Fatty Acid Esters of Sitosterol 3 β -Glucoside from Soybeans and Tempe (Fermented Soybeans) as Antiproliferative Substances

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Summary In the present study we isolated and identified two mixtures of fatty acid esters of β -sitosterol glucoside from soybeans and its fermented product, tempe. The mixtures of glucolipids occurring in soybeans and tempe are unseparable and possess the following structures: sitosterol-3-O- β -D-(6'-O-palmitoyl/-linoleoyl)- and sitosterol-3-O- β -D-(6'-O-palmitoyl/-stearoyl)-glucopyranoside, respectively. Since tempe constituents show antitumor activity, we tested these glucolipids against mouse myeloma cells (tumor cells). The tempe glucolipid mixture showed higher inhibition (95.3%) at the 50 μ g/ml concentration than the soybean one. The maximum activity of the soybean glucolipid mixture was (96%) at 100 μ g/ml.

Key Words: fermented soybean, sitosterol, sitosterol glucoside, sitosterol-3-O- β -D-(6'-O-palmitoyl)-glucopyranoside, sitosterol-3-O- β -D-(6'-O-stearoyl)-glucopyranoside, sitosterol-3-O- β -D-(6'-O-linoleoyl)-glucopyranoside, mouse myeloma cells X63-Ag8.653, antitumor activity

Apart from being a very nutritious food, soybeans (*Glycine max*) contain many secondary metabolites of significant physiological importance [1–5]. Some of these metabolites are biotransformed by treating soybeans with the mycelium of *Rhizopus oligosporus* for 36 h at 30–35°C [6, 7]. This fermented product constitutes a very popular food in southeast Asia, specially Indonesia, and is known as tempe kedele [8]. In an exhaustive study to investigate the physiological activity of the secondary metabolites of tempe they were isolated, purified and analyzed with the help of spectroscopic methods to elucidate their chemical structures. Thereafter

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they were used for different tests to evaluate their biological activity.

MATERIALS AND METHODS

Plant material. Soybeans of Canadian origin were procured from a local market. In a joint research project between the Federal Republic of Germany and the Indonesian Republic tempe was obtained from Jakarta, Indonesia, and quickly transported to Germany at 0°C.

Chemicals. β -Sitosterol glucoside (SiMG) was isolated from soybeans. Sitosterol and dimethyl sulfoxide (DMSO) were from Merck, Darmstadt, Germany. Linoleic and palmitic acids were from Sigma, Munich, Germany. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) for cell culture were from Biochrom KG, Berlin, Germany.

TLC analysis. TLC was carried out on a silica gel 60 F₂₅₄ plate (Merck, Darmstadt, Germany) with cyclohexane-CH₂Cl₂-formic acid-methyl formate (35:30:30:5, v/v/v/v) used as solvent. The spots on TLC plates were visualized by heating at 120°C for 2 min after spraying with 10% H₂SO₄.

¹H-NMR. Spectra were recorded on a Bruker AMX 500 X32 data calculator (CD₃Cl 500 MHz).

GC/MS measurements. Finnigan/Mat 1020 B gas chromatograph; column length: 30 m×0.32 mm; temp. program: 150°C for 2 min and then raised to 250°C at a rate of 5°C/min; carrier gas: helium; flow rate: 6 cm/s; injection type: split 1:20. FAB-MS were recorded with a VG Analytical ZAB HF mass spectrometer with reverse geometry, equipped with a FAB-Gun.

Cell culture. Mouse myeloma cells X63-Ag8.653 were cultured in DMEM supplemented with 10% heat-inactivated FCS in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells were seeded at 2×10⁵ cells/ml medium in 6 cm diameter plastic culture dishes. The final volume in the culture dishes was 5 ml. The isolated compounds in different concentrations of 10, 50, and 100 µg/ml (from a 0.1 M stock solution in DMSO) were added to the culture dishes. DMSO at the same dilution was added to parallel cultures as the control. Cells were incubated for 6 days and were counted with a hemocytometer after trypan blue staining for viability testing. Inhibition of cell growth was calculated according to the following formula:

$$\% \text{ Inhibition} = 100 \times \left(1 - \frac{\text{treated viable cells}}{\text{DMSO control viable cells}} \right).$$

RESULTS

Isolation and structure elucidation of the glucolipids

From soybeans: Soybean seeds (1 kg) were powdered and extracted with 4 liters of MeOH. After vacuum evaporation of the filtrate, the residue (70 g) was

dissolved in 200 ml MeOH and adsorbed on 200 g of silica gel by evaporating the solution under reduced pressure. The dry mass was chromatographed on a silica gel column (8 $\times$ 20 cm; CHCl₃) with a gradient system of CHCl₃/MeOH (7 liters), CHCl₃/MeOH/H₂O (2 liters) to give 48 fractions (each 150 ml). A TLC investigation revealed that the fractions 11–30 contained a spot that showed a violet color on spraying with 10% H₂SO₄ and subsequent heating (120°C).

These fractions were evaporated together, and the residue (620 mg) was chromatographed again on silica gel (column 3 $\times$ 40 cm) with CH₂Cl₂ with gradually increasing amounts of MeOH used as the eluent. The aforesaid substances were eluted with CH₂Cl₂/MeOH (100 : 60, v/v), and the solvent was evaporated to dryness to furnish a brown gummy mass (30 mg). Several attempts at crystallization of the substance remained unsuccessful; so 15 mg of it was acetylated with Ac₂O-pyridine for 24 h at room temperature. The acetate (18 mg) was recrystallized thrice from MeOH and was used for spectroscopic investigations.

From tempe: Fresh tempe (1.55 kg) was extracted with 4 liters of methanol. Evaporation of the solvent under vacuum yielded 178 g of extract. This was dissolved in 300 ml of methanol and adsorbed onto 500 g of silica gel by evaporating the solution under reduced pressure. The dry mass was fractionated on a silica gel column (8 $\times$ 20 cm) by use of the same gradient system as employed for the soybean extract, and 65 fractions (each 100 ml) were collected.

Fractions 5–11 showed, after TLC investigation and visualizing the spots with 10% H₂SO₄, one violet spot with the same *R_f* value as obtained in the case of soybean. These fractions were evaporated, and the residue was rechromatographed as described above. The purified substance (38 mg) did not crystallize either; and so a part of it (20 mg) was acetylated with acetic anhydride in pyridine. After recrystallization from methanol the acetate (24 mg) was examined by spectroscopic methods.

After ¹H-NMR, FAB-MS, and GC/MS investigation the soybean glucolipids were determined as a mixture of sitosterol-3-O- β -D-(6'-O-palmitoyl)-glucopyranoside and sitosterol-3-O- β -D-(6'-O-linoleoyl)-glucopyranoside and the structure of the tempe glucolipid as a mixture of sitosterol-3-O- β -D-(6'-O-palmitoyl)-gluco-

R = palmitoyl; stearoyl; linoleoyl

Fig. 1. Structure of the fatty acid ester of sitosterol 3 β -glucoside.

pyranoside and sitosterol-3-O- β -D-(6'-O-stearoyl)-glucopyranoside.

The $^1\text{H-NMR}$ spectra of the two acetates showed three signals at δ_{H} 2.00, 2.01, and 2.04 ppm which were assigned to the three acetyl groups of the glucose moiety. Other peaks at δ_{H} 5.20 (H, t, 3'-H), 5.03 (H, dt, 4'-H), 4.96 (H, dd, 2'-H), 4.23 (H, m, 6'_{1-H}), 4.12 (H, m, 6'_{2-H}), and 3.68 (H, m, 5'-H) were also assigned to the glucose. The anomeric proton of the glucose (1'-H) was present as a doublet at δ_{H} 4.58 with a $J_{1,2}=8.4$ Hz due to axial-axial coupling, thus showing that the glucose was β -linked to the aglycone.

The occurrence of only three acetyl signals and the position of the 6'-H protons in the $^1\text{H-NMR}$ spectrum showed that the 6'-O-position of the glucose was differently occupied. The peak at δ_{H} 5.32 (H, m) and 3.46 (H, m) could be assigned to the steroidal 3-H and 6-H, respectively.

Several signals ($-\text{CH}_2-$, $-\text{CH}_3$ protons) in the aliphatic area of the $^1\text{H-NMR}$ spectrum (δ_{H} 0.75–1.6 ppm) and a peak at δ_{H} 2.31 (2H, t), which is characteristic of a methylene attached to carbonyl group, supported the hypothesis of the occurrence of a fatty acid group in the 6'-O-position of the glucose.

The FAB-MS spectrum of the acetate of the soybean glucolipid showed molecular peaks $[\text{M}+\text{Na}]^+$ of 964 and 988. The base peak at m/z 397 $[\text{C}_{29}\text{H}_{49}]^+$ suggested the aglycone part of the mixture as sitosterol. However, two peaks appearing at m/z 527 $[\text{C}_{28}\text{H}_{47}\text{O}_9]^+$ and 551 $[\text{C}_{30}\text{H}_{47}\text{O}_9]^+$ were derived from the loss of the aglycone molecule $[\text{M}-23-\text{M}_{\text{Ag1}}(414)]^+$ and indicated the acylated glucose moiety of the soybean glucolipid mixture.

The FAB-MS spectrum of the acetate from tempe glucolipid showed molecular peaks $[\text{M}+\text{Na}]^+$ of 964 and 992, m/z 397 $[\text{C}_{29}\text{H}_{49}]^+$, m/z 527 $[\text{C}_{28}\text{H}_{47}\text{O}_9]^+$, and 555 $[\text{C}_{30}\text{H}_{51}\text{O}_9]^+$. The latter two indicated the acylated glucose moiety of the glucolipid mixture.

The acetylated compounds (3 mg) were transesterified with 2 ml of 1 M methanolic sodium methoxide for 24 h at room temperature. After acidification with dil. HCl and evaporation of the MeOH and subsequent addition of 2 ml of H_2O , the methyl esters were extracted with *n*-hexane (2×5 ml). The hexane layer was dried over anhydrous sodium sulphate, and the solvent removed by evaporation.

Palmitic acid and linoleic acid methylesters of the soybean glucolipid and palmitic acid and stearic acid methylesters of the tempe glucolipid were identified by capillary gas chromatography and GC/MS by comparison with authentic samples (Figs. 2, 3).

The steroidglycoside obtained from the above hydrolysis was identified as sitosterol 3 β -glucose ($^1\text{H-NMR}$, TLC comparison with authentic samples as well as by acid hydrolysis to sitosterol).

Effect of the glucolipid mixtures, sitosterol, sitosterol glucoside (SiMG) and the free palmitic and linoleic acid on growth of X63-Ag8.653 mouse myeloma cells

Figures 4, 5, and 6 show the growth curves of the cells incubated with various

Fig. 2. GC (top) and MS (middle, bottom) charts of the fatty acid methyl esters from soybean glucolipids; 1=methyl palmitate, 2=methyl linoleate.

Fig. 3. GC (top) and MS (middle, bottom) charts of the fatty acid methyl esters from tempe glucolipids; 1=methyl stearate, 2=methyl palmitate.

concentrations of the two glucolipid mixtures, sitosterol glucoside, sitosterol, and the free palmitic and linoleic acids. A dose-dependent inhibition of growth was observed between 10 and 100 $\mu\text{g}/\text{ml}$ concentration. On day 6 the number of viable cells was determined with a hemocytometer after trypan blue staining. The data for the inhibition of cell growth have been calculated with the mean values of 5 repetitions for each concentration and in case of each substance. In a duplicate experiment only a difference of less than 1% in the values was observed.

Fig. 4. Effect of increasing doses of soybean and tempe glucolipid mixtures on the proliferation of the myeloma cells. □, tempe-glucolipid; ▨, soybean-glucolipid.

Fig. 5. Effect of increasing doses of sitosterol and sitosterol glucoside (SiMG) on the proliferation of the myeloma cells. □, SiMG; ▨, sitosterol.

Fig. 6. Effect of increasing dose of palmitic and linoleic acid on the proliferation of the myeloma cells. □, linoleic acid; ▨, palmitic acid.

DISCUSSION

The glucolipids from soybeans and tempe inhibit the growth of myeloma cells. Of both of them the tempe glucoside mixture, sitosterol-3-O- β -D-(6'-O-palmitoyl/stearoyl)-glucopyranoside, was more active, showing 95.3% inhibition at 50 μ g/ml concentration. At a higher concentration (100 μ g/ml) the activity might be reduced due to precipitation of the glucolipid as fine oily particles on the upper layer of the medium and thereby not miscible with the medium. The solubility of the soybean glucolipid mixture, sitosterol-3-O- β -D-(6'-O-palmitoyl/linoleoyl)-glucopyranoside, was much better; and the mixture showed its maximum activity at the higher concentration of 100 μ g/ml (Fig. 4).

In order to know which part of the glucolipid is essential for the activity we also tested sitosterol and sitosterol glucoside. As representatives of a saturated and unsaturated fatty acid compound, palmitic and linoleic acid, respectively, were included in this test.

Figure 5 reveals that sitosterol aglycone is not a good inhibitor of the tumor cell growth in our system. The activity increased after glucosylation of sitosterol, with the maximum at 50 μ g/ml. The esterification of the 6'-hydroxyl group of glucose enhanced the activity at higher concentration, although the activity remained the same as that of the glucoside at 50 μ g/ml. The palmitic and linoleic acid alone showed very high activity already at the 50 μ g/ml concentration (Fig. 6). Although the higher inhibition in the case of linoleic acid indicates a beneficial effect of the unsaturated fatty acid for human health, at this concentration the untoward taste and the toxicity towards normal cells preclude the use of these substances as anticarcino agents. In the acyl glucoside of sterol the fatty acids are incorporated in the compound which are released subsequently by enzymatic hydrolysis to be available to the organism. Moreover the moieties like sitosterol glucoside and sitosterol, besides showing antitumor activities, are of other biological value [5, 9-11].

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