucosidic conjugates composed of steryl glucosides (SGs) and acylated (also termed as esterified) steryl glucosides (ASGs) [2]. In SGs, one glucose moiety binds at the 3β-hydroxy group of the sterol core, forming β-glucosides. Conjugates with other monosaccharides, (e.g., galactose, xylose, and mannose) and oligosaccharides have also been described [3]. When SGs are esterified by a fatty acid presumably at the C6 position of the sugar moiety, it gives rise to ASGs [4]. The structural diversity of SGs and ASGs has been reviewed extensively [3]. Several biological effects of these glucosides in human and animals are known, including anti-inflammatory [5] and anti-diabetic effects [6] as well as reduction of intestinal cholesterol absorption [7-9], modulation of immunoregulatory functions [10], and anticancer effects [11], suggesting their therapeutic application in some chronic diseases.

Rice (*Oryza sativa*), one of the world's three major grains, serves as the staple food for almost half of the global population, usually in the form of polished rice. Nonetheless, brown rice (BR) is recognized for its nutritional superiority over that of polished rice because BR contains both embryo and bran, which have various nutritional and bioactive compounds, e.g., vitamin Bs (vitamin B_2, niacin, pantothenic acid, vitamin B_6 and biotin), minerals (sodium, calcium, phosphorus, and iron), phytic acid, ferulic acid, γ-oryzanol, fat, dietary fiber, good vegetable proteins, as well as phytosterols. Therefore, many nutritionists recommend consuming BR, rather than polished rice. Although BR has superior nutritional

properties, many people prefer polished rice because of its cooking ease and superior texture. To overcome these problems of BR, rice bran (RB, the outer coating of the rice kernel)-based fermented food (FBRA) was introduced. The FBRA is produced by solid-state fermentation of a mixture of RB (main material) and BR with *Aspergillus oryzae* (*A. oryzae*) and is mainly consumed in Japan and less commonly in Korea, Malaysia, Canada, and the United States. The FBRA has a much better eating-quality than BR and has been reported to have preventive properties in experimental colitis induced by sodium dextran sulfate [12] as well as to increase the number of lactobacilli species in the rat intestine [13]. FBRA also influences the development of acute hepatitis in Long-Evans Cinnamon rats [14], as well as chemical or inflammation-related carcinogenesis in rodents [15], and induces apoptotic death of colorectal tumor HCT 116 cells [16] and human acute lymphoblastic leukemia cells [17]. These beneficial effects along with the biological effects of SGs and ASGs described above have led to the widening consumption of FBRA for people especially outside of Japan with the hope of preventing chronic disease due to the consumption of Western-style diet, characterized by high intake of red meat, animal fat, sweets, and desserts and low intake of fresh fruits and vegetables and low-fat dairy products.

However, the exact mechanisms responsible for these biological actions of FBRA still remain to be solved. Therefore, it is important to examine the chemical profile of various beneficial compounds in FBRA for verification of the wealth of functional ingredients generated by fermentation, and to insure quality control during FBRA preparation for a marketed product.

In our previous studies, it was shown that the amounts of some bioactive and health-enhancing chemicals, such as ferulic acid (which is known as a strong antioxidant), increased as a result of fermentation [18, 19]. We also demonstrated that the content of spermidine (which is known to have a preventive effect in age-related diseases) increased 158% after

fermentation with *A. oryzae*, whereas the contents of analogous polyamines, such as putrescine and spermine, decreased [20].

During the last decade, solid-state fungal fermentation process of RB was extensively studied [21]. The main results achieved concerned the increase of protein content and antioxidant activity. Apart from proteins, fibers and minerals, RB is a good source of lipids, which can reach up to 20% of its weight. Fermentation with *A. oryzae* or *Rizopus oryzae* significantly increased palmitic and linoleic acid contents, causing a decrease in saturated fatty acids and an increase in unsaturated fatty acids [22, 23], thus improving the overall nutritional quality. These findings along with our previous observations led us to wish to be able to measure the changes in the contents of SGs and ASGs by fermentation with *A. oryzae*, because the fermentation has been proven as a very feasible option to enhance nutritional and functional features of RB. We therefore, aimed to analyze the contents of SGs and ASGs in FBRA and RM.

To date, liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) is widely used for the identification and quantification of SGs in foods due to its high sensitivity and selectivity [24-26]. The SGs and ASGs in FBRA have never been isolated and characterized. Here we describe the quantification of glucosidic conjugates of three major sterols (Fig. 1) in FBRA and raw material (RM) by LC/ESI-MS/MS. The changes in the ASG contents after fermentation were also examined by carrying out an alkaline hydrolysis prior to LC/ESI-MS/MS.

2. Experimental

2.1. Materials

Stigmasterol (purity 97.0%), β-sitosterol (purity 97.0%), and campesterol (purity 97.0%) were purchased from Tama Biochemical Co. Ltd. (Tokyo, Japan). Cholesterol (purity 96%) was obtained from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide was obtained from Toronto Research Chemicals (Toronto, Canada). Cadmium carbonate was freshly prepared [27] and dried under vacuum before use. Silica gel plates (Merck F254) and silica gel 60 (spherical, neutral) from Kanto Chemical Co., Inc. (Tokyo) were used for thin layer and column chromatography, respectively. 2-Propanol, methanol and ammonium formate of HPLC grade were purchased from Kanto Chemical Co. Inc. An Oasis® HLB cartridge (adsorbent weight, 60 mg) was provided by Waters Co. (Milford, MS, USA) and was successively conditioned by washing with methanol and H_2O prior to use. All other chemicals and solvents were of analytical grade and obtained from Nacalai Tesque Inc. (Kyoto, Japan). Water from a Millipore water filtration system (Milli Q UV Plus, Darmstadt, Germany) was used to prepare the aqueous solutions described below.

2.2. Apparatus

Melting points (mp) were determined on a micro hot-stage apparatus and are uncorrected. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra of syteryl glucoside tetra-acetates were recorded on a JNM-ECA-500 (LEOL Ltd., Tokyo) at 500 MHz and at 125 MHz with $CDCl_3$, respectively. ^{1}H- and ^{13}C-NMR spectra of syteryl glucosides were recorded on an AMX500 (Bruker Inc., Rheinstetten, Germany) at 500 MHz and 126 MHz with pyridine-D_5 were recorded, respectively; chemical shifts are expressed as δ ppm relative

to tetramethylsilane. The ^{13}C distortionless enhancement by polarization transfer spectra were also measured to determine the ^{1}H signal multiplicity and to differentiate between CH_3, CH_2, CH, and C based on their proton environments. The ^{1}H- and ^{13}C-NMR chemical shift assignments of SGs and their acetates are tabulated in Tables S1 - S4. Electron ionization (EI) low-resolution mass spectra (LR-MS) were obtained using an Auto Spec 3000 mass spectrometer (Micromass UK Limited, Wythenshawe, Manchester, UK). High-resolution (HR)-mass spectrometry measurements were performed on an Orbitrap mass spectrometer (Thermo Scientific Exactive, San Jose, CA, USA). Measurement conditions of ESI-Orbitrap-MS were as follows: ion source voltage, 3 kV; capillary temperature, 300°C; capillary voltage, 27.5 V; tube lens voltage, 110V; skimmer voltage, 45V; sample injection, flow-injection (flow rate of 100 μL/min); solvent, methanol.

2.3. LC/ESI-MS/MS

LC/ESI-MS/MS was performed using a Sciex 4000QTRAP LC/MS/MS System (Sciex, Concord, Ontario, Canada) equipped with an ESI interface and an Agilent 1200 LC-chromatograph (Agilent Technologies, Palo Alto, CA). LC separations were conducted using a reversed-phase semi-micro column, Kinetex XB-C18 (2.6 μm particles, 50 mm × 2.1 mm i.d.) from Phenomenex (Torrance, CA, USA) by an isocratic elution with methanol-2-propanol-H_2O (9:1:1, v/v/v) containing 5 mM ammonium formate at a flow rate of 350 μL/min. The SGs were analyzed in the positive-ion mode. The MS/MS conditions were as follows: ion spray voltage, 4.5 kV; collision energy, 21 V (β-sitosteryl glucoside) or 19 V (stigmasteryl glucoside, campesteryl glucoside, and cholesteryl glucoside [internal standard (I.S.)]); nebulizer gas (N_2), source gas 1, 30 psi, source gas 2, 60 psi and curtain gas setting, 20 psi; ion source temperature, 200°C. The selected reaction monitoring (SRM) transitions (precursor and product ions) were *m/z* 594 → 397 (β-sitosteryl glucoside), *m/z* 592 → 395

(stigmasteryl glucoside), *m/z* 580 → 383 (campesteryl glucoside), and *m/z* 566 → 369 (I.S.).

2.4. Syntheses of authentic steryl glucoside standards and cholesteryl glucoside

2.4.1 General method

SGs and cholesteryl glucoside were synthesized using the Köenigs-Knorr reaction [25].

To a solution of sterol (0.07-0.19 mmol) in benzene (3 - 4 mL) was added cadmium carbonate (0.36 mmol) as catalyst and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.18 mmol), and the mixture was refluxed for 2 h. After filtration of the mixture, the solvent of the filtrate was removed in vacuo and the crude product obtained was purified by column chromatography on a silica gel using *n*-hexane-ethyl acetate (3:1, v/v) or benzene-ethyl acetate (6:1, v/v) as an eluant. This was followed by deacetylation with 2 M KOH in a mixed solvent of methanol (3 mL) and tetrahydrofuran (3 mL) by refluxing for 6 hr. After neutralization with 2 M HCl and dilution with water, the product was extracted with ethyl acetate, washed with saturated solution of NaCl, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product was purified by column chromatography on a silica gel using $CHCl_3$-methanol (7:1, v/v) as an eluant. The structures of the synthesized authentic glucosides were verified by ^{1}H- and ^{13}C-NMR spectra (Supporting Information Tables S1-S4) and HR-MS. The data of the synthesized β-sitosteryl glucoside, stigmasteryl glucoside, and cholesteryl glucoside were in line with those of the literatures [25, 28, 29].

*2.4.2. β-Sitosteryl glucoside (**1**)*

The compound (**1**) was obtained from β-sitosterol as a colorless solid (methanol): yield 35 mg (51%). Mp, 254.1-257.3°C (decomp.); LR-MS: *m/z* 396.4 $[M\text{-glucose}]^+$, 255.2 $[M\text{-glucose-side chain}]^+$, 147.1; HR-MS: *m/z* 599.42859 (found) *m/z* 599.42821 (calcd. for

$C_{35}H_{60}O_6Na$ $[M+Na]^+$)

*2.4.3. Stigamasteryl glucoside (**2**)*

The compound (**2**) was obtained from stigmasterol as a colorless solid (ethanol): yield 20 mg (18%). Mp, 254.1-253.3°C (decomp.); LR-MS: *m/z* 394.4 $[M\text{-glucose}]^+$, 255.2 $[M\text{-glucose-side chain}]^+$; HR-MS: *m/z* 597.41288 (found); *m/z* 597.41256 (calcd. for $C_{35}H_{58}O_6Na$ $[M+Na]^+$)

*2.4.4. Campesteryl glucoside (**3**)*

The compound (**3**) was obtained from campesterol as a colorless solid (ethanol): yield 20 mg (57%). Mp, 261.5°C (decomp.); LR-MS: *m/z* 382.4 $[M\text{-glucose}]^+$, 255.2 $[M\text{-glucose-side chain}]^+$, 147.1; HR-MS: *m/z* 585.41295 (found), *m/z* 585.41256 (calcd. for $C_{34}H_{58}O_6Na$ $[M+Na]^+$).

*2.4.5. Cholesteryl glucoside (**4**)*

The compound (**4**) was obtained from cholesterol as a colorless solid (methanol): yield 45 mg (31.2%). Mp, 261.5°C (decomp.); LR-MS: *m/z* 368.3 $[M\text{-glucose}]^+$, 255.2 $[M\text{-glucose-side chain}]^+$, 147.1; HR-MS: *m/z* 571.39661(found) *m/z* 571.39691 (calcd. for $C_{33}H_{56}O_6Na$ $[M+Na]^+$):

2.5. FBRA and RM samples

The BR was harvested by contracted farmers in Hokkaido, Japan in 2018, and RB was manufactured by a factory in Hokkaido. The FBRA was produced by the fermentation of blended RB and BR with *A. oryzae* in Koken Co. Ltd. (Tobetsu, Hokkaido) according to the established procedure: see reference [20] for further details. The RM sample contained

only the unfermented RB and BR.

2.6. Analysis of SGs and ASGs in FBRA and RM

The sample (FBRA and RM, 10 mg each) was subjected to an extraction in $CHCl_3$-methanol (2:1, v/v) (1 mL) by stirring at ambient temperature (ca. 20°C) for 24 h. After centrifugation (2000 × *g*, 3 min), the supernatant was diluted with methanol (10 mL) and was further diluted to a tenth part with methanol. The resulting diluted solution (named RBRA-extract or RM-extract) was used for analysis of intact SGs and total SGs (intact SGs plus SGs released by alkaline hydrolysis of ASGs) as described below.

2.6.1 Analysis of intact SGs

A 200 μL aliquot of the FBRA-extract or RM-extract (equivalent to FBRA and RM, 20 μg each) was mixed with a 20 μL aliquot of I.S. solution, and the solvent was evaporated to dryness under a gentle stream of N_2 gas at room temperature. The residue was suspended in H_2O, and then applied to an Oasis HLB® cartridge. After washing with H_2O (4 mL), the SGs and I.S. were eluted with methanol (4 mL). After evaporation of the solvents under a gentle stream of N_2 gas at room temperature, the residue was redissolved in 50 μL of mobile phase, and a 1 μL aliquot was injected to the LC/ESI-MS/MS system.

2.6.2 Analysis of total SGs (sum of SGs and ASGs)

A 100 μL aliquot of the FBRA-extract or RM-extract (equivalent to FBRA and RM, 10 μg each) was mixed with a 20 μL aliquot of I.S. solution, and the resulting solution was treated with 1 M KOH in methanol (250 μL) at 37 °C for 1h [29]. After evaporation of methanol, the residue suspended in H_2O was neutralized with 1 M HCl, and then applied to solid phase extraction using an Oasis HLB® cartridge. After the usual work-up described

above, a 1 μL aliquot was injected into the LC/ESI-MS/MS system.

2.7. Method validation

Stock solutions of each SG were prepared using methanol as solvent at the concentration of 100 nmol/mL. Standard solutions were prepared by dilution of the stock solutions with methanol to give concentrations of 1, 2, 5, 10, 20, 50, 100, and 200 pmol/mL. The I.S. solution at a concentration of 100 pmol/mL was prepared gravimetrically in methanol. All the solutions were stored at -4°C or below, and allowed to equilibrate at room temperature for at least 15 min before use. In the calibration study, a 100 μL aliquot of each standard solution was mixed with 20 μL of the I.S. solution; the mixture was evaporated to dryness under a gentle stream of N_2 gas. The residue was redissolved in 100 μL of mobile phase, and 1 μL of this solution was injected into the LC/ESI-MS/MS system. The calibration curves were constructed by plotting the peak area ratio of each SG to I.S. against the amount of SG. As the lower limit of quantification (LLOQ) was defined as the lowest amount of calibration (ICH-guideline) [30], the LLOQ was calculated on the most intense transition, with the criterion of a signal-to noise ratio exceeding 10.

In order to assess matrix effects, FBRA samples were extracted using the assay procedure described above. After extraction, the extracts were spiked with defined amounts of standard solution (400 or 2000 nmol/g samples). Matrix effects were assessed by comparing the peak areas of spiked extract samples to those of samples containing only standard solutions. Matrix effects were defined as $(A\text{-}A_0 / B) \times 100$ (%), where A is the peak area of SGs in the spiked extract sample, A_0 is the peak area of SGs in the unspiked extract sample and B is the peak area of SGs in standard solutions.

Analytical recoveries (%) were obtained with the test samples (n = 5), in which the test samples were prepared as follows; the FBRA extract was diluted to fourfold with methanol,

and then 10, 50, 100 pmol of reference SGs and I.S. (20 pmol) was added to the resulting solution (1 mL). A 100 μL aliquot of test sample was subjected to LC-ESI-MS/MS analysis according to the method described in Section 2.6.2. The amounts of the SGs in the added and nonadded samples were defined as F and F_0, respectively, and the analytical recoveries were calculated as [F / {(F_0+ amount of added SG}] × 100 (%).

3. Results and Discussion

3.1. Authentic SG standards

For the ongoing characterization of SGs, standard reference specimens were a crucial prerequisite. Although all the SGs are commercially available, they are very expensive and campesteryl glucoside is available as a mixture of four SGs. We, therefore, synthesized the selected SGs, β-sitosteyl, stigmasteryl, campesteryl, and cholesteryl glucosides (I. S.) (Fig. 1) using pure sterols as starting materials to secure sufficient amounts of reference SGs for not only analysis in this study but also future experiments on the pharmacological and biological study according to the reported method [25]. These SGs were obtained in a satisfactory yield and good purity by two-step synthesis from free sterols. Common reaction byproducts (orthoester), remaining substrates, and excess reagents were separated by column chromatography using silica gel from the crude mixtures to yield pure peracetylated β-glucosides. The β-glucosidic character of these intermediates was proven by ^{1}H-NMR analysis to give a characteristic doublet for the anomeric proton H-1' (J = 7.6 Hz) (Tables S1-S2). Final deacetylation for the sugar moieties was achieved with methanolic KOH in an almost quantitative manner [29]. In total, three SGs as well as I.S. were synthesized and unequivocally verified by spectroscopic and spectrometric means (NMR, LR- and HR-MS). The NMR data for SGs are given in the Supporting Information (Tables S3 - S4). All the SGs were very unstable under EI-MS; the glucose residue was readily eliminated. The

fragment carbocation derived to scission C9/C11 leading to the formation of fragment ion at *m/z* 147 which may be rationalized by a charge-remote [2+2+2]-cycloreversion reaction of the C-ring, which was also found in the study using ESI [26]. As reported, the formation of the fragment ion at *m/z* 255 was rationalized by elimination of side chain from the fragment carbocation [26].

The formation of major product ions of the SGs under positive-ion ESI-MS/MS on the QTRAP mass spectrometer was also interpreted. The typical collision-induced dissociation spectra of the SGs are shown in Fig. 2. The spectra of all the SGs showed $[M\text{-glucose}]^+$ as the base peak along with a moderate cationized molecules with the ammonium adduct ($[M+NH_4]^+$) and weak protonated molecule ($[M+H]^+$), indicating the covalently condensed product of sterol and glucose.

3.2. LC-ESI-MS/MS analysis of SGs

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetrical peak shapes for the analytes, as well as minimizing the run time. Modifiers such as ammonium acetate and ammonium formate alone or with combination in different concentrations, were added. It was found that an isocratic elution with methanol-2-propanol-H_2O (9:1:1, v/v/v) containing 5 mM ammonium formate could achieve this purpose and it was finally adopted as a mobile phase. The concentration of ammonium formate was optimized to maintain well-resolved peaks while being consistent with good ionization in the mass spectrometer. After careful comparison of several columns, a Kinetex XB-C18 column (2.6 μm, 50 mm x 2.1 mm i.d.) was finally used with a flow rate of 350 μL/min to produce good peak shapes and permit a run time of 3.0 min. Fig. 3A shows typical SRM chromatograms of authentic SGs. All the SGs were well separated.

The calibration curves were constructed with 8 points according to ICH guidelines [30]. Each calibration curve, as determined by linear regression analysis, exhibited good linearity with correlation coefficients of 0.999 within the range of 1- 200 pmol/mL. The slope of the regression lines was reproducible with the relative standard deviation (R.S.D.) values less than 1.4%. The LLOQ of each SG was 1 pmol/mL.

To examine the matrix effects, the post extraction addition experiment was performed. The matrix effects were assessed by comparing the peak areas of spiked FBRA extracts to those of samples containing only standard stock solutions. The relative peak areas of SGs were 96.3 - 105.0% with R.S.D values of less than 6.3% (Table 1). The results showed that no significant ion suppression or enhancement effects were observed.

A solid-phase extraction protocol was used for the extraction of SGs with or without alkaline hydrolysis prior to LC/ESI-MS/MS analysis, as has been used by others for this purpose [24, 25]. The recovery rates through the assay procedure described in Section 2.6.1. were tested by adding known amounts of SGs to FBRA-extract. The analytical recoveries of SGs were 94.6% - 102.4% of the added amounts of their standard samples with R.S.D. value less than 7.8% (Table 2) and the absolute recovery rate of the I.S. from FBRA-extract was 94.4 ± 2.9 % (Mean ± S.D., n=10).

3.3. Application to FBRA and RM samples

The developed method was applied to FBRA and RM samples. Typical SRM chromatograms of a sample obtained from FBRA are shown in Fig. 3B. All SGs were detected and well separated without any interferences. The results of assay are summarized in Table 3. The total and intact glucoside contents in FBRA and RM did not significantly differ from lot to lot, where the quantitatively most important SG was β-sitosteryl glucoside followed by stigmasteryl glucoside and campesteryl glucoside. The overall composition in

FBRA, with 74.8% β-sitosteryl glucoside, 15.0% stigmasteryl glucoside, 10.2% campesteryl glucoside, were comparable with 76%, 15.2%, and 8.8% for those in RM. Particular interest is the observation of the decreased SG contents in FBRA compared to RM (Fig. 4A). In contrast to SGs, the calculated amounts of ASGs (total SGs minus intact SGs) in FBRA were significantly higher than those of RM (Fig. 4B), suggesting the acylation of the 6'-hydroxy group of glucose core in SGs with various molecular species of fatty acids during fermentation. Thus, the contained amounts of SGs and ASGs were symmetrically changed by the fermentation, and the total contents of glucosides (sum of SGs and ASGs) were not significantly different between FBRA and RM. These results suggested that acylation of SGs occurred during the fermentation by acyltransferase excreted from *A. oryzae*.

It has been reported that ASGs lower cholesterol absorption in mice [7], and reduce blood cholesterol levels and the risk of arteriosclerosis in obese Japanese men [8] and post-menopausal Vietnamese women [9]. Furthermore, it has been confirmed that the molecular species of ASG isolated from pre-germinated BR stimulate Na^+/K^+ ATPase activity, which is an important contributing role in the anti-diabetes, and the SGs liberated by alkaline hydrolysis of ASGs lose this enzyme activator activity [4]. It has also shown that ASGs may affect pancreatic β-cells through the activation of an insulin-like growth factor 1-dependent mechanism, together with the activation of homocysteine-thiolactonase activity [6]. These findings suggest that the FBRA might have much more cholesterol lowering effect and stronger anti-diabetic properties than the RM (RB) because the ASG contents are enriched by the fermentation with *A. oryzae*.

4. Conclusions

In this study, we determined three major SGs in FBRA and RM by the newly developed LC/ESI-MS/MS method with the aid of synthesized standard samples. The contents of

ASGs were also determined using an alkaline hydrolysis step. The results showed that the quantitatively most important SG was β-sitosteryl glucoside followed by stigmasteryl glucoside and campesteryl glucoside. Decreases of SGs and increases of ASGs after fermentation of RB-based material with *A. oryzae* were observed. ASGs have been reported to have more potent cholesterol-lowering and antidiabetic effects than SGs [6, 8, 9]. The results obtained in this study indicate that the fermentation of RB with *A. oryzae* is an approach to enrich the ASG contents, therefore, the FBRA might be a new functional food for prevention of lifestyle-related diseases. Further studies to clarify an unknown peak appeared in SRM chromatogram (*m/z* 592 → 395), which could be one of monosaccharide-conjugated phytosterols, will be conducted in a near future.

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Abbreviations

SGs, steryl glucosides; ASGs, acylated steryl glucosides; RB, rice bran; FBRA, rice bran-based fermented food; BR, brown rice; *A. oryzae*, *Aspergillus oryzae*; LC/ESI-MS/MS, liquid chromatography/electrospray ionization-tandem mass spectrometry; mp, melting points; ^{1}H-NMR, proton nuclear magnetic resonance; SRM, selected reaction monitoring; RM, raw material; EI, electron ionization; LR-MS, low-resolution mass spectra

Conflict of interest

The authors declare no conflict of interest.

Authors' contribution

SI conceived the project and supervised research work, and manuscript preparation. TM, MI, YH carried out experimental work, data collection, evaluation. SJ performed NMR studies. TH helped in study design and drafted the final version. All authors read and approved the final manuscript for publication.

Supporting Information for Manuscript:

Analysis of steryl glucosides in rice bran-based fermented food by LC/ESI-MS/MS

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Supplementary data for ^{1}H and ^{13}C chemical shifts of glucosides are available as Table S1, S2, S3 and S4 with free of charge on the Web.

Figure legends

Figure 1. Chemical structures of β-sitosteryl glucoside (1), stigmasteryl glucoside (2), campesteryl glucoside (3), and cholesteryl glucoside (4, I.S.).

Figure 2. Product ion spectra obtained by CID of the $[M+NH_4]^+$ of β-sitosteryl glucoside (1), stigmasteryl glucoside (2), campesteryl glucoside (3), and cholesteryl glucoside (4, I.S.).

Figure 3. Typical SRM chromatograms of standard SGs (A) and SGs in FBRA (B).

Figure 4. Changes in the contents of SGs (A) and ASGs (B) in RM and FBRA. Bars represent the mean of 5 lots. A paired t-test was used for statistical significance.

Highlights

- Four steryl glucosides (SGs) were synthesized using Köenigs-Knorr reaction.
- An LC/ESI-MS/MS method was developed for determination of SGs.
- SGs in rice bran-based fermented food were determined by the developed method.
- Fermentation brought the decrease of steryl glucoside contents.
- Fermentation brought the increase of acylated steryl glucoside contents.

Table 1

Matrix effects for SGs and I.S.

Glucoside	Content nmol/g	Relative Peak Area (%, Mean ± S.D., n = 6)
β-Sitosteryl glucoside	400	96.3 ± 3.2 (3.3)
	2000	98.4 ± 4.6 (4.7)
Stigmasteryl glucoside	400	98.8 ± 6.3 (6.4)
	2000	102.5 ± 4.3 (4.2)
Campesteryl glucoside	400	104.6 ± 6.1 (5.8)
	2000	105.0 ± 3.2 (3.0)
Cholesteryl glucoside (I.S.)	800	101.9 ± 4.0 (3.9)

S.D., standard deviation. Values in parentheses represent R.S.D. (%)

Table 2

Analytical recoveries of SGs from FBRA-extracts

	Recovery (%, Mean ± S.D., n = 5)		
	Content 400 nmol/g	Content 2000 nmol/g	Content 4000 nmol/g
β-Sitosteryl glucoside			

Intra-assay	97.1 ± 7.6 (7.8)	97.7 ± 5.7 (5.8)	97.5 ± 3.3 (3.4)
Inter-assay	95.7 ± 1.8 (1.9)	97.9 ± 2.5 (2.6)	98.6 ± 4.6 (4.7)
Stigmasteryl glucoside			
Intra-assay	96.6 ± 3.8 (3.9)	96.4 ± 4.7 (4.9)	101.1 ± 3.3 (3.3)
Inter-assay	94.6 ± 1.6 (1.7)	97.3 ± 0.5 (0.5)	102.4 ± 3.9 (3.8)
Campesteryl glucoside			
Intra-assay	99.0 ± 5.1 (5.2)	98.4 ± 4.3 (4.4)	101.3 ± 3.9 (3.8)
Inter-assay	95.0 ± 2.4 (2.5)	97.8 ± 0.7 (0.7)	101.0 ± 1.0 (1.0)

S.D., standard deviation. Values in parentheses represent R.S.D. (%).

Table 3

Contents (nmol/g) of SGs and ASGs in FBRA and RM

Lot		β-Sitosteryl			Stigmasteryl		
		Glucoside		Acylated	Glucoside		Acylated
		A	B	Glucoside	A	B	Glucoside
1	FBRA	660.1	250.3	409.8	128.7	51.2	77.5
	RM	829.7	564.6	265.1	158.4	105.9	52.5
2	FBRA	650.0	266.2	383.8	131.2	50.0	81.2
	RM	555.5	343.2	212.3	113.6	70.2	43.4
3	FBRA	643.1	243.0	400.1	123.6	55.1	68.5

	RM	691.4	383.7	307.7	146.9	83.3	63.6
4	FBRA	759.0	319.7	439.3	144.0	61.7	82.3
	RM	717.1	421.4	295.7	137.4	81.5	55.9
5	FBRA	802.7	292.0	510.7	146.6	58.1	88.5
	RM	719.6	408.1	311.5	142.8	79.9	62.9
	FBRA	703.0 ± 65.3	274.2 ± 28.3	428.7 ± 50.1	134.8 ± 8.9	55.2 ± 4.3	79.6 ± 7.4
Mean ± SD							
	RM	702.7 ± 87.7	424.2 ± 75.1	278.5 ± 41.2	139.8 ± 14.8	84.2 ± 11.8	55.7 ± 8.3

A, Content of total SG; B, Content of intact SG; Content of ASG = A – B.

Fig. 1.

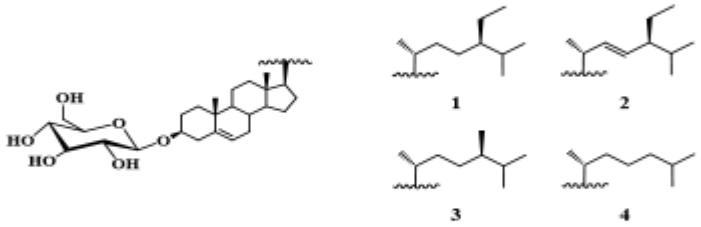

Fig. 2

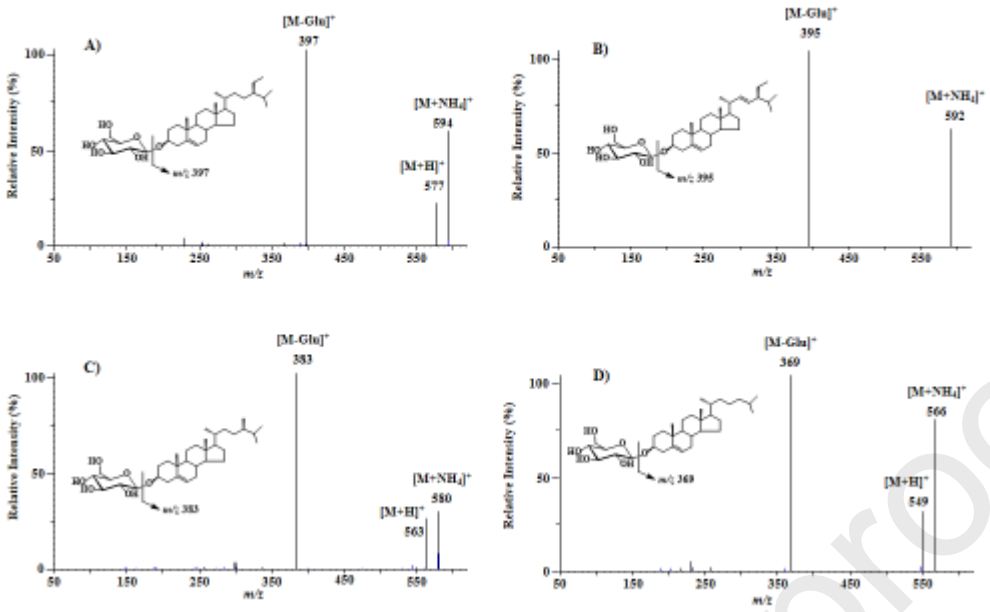

Fig. 3

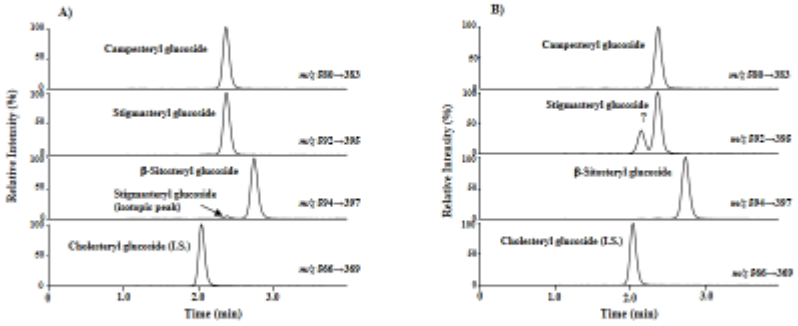

Fig. 4