Metabolism of Soyasaponin I by Human Intestinal Microflora and Its Estrogenic and Cytotoxic Effects

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Abstract – Metabolites of Soyasaponin I, a major constituent of soybean, by human intestinal microflora were investigated by LC-MS/MS analysis. We found four peaks, one parental constituent and three metabolites: m/z 941 $[M-H]^-$, m/z 795 $[M-rha-H]^-$, m/z 441 $[aglycone-H_2O+H]^+$, and m/z 633 $[M-rha-gal-H]^-$, which was an unknown metabolite, soyasapogenol B 3- β -D-glucuronide. When soyasaponin I was incubated with the human fecal microbial fraction from ten individuals for 48 h, soyasaponin I was metabolized to soyasapogenol B via soyasaponin III and soyasapogenol B 3- β -D-glucuronide or via soyasaponin III alone. Both soyasaponin I and its metabolite soyasapogenol B exhibited estrogenic activity. Soyasaponin I increased the proliferation, mRNA expression of c-fos and pS2, in MCF7 cells more potently than soyasapogenol B. However, soyasapogenol B showed potent cytotoxicity against A549, MCF7, HeLa and HepG2 cells, while soyasaponin I did not. The cytotoxicity of soyasapogenol B may prevent its estrogenic effect from increasing dose-dependently. These findings suggest that orally administered soyasaponin I may be metabolized to soyasapogenol B by intestinal microflora and that soyasapogenol B may express a cytotoxic effect rather than an estrogenic effect.

Keywords: Soyasaponin I, Metabolism, Phytoestrogen, Cytotoxicity, Soyasapogenol B

INTRODUCTION

Most herbs are orally administered to human. Their components are therefore inevitably brought into contact with intestinal microflora in the alimentary tract. Most of components may be transformed by the intestinal bacteria before absorption from the gastrointestinal tract. Studies on the metabolism of the components by human intestinal microflora are of a great importance to an understanding of their biological effects (Kobashi and Akao, 1997; Kim, 2002).

Soy contains numerous phytochemicals including isoflavones, phytic acid, phytosterols, and saponins (Okubo, 1992; Kim *et al.*, 2006). Saponins are a family of steroid or triterpenoid glycosides found in a wide variety of plants (Oakenfull, 1981; Price *et al.*, 1987). Soyasaponins are triterpenoid glycosides with one or two polysaccharide chains. The soyasaponins are mainly present in legumes, and significant amounts of soyasaponins are found in soybeans

and soy products (John *et al.*, 2004). The potential relationship of soyasaponins to health effects has been suggested (Cheeke, 1976; Potter *et al.*, 1979; Messina *et al.*, 1994; Messina, 1995; Joel and Zhang, 2008). Indeed, soyasaponins have anti-carcinogenic, hepatoprotective and antiviral activities, and soyasapogenol N exhibits antigenotoxic, hepatoprotective, and cytotoxic activities. The pharmacological effects of these soyasaponin, or triterpenoid glycoside, constituents of natural products may depend on their metabolism by intestinal bacteria (Kobashi and Akao, 1997; Kim, 2002). Hu *et al.* (2004) reported that human fecal microflora potentially hydrolyzed soyasaponin I via soyasaponin III to soyasapogenol B. However, they did not find other metabolite(s). In addition, Rowlands *et al.* reported that soyasapogenol B exhibits estrogenic effects, but they did not compare the estrogenic effect of soyasaponin I with that of soyasapogenol B (Rowlands *et al.*, 2002).

In order to understand the relationship between the intestinal bacterial metabolism and the pharmacological effect of soyasaponin I, we investigated the metabolic pathway of soyasaponin I by human intestinal microflora and its estro-

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genic and cytotoxic effects against some tumor cells.

MATERIALS AND METHODS

Materials

Sulforhodamine B (SRB), NP40, 17 β -estradiol, Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS) and charcoal dextran stripped FBS (CD-FBS) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). The general anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). The tryptic soy broth (TS) and other media were purchased from Difco Co. (Livonia, MI, USA). All other chemicals were of analytical reagent grade, and all solutions used after redistillation.

Isolation of soyasaponin I and its metabolite soyasapogenol B by human intestinal microflora

Fresh human feces (5 g) were suspended in 100 ml of anaerobic dilution medium (Bae *et al.*, 2003), centrifuged at 500 $\times$ g for 10 min, the resulting supernatant centrifuged at 10,000 $\times$ g for 30 min, and then washed twice with anaerobic dilution medium. The resulting precipitate was suspended in 100 ml of 20 mM phosphate buffer (pH 7.0), containing 50 mg of soyasaponin I (purity, >95%), incubated for 24 h at 37°C, and extracted with n-butanol. The n-butanol fraction was subjected to chromatography on a MPLC [column, Silica gel 100 C18-reverse phase (Fluka Co., USA); elution solvent, a linear-gradient applied by 10% CH₃CN in H₂O to 70% CH₃CN in H₂O; a flow rate, 4 ml/min over 4 h], to isolate soyasapogenol B (12 mg; purity, >95%). The isolated soyasaponin I and soyasapogenol B were identified by comparison to authentic standards by instrumental (FAB-MS, ¹H-NMR, ¹³C-NMR) analysis with that of the previously reported literatures (Kitagawa *et al.*, 1988; Kudou *et al.*, 1992, 1993).

The purities of the isolated soyasaponins were assayed by HPLC [Hitachi HPLC system: column, Lichrosorb C₁₈ (25 $\times$ 0.4 cm, 5 mm, Merck Co.); eluted with mixtures of solvent (methanol/MeOH=30:70); Detector, ELSD (Altech Co., USA).

Assay of metabolic activity of soyasaponin I by human intestinal bacteria

The reaction mixture containing 5 ml of 1 mM soyasaponin I, 2.5 ml of the above fecal microflora suspension (0.5 g wet weight/ml) and 2.5 ml of GAM (Bae *et al.*, 2003) was anaerobically incubated for 6 h at 37°C. The reaction mixture (1 ml) was taken out, adjusted to pH 2 with 0.1 N HCl, extracted with EtOAc, evaporated and assayed by LC-

MS/MS.

The supernatant of the reaction mixture was deproteinized with CH₃CN/MeOH. For the analysis of soyasaponin I metabolites, an Agilent G6410 Triple Quadrupole Mass Spectrometer equipped from Agilent Technologies, Inc. (USA) with an automatic sample injector was used. Soyasaponin I and its metabolites were analyzed on a column of ZORBAX XDB-C18 column (Rapid Resolution HT 2.1 $\times$ 50 mm, i.d., 1.8 μ m, Agilent) using the mobile phase of 0.1% aqueous formic acid and acetonitrile at a flow rate of 0.3 ml/min. The column was maintained at a temperature of 40°C during HPLC analysis and the injection volume was set to 5 μ l. MS experiments were performed on Micromass Quattro API. The mass spectrometer was operated in electrospray positive and negative ionization modes (cone voltage: 19 V). Multiple reaction monitoring (MRM) data acquisition for each transitions mass of soyasaponin I and its metabolites was achieved with a collision energy of 15 eV and a dwell time of 0.50 s. The ESI/MS source was set as follows: capillary temperature 350°C; spray voltage 5 kV; capillary voltage 4 kV; gas flow rate 10 L/min. The flow rate of the nebulizer gas (nitrogen) was 5 L/min. Spectra were acquired in negative and positive-ion modes.

Culture of MCF7 cells and E-screen assay

MCF7 cells were maintained in DMEM containing 10% FBS. Cells were grown at 37°C in a humidified 95% air and 5% CO₂ atmosphere. The cells were washed with phosphate-buffered saline (PBS), and then cultured in phenol red-free DMEM, with 10% CD-FBS, for 2 days to eliminate any estrogenic source prior to treatment. The cultured cells (5 $\times$ 10³ cells/well) were seeded in culture flasks, with the medium exchanged 24 h later. The fresh phenol red-free medium contained 5% CD-FBS and the indicated test compounds. After a further 96 h, the phenol red-free medium was exchanged again, and the cells harvested after 144 h (Park *et al.*, 2006; Shin *et al.*, 2006). The viable cell density numbers were determined using the SRB assay (Voigt, 2005).

In vitro cytotoxicity assay

The cytotoxicity of test agents for HepG2, A549, HeLa and MCF7 cells (Korea Cell Line Bank, Seoul, Korea) was measured by SRB assay (Voigt, 2005). Cells cultured in RPMI medium were harvested, counted, inoculated (160 μ l volume, 1 $\times$ 10⁴ cells/well) into 96-well microtiter plates, cultured for 24 h in media with bovine serum and then treated with the samples. Cells were exposed to test agents (soyasaponin I and Soaysapogenol B) for 2 days at 37°C. Then cells were fixed by addition of 50 μ l of cold 40% TCA (4°C)

Fig. 1. LC-MS/MS chromatogram of metabolites of soyasaponin I by human intestinal microflora. Soyasaponin I was anaerobically incubated with human fecal suspension for 48 h at 37°C, extracted with butanol, and analyzed by LC-MS/MS. (A) Negative and positive electrospray ion currents of soyasaponin I metabolites of eight subjects before (b) and after (b) incubation with human intestinal microflora. (B) MS spectrum of four main peaks of the incubation mixture: (a) soyasaponin I (p, m/z 941.3 $[M-H]^-$), (b) m1 (m/z 795.3 $[M-rhm-H]^-$), (c) m2 (m/z 633.2 $[M-rhm-gal-H]^-$), (d) m3 (m/z 441.2 $[aglycone-H_2O+H]^+$).

Fig. 2. Negative and positive electrospray ion currents of soyasaponin I metabolites by fecal microflora of ten subjects. The fecal suspension of each subject was incubated with soyasaponin I at 37°C for 48 h. The reaction mixture was periodically [1 h (upper), 24 h (middle) and 48 h (down) after incubation] extracted with n-butanol.

into the growth medium for 1 h and washed with distilled water 5 times. Plates were allowed to dry in air then 100 μ l of 4% (w/v) SRB dissolved in 1% acetic acid in water was added to each well and allowed to stand for 30 min and washed four times with 1% acetic acid. The plate was air-dried and 150 μ l of 10 mM aqueous Tris base was dissolved, and the optical density was read at 488/540 nm in a microplate reader (Bio, Tek instrument Inc., USA).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

MCF-7 cells were cultured with phenol red-free DMEM containing 10% CD-FBS. After 48 h, the cells were treated with each agent or 17 β -estradiol. The treated cells were washed in PBS, and the total RNA isolated using an RNeasy[®] Mini Kit (Qiagen, USA), according to the manufacturer's instruction (Park *et al.*, 2006; Shin *et al.*, 2006). To synthesize cDNA, AccuPower RT-PCR Premix (Bioneer, Korea) was used. The sequences of the c-fos primers were 5'-TCCCAGAGGAGATGTCTGTG-3' and 5'-GGCTCCAGC TCTGTGACCAT-3', and those of GAPDH were 5'-GCCA CAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTTG CTGTA-3'. The PCR products for c-fos and GAPDH were 330 bp and 452 bp, respectively. The reactions were initiated by 5 min of denaturation at 94°C, followed by amplification at 94°C for 45 s and 55°C for 45 s; with 30 cycles for c-fos and 32 for GAPDH. The PCR reactions were terminated by elongation at 72°C for 5 min. The PCR products were analyzed using 2% agarose gel electrophoresis.

Statistical analysis

All data are expressed as the mean $\pm$ standard deviation, with statistical significance analyzed using one-way ANOVA followed by a Student-Newman-Keuls test ($p < 0.05$).

RESULTS

Metabolism of soyasaponin I

To investigate the metabolism of soyasaponin I by human intestinal microflora, soyasaponin I was incubated with human fecal suspensions for 48 h and the metabolites were investigated by LC-MS/MS analysis (Fig. 1). We found four peaks: one parental constituent and three metabolites. The parental constituent soyasaponin I showed a peak at m/z 941 [M-H]⁻ ion. Of these metabolites, M1 and M3 were soyasaponin III and soyasapogenol B, respectively, as previously reported (Hu *et al.*, 2004). The scanned spectra showed peaks at m/z 795 [M-rha-H]⁻ ion and m/z 441 [aglycone-H₂O + H]⁺ ion. ESI spectra of these metabolites were in agreement with previous data. The MS spectral data

of M2 showed a peak at m/z 633 [M-rha-gal-H]⁻, which matched that of soyasapogenol B 3- β -D-glucuronide.

To understand the metabolic pathway of soyasaponin I, we incubated soyasaponin I with the human fecal microbial fraction of ten individuals for 48 h incubation and periodically measured its metabolites (Fig. 2). All ten human fecal specimens showed soyasaponin I hydrolyzing activity. However, its activity not only significantly varied between individuals, but its metabolic pathway was also different between individuals.

Estrogenic and cytotoxic effects of soyasaponin I and soyasapogenol B

The estrogenic activity of soyasaponin I and its metabolite soyasapogenol B was investigated (Fig. 3). These constituents exhibited estrogenic activity. At 0.1 μ M, soyasaponin I and soyasapogenol B increased the proliferation of MCF7 cells by 1.3-fold, and 1.2-fold, respectively. However, at more than 2 μ M, soyasapogenol B showed cytotoxicity against MCF7 cells, although soyasaponin I dose-dependently increased their proliferation.

Fig. 3. Estrogenic effect of soyasaponin I (SG), soyasapogenol B (SA) and 17 β -estradiol (ED) in MCF7 cells. (A) E-screen assay. Samples were dissolved in 0.2% DMSO and treated with 0.1 μ M, 1 μ M or 10 μ M. (B) Effect on c-fos and pS2 mRNA expression. MCF7 cells were treated with vehicle alone and 1 μ M SG (circle), SA (square) or ED (triangle). Total RNA, prepared from each treatment, was analyzed for mRNA levels of c-fos and pS2 by RT-PCR.

To evaluate the potential of soyasaponin I and soyasapogenol B as activators of estrogen-responsive genes, mRNA induction of c-fos and pS2 in MCF7 cells was examined after treatment with these soyasaponins. The mRNA expression levels of c-fos and pS2 were measured by RT-PCR from the total RNA of MCF7 cells treated with these saponins; constitutively expressed GAPDH mRNA was used as an internal control. The mRNA expression of c-fos and pS2 was induced after 17 β -estradiol treatment. At 1 μ M, soyasaponin I and soyasapogenol B activated the transcription of c-fos and pS2 genes, although the effects were not as prominent as those of 17 β -estradiol. However, soyasapogenol B showed potent cytotoxicity against A549, MCF7, HeLa and HepG2 cells, although soyasaponin I did not inhibit the proliferation of these cells (Fig. 4).

DISCUSSION

Soybean, which is frequently consumed by humans in Korea, Japan and China (Kim *et al.*, 2006), and its constituents, isoflavones and soyasaponins, are reported to be beneficial in the treatment of bone loss and menopause. In Western countries, soy is used as a healthy functional food for postmenopausal women. Many researchers have reported that soybean, isoflavones, glycinol, and soyasaponins exhibit estrogenic activity (Tham *et al.*, 1998; Rowlands *et al.*, 2002; Lehmann *et al.*, 2005; Park *et al.*, 2006; Boué *et al.*, 2009; Cederroth and Nef, 2009). The isoflavones also activate estrogen-responsive genes and regulate the growth of human breast cancer cells. Most studies have focused on the estrogenic effect of genistein, daidzein and soyasapogenols A and B. However, the glycoside of soyasapogenol B, soyasaponin I, has not been thoroughly studied. There-

Fig. 4. The cytotoxicity of soyasaponin I (SG) and its metabolite soyasapogenol B (SA) against tumor cells. HeLa, MCF7, HepG2 and A549 cells (1×10^4 cells, each) were incubated with SG or SA at concentrations of 0 ($\square$), 6.25 ($\blacksquare$), 12.5 ($\square$), 25 ($\square$), 50 ($\square$) and 100 μ M ($\blacksquare$). *Significantly different vs non-treated group ($p < 0.05$). The cell viability was measured by the sulforhodamine B methods.

Fig. 5. Proposed metabolic pathway of soyasaponin I by human intestinal bacteria. Thick arrow, main pathway; thin arrow, minor pathway.

fore, we measured the estrogenic activity of soyasapogenol B and soyasaponin I in an E-Screen assay. Both soyasaponin I and soyasapogenol B showed estrogenic effects. Soyasaponin I dose-dependently increased the proliferation of MCF7 cells. However, soyasapogenol B at concentrations of more than 1 μ M exhibited cytotoxicity against MCF7 cells. At more 1 μ M, soyasaponin I showed more potent estrogenic effects than soyasapogenol B. The potential of soyasaponin I as an activator of the estrogen-responsive genes, c-fos and pS2, in MCF-7 cells was compared with that of soyasapogenol B. The positive agent, 17 β -estradiol, induced c-fos and pS2 mRNA expression. The saponins also activated the transcription of the c-fos and pS2 genes, although its effect was very weak compared to that of 17 β -estradiol. Soyasaponin I more potently induced PR protein, which is an endogenous estrogen responsive gene, than soyasapogenol (data not shown). Beck *et al.* (2005) and Shutt and Cox (1972) reported that the isoflavones, genistin, genistein, and daidzein, activated ER-mediated transcription, without direct receptor interaction. However, soyasapogenol B exhibited potent cytotoxicity against various tumor cells, such as HepG2, A549, HeLa and MCF7 cells, although its glycoside soyasaponin I barely showed any cytotoxicity. These results suggest that its cytotoxicity may prevent the estrogenic effect of soyasapogenol B from increasing in a dose-dependent manner. Hu *et al.* (2005) reported that soyasaponin I is metabolized to soyasapogenol B via soyasaponin III by intestinal microflora. In the present study, we also observed that the soyasaponin I metabolism varied significantly between human fecal specimens and the main metabolites of soyasaponin I by human intestinal microflora were soyasaponin III and soyasapogenol B. Furthermore, we identified a new metabolite, soyasapogenol B 3- β -D-glucuronide, in six of the ten human fecal specimens. These results suggest that the metabolism of soyasaponin I may proceed to soyasapogenol B via soyasaponin III and soyasapogenol B 3- β -D-glucuronide or via soyasaponin III alone (Fig. 5).

Therefore, orally administered soyasaponin I may be metabolized to soyasapogenol B in the intestine and the pharmacological activities of soyasaponin I may be dependent on its metabolite(s). Thus, soyasaponin I may express cytotoxicity against tumor cells rather than estrogenicity.

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