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Short communication

Liquid chromatography/mass spectrometry-based structural analysis of soyasaponin Ab metabolites by human fecal microflora

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ABSTRACT

Soyasaponin Ab, a major constituent of soybean by human intestinal microflora, was anaerobically incubated with fecal suspensions from ten individuals for 48 h and its metabolites were measured by LC–MS/MS analysis. Ten metabolites were detected. The spectra of the parental constituent soyasaponin Ab showed a peak at m/z 1435 $[M-H]^-$ ion and those of its nine metabolites showed peaks at m/z 1310.0 $[M\text{-}3C_2H_2O\text{-}H]^-$ ion, m/z 1267.9 $[M\text{-}4C_2H_2O\text{-}H]^-$ ion, m/z 1105.3 $[M\text{-Glc-}4C_2H_2O\text{-}H]^-$ ion, m/z 973.2 $[M\text{-Glc-Ara-}4C_2H_2O\text{-}H]^-$ ion, m/z 943.4 $[M\text{-2Glc-}4C_2H_2O\text{-}H]^-$ ion, m/z 811.1 $[M\text{-2Glc-Ara-}4C_2H_2O\text{-}H]^-$ ion, m/z 781.2 $[M\text{-2Glc-Gal-}4C_2H_2O\text{-}H]^-$ ion, m/z 649.0 $[M\text{-2Glc-Gal-Ara-}4C_2H_2O\text{-}H]^-$ ion, and m/z 458.8 $[\text{Soyasapogenol A}+H]^+$ ion. Metabolic activity varied significantly between individuals. The metabolic pathway was classified into two groups: the first group potently produced soyasapogenol A and the second group accumulated soyasapogenol A 3-β-D-glucuronide.

1. Introduction

Soy contains numerous phytochemicals including isoflavones, phytic acid, phytosterols, and saponins [1,2]. Saponins are a family of steroid or triterpenoid glycosides found in a wide variety of plants [3,4]. The saponins in the mature soybean are divided into group A and group B soyasaponins based on their aglycone structures [2,5–9]. The group B soyasaponins appear to exist in intact plant tissue as conjugates of 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) at the 22-hydroxyl position. DDMP conjugates are relatively labile and are easily degraded, most likely resulting in the formation of the non-DDMP group B soyasaponins. The other various forms of the group B soyasaponins arise from alternate sugars in the oligosaccharide attached to the 3-hydroxyl position of the aglycone. The group A soyasaponins are didesmosidic with alternate sugar compositions in both sets of oligosaccharides attached to the aglycone at the 3- and 21-hydroxyl positions. The biological effects of soyasaponins have been suggested [10–15]. Indeed, soyasaponins have anti-carcinogenic, hepatoprotective and antiviral activities, and soyasapogenols exhibit antigenotoxic, hepatoprotective, and cytotoxic activities.

As with ginseng saponins [16,17], the pharmacological effects of these soyasaponins may depend on their metabolism by intestinal bacteria. Hu et al. and Chang et al. reported that soyasaponin I, which is also called soyasaponin B, was metabolized to soyasapogenol B by human fecal microflora [18,19]. Soyasapogenol B exhibited more potent estrogenic and cytotoxic effects than soyasaponin I. Therefore, to understand the biological effects of soy and its constituents, the metabolic pathways of group A and B soyasaponins should be studied. Nevertheless, the metabolic pathway of group A soyasaponin has not been studied.

Therefore, soyasaponin Ab, a major constituent among group A soyasaponins in soybeans was isolated, and its metabolism by human intestinal microflora was investigated.

2. Experimental

2.1. Reagents and chemicals

Soybeans (5 kg) harvested during the 2007 growing season were provided by a local grower from Chungchungbuk-Do, Korea. The anaerobic media were purchased from Difco Co. (USA). All other chemicals were of analytical reagent grade, and all solutions were used after redistillation.

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2.2. Soy sample preparation and isolation of soyasaponin Ab

Dried soybeans (3 kg) were ground to a fine powder in a commercial coffee mill, defatted with hexane (10 l), allowed to dry, extracted with MeOH (10 l) twice and concentrated in vacuo (65 g). The extract was suspended in water, extracted with *n*-butanol (15 l), evaporated to dryness under reduced pressure (40 g). The residue was then dissolved in MeOH and adsorbed on silica gel (40 g). The adsorbed material was transferred to a silica gel column (550 g, 6 × 40 cm). The column was eluted with $CHCl_3$-MeOH-H_2O (65:35:10, v/v/v) to afford 6 subfractions (FA1–FA6). Subfraction FA3 was successively subjected to medium pressure liquid chromatography (MPLC) to afford soyasaponin Ab (25 mg). Chromatographic separation was carried out on a Ultra-Pak C_{18} column (300 mm × 37 mm, Yamazen Co., Ltd., Japan), which was eluted with a gradient system of 5% acetonitrile and 100% acetonitrile for 2 h. The flow rate was 4 ml/min. The isolated soyasaponin Ab was identified by comparison of an authentic standard in instrumental (FAB-MS, ^{1}H-NMR, ^{13}C-NMR) analysis with that previously reported [2,5,8].

The purity of the isolated soyasaponin Ab was assayed by HPLC (YoungLin Co., Seoul, Korea). Chromatographic separation was carried out on a Lichrosorb C_{18} column (25 cm × 0.4 cm, 5 mm, Merck Co.). The elution solvent was a mixture of methanol and MeOH (30:70, v/v). The detector was ELSD (Altech Co., USA). The detection temperature was 92 °C. The flow rate of the nebulizing gas (nitrogen) was 2.0 l/min.

2.3. Isolation of a soyasaponin metabolite, soyasapogenol A, by human intestinal microflora

Fresh human feces (5 g) were obtained from ten individuals including 5 women (23–35 years old) and 5 men (25–34 years old) who had not eaten soy for 3 days before the experiment, Each specimen (5 g) was suspended in 100 ml of anaerobic dilution medium, as previously reported [20] and centrifuged at 500 × *g* for 10 min. The resulting supernatant was centrifuged at 10,000 × *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 Ab, incubated for 24 h at 37 °C to isolate diverse metabolites, and then extracted with *n*-butanol (200 ml) twice. The *n*-butanol extract was subjected to MPLC, to afford soyasapogenol A. Chromatographic separation was carried out on a Ultra-Pak C_{18} column (300 × 37 mm, Yamazen Co., Ltd., Japan), which was eluted with a linear gradient of 10% acetonitrile and 70% acetonitrile for 4 h. The flow rate was 4 ml/min.

The isolated soyasaponin Ab and soyasapogenol A were identified by comparison of authentic standards in instrumental (FAB-MS, ^{1}H-NMR, ^{13}C-NMR) analysis with that of previous reports [5,6].

Soyasaponin Ab (purity, >92%) white amorphous powder. mp 253–256 °C. FAB-MS: *m*/*z* 763.5 $[M-H]^-$.

Soyasapogenol A (purity, >95%) white amorphous powder. mp 307–310 °C. FAB-MS: *m*/*z* 458.8 $[M+H]^+$

2.4. Assay of metabolites of soyasaponin Ab by human fecal microflora

The reaction mixture containing 5 ml of 1 mM soyasaponin Ab, 2.5 ml of the above fecal microflora suspension (0.5 g wet weight/ml) and 2.5 ml of the anaerobic dilution medium was anaerobically incubated for 6 h at 37 °C. The reaction mixture (1 ml) was taken out, extracted with EtOAc (5 ml) twice, evaporated under reduced pressure and assayed by LC–MS/MS.

The supernatant of the reaction mixture was deproteinized with CH_3CN/MeOH. For the analysis of soyasaponin B metabolites, an Agilent G6410 Triple Quadrupole Mass Spectrometer from Agilent Technologies, Inc. (USA) equipped with an automatic sample injector was used. Soyasaponin Ab and its metabolites were analyzed on a ZORBAX XDB-C18 column (Rapid Resolution HT 2.1 mm × 50 mm, i.d., 1.8 μm, 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 5 μl. 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 was used for the transitions mass. 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 positive-ion mode. Soyasaponin Ab and its metabolites were analyzed with a collision energy of 15 eV and a dwell time of 0.50 s. Nitrogen (set at approximately 550 l/h) was used for the nebulizing and desolvation gases, and the desolvation temperature was set at 350 °C.

3. Results and discussion

To investigate the metabolism of soyasaponin Ab by human intestinal microflora, soyasaponin Ab was incubated with human fecal suspensions for 48 h and the metabolites were investigated by LC–MS/MS positive and negative mode analysis (Fig. 1). We found ten peaks: one parental constituent and nine metabolites. The parental constituent and eight metabolites were well detected

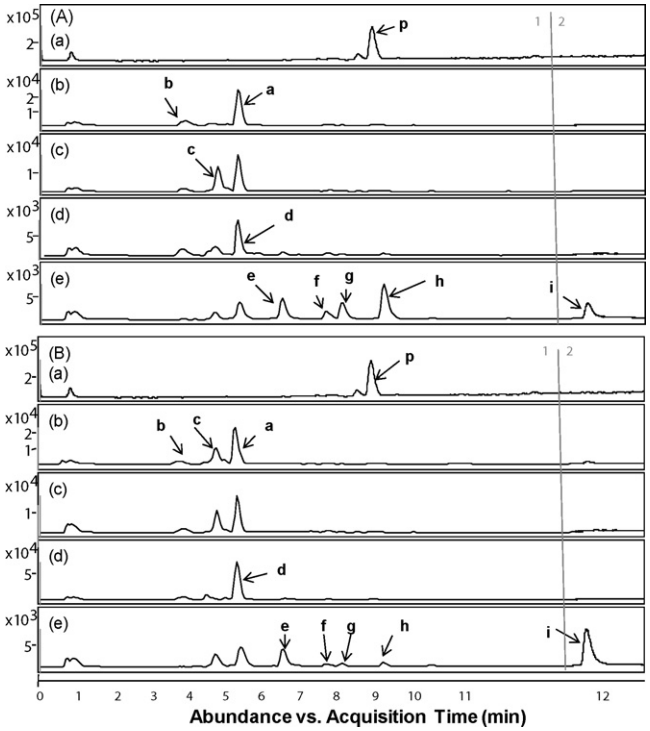

Fig. 1. LC–MS/MS chromatogram of metabolites of soyasaponin Ab by human intestinal microflora. Soyasaponin Ab was anaerobically incubated with human fecal suspension for 48 h at 37 °C, extracted with butanol, and analyzed by LC–MS/MS: column, ZORBAX Eclipse XDB-C18 (50 mm × 2.1 mm, i.d., 1.8 μm), Agilent; elution solvent, a linear gradient from 20% to 95% B in A for 13 min (solvent A was 0.1% aqueous formic acid; solvent B was acetonitrile). (A) and (B), negative (1) and positive electrospray ion currents (2) of soyasaponin Ab metabolites before (a) and after incubation with two representative human fecal specimens for 0.5 (b), 1 (c), 5 (d), and 24 h (e). Peak p is a parental compound, soyasaponin Ab, and peaks a–i are metabolites.

by LC–MS/MS negative mode analysis; the aglycone soyasapogenol A was detected well by LC–MS/MS positive mode analysis. The parental constituent soyasaponin Ab showed a peak at *m/z* 1435 $[M-H]^-$ ion. The scanned spectra of its metabolites, a–i, contained peaks at *m/z* 1310.0 $[M\text{-}3C_2H_2O\text{-}H]^-$ ion (rt 4.106 min), *m/z* 1267.9 $[M\text{-}4C_2H_2O\text{-}H]^-$ ion (rt 2.949 min), *m/z* 1105.3 $[M\text{-Glc-}4C_2H_2O\text{-}H]^-$ ion (rt 3.632 min), *m/z* 973.2 $[M\text{-Glc-Ara-}4C_2H_2O\text{-}H]^-$ ion (rt 5.937 min), *m/z* 943.4 $[M\text{-2Glc-}4C_2H_2O\text{-}H]^-$ ion (rt 4.17 min), *m/z* 811.1 $[M\text{-2Glc-Ara-}4C_2H_2O\text{-}H]^-$ ion (rt 6.305 min), *m/z* 781.2 $[M\text{-2Glc-Gal-}4C_2H_2O\text{-}H]^-$ ion (rt 5.041 min), *m/z* 649.0 $[M\text{-2Glc-Gal-Ara-}4C_2H_2O\text{-}H]^-$ ion (rt 8.220 min), and *m/z* 458.8 $[\text{Soyasapogenol A}+H]^+$ ion (rt 11.307 min), respectively (Fig. 2).

To understand the metabolic pathway of soyasaponin Ab, soyasaponin Ab was incubated with the human fecal microbial fraction of ten individuals for 48 h and the metabolites were periodically measured (Fig. 3). Early on, soyasaponin Ab was hydrolyzed to tetradeacetylated metabolite b via trideacetylated metabolite a. Then, 3'-O-*β*-D-glucopyranosyl moiety of metabolite b was hydrolyzed to metabolite c, which was metabolized to soyasapogenol A 3-β-D-glucuronide via metabolites d and e or f and g. Then the soyasapogenol A 3-β-D-glucuronide was transformed to soyasapogenol A within 24 h after incubation. Soyasapogenol A was successively produced by 48 h incubation. However, soyasaponenol A was not transformed to any more compounds within 48 h (Fig. 4). Soyasaponin Ab hydrolyzing activity was detected in all ten human fecal specimens. However, the activity varied significantly between individuals, as did the metabolic pathway.

The metabolic pathway between individuals was classified into two groups: the first group potently produced soyasapogenol

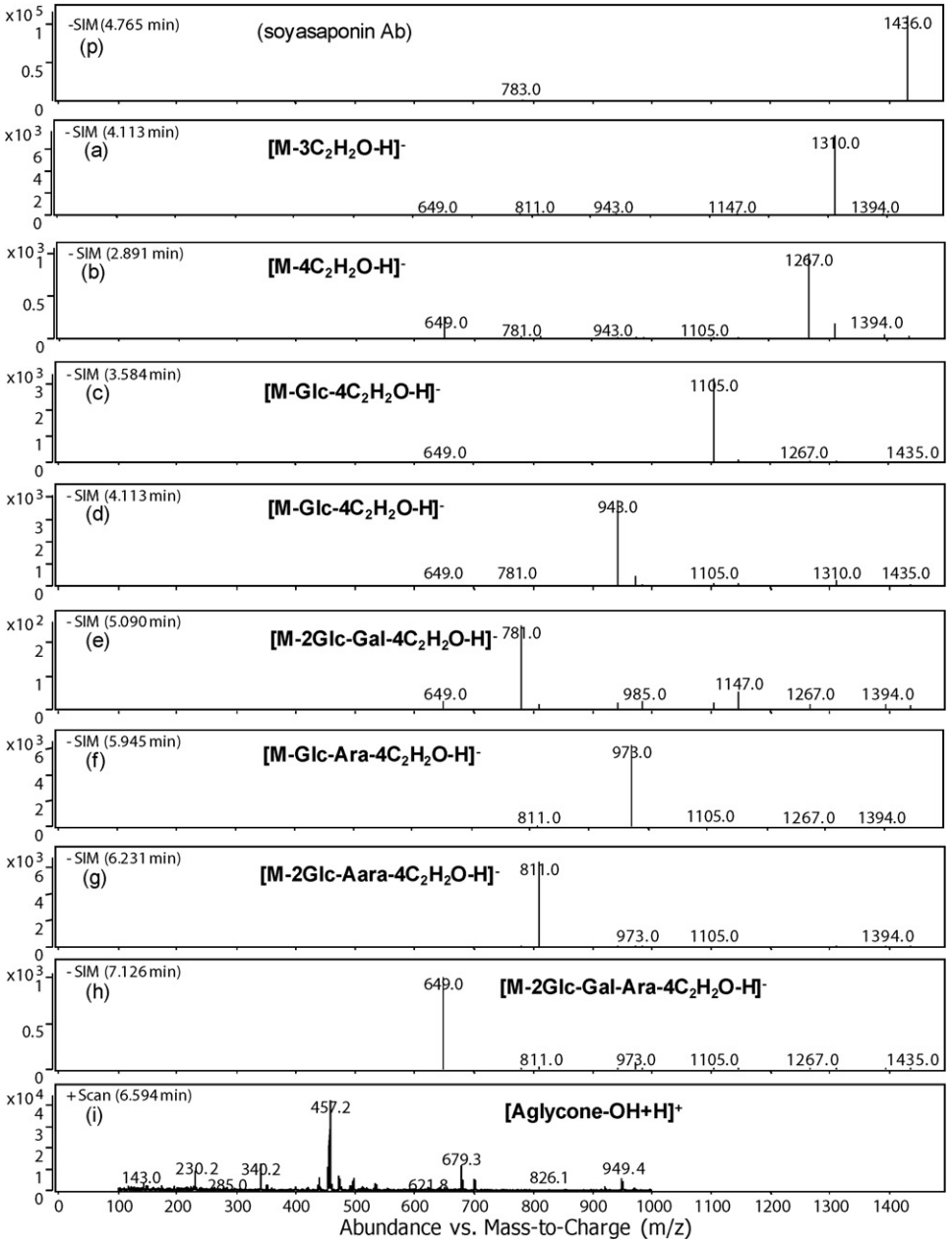

Fig. 2. The ESI mass spectra of ten main peaks in the incubation mixture of soyasaponin Ab and its metabolites. Mass spectra were acquired in: p (parental compound, soyasaponin Ab), *m/z* 1435.0 $[M-H]^-$ ion; (a), *m/z* 1310.0 $[M\text{-}3C_2H_2O\text{-}H]^-$ ion; (b), *m/z* 1267.9 $[M\text{-}4C_2H_2O\text{-}H]^-$ ion; (c), *m/z* 1105.3 $[M\text{-Glc-}4C_2H_2O\text{-}H]^-$ ion; (d), *m/z* 973.2 $[M\text{-Glc-Ara-}4C_2H_2O\text{-}H]^-$ ion; (e), *m/z* 943.4 $[M\text{-2Glc-}4C_2H_2O\text{-}H]^-$ ion; (f), *m/z* 811.1 $[M\text{-2Glc-Ara-}4C_2H_2O\text{-}H]^-$ ion; (g), *m/z* 781.2 $[M\text{-2Glc-Gal-}4C_2H_2O\text{-}H]^-$ ion; (h), *m/z* 649.0 $[M\text{-2Glc-Gal-Ara-}4C_2H_2O\text{-}H]^-$ ion; (i), *m/z* 458.8 $[\text{soyasapogenol A}+H]^+$ ion. ESI scan mode (acquired range *m/z* 100–1500).

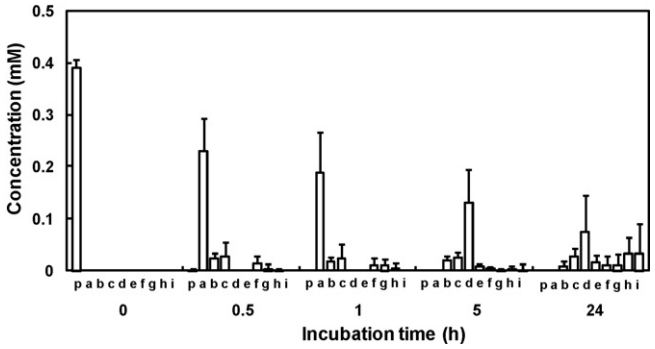

Fig. 3. Periodical change of the metabolites of soyasaponin Ab by intestinal microflora of ten subjects. The fecal suspension of ten subjects were incubated with soyasaponin Ab at 37 °C for 48 h, periodically extracted with butanol, and analyzed by LC–MS/MS: soyasaponin Ab metabolites were analyzed by full scan negative (1) and positive (2) ion mode (acquired range *m*/*z* 150–2000). The quantitative analysis of soyasapogenin Ab and soyasapogenol A was performed by using the commercial standards and the other metabolites were by using soyasapogenin Ab instead of the metabolites. p, *m*/*z* 1435.0; (a), *m*/*z* 1310.0; (b), *m*/*z* 1267.9; (c), *m*/*z* 1105.3; (d), *m*/*z* 973.2; (e), *m*/*z* 943.4; (f), *m*/*z* 811.1; (g), *m*/*z* 781.2; (h), *m*/*z* 649; (i), *m*/*z* 458.8.

A, and the second group accumulated soyasapogenol A 3-β-D-glucuronide.

Like other foods and herbs, soybeans are orally administered to humans. Their hydrophilic constituents, group A and B soyasaponins, may be resistant to stomach acid, like many glycosides, such as ginsenoside Rb1, baicalin, and glycyrrhizin; however, some constituents isolated from natural products may be unstable under stomach acid [18,19]. These soyasaponins are inevitably brought into contact with intestinal microflora in the alimentary tract and may be transformed by the intestinal bacteria before absorption from the gastrointestinal tract [18,19]. Studies of the metabolism of the components by human intestinal microflora are of great importance to understanding their biological effects. Hu et al. reported that soyasaponin B is metabolized to soyasapogenol B via soyasaponin III by intestinal microflora [21]. We also observed that soyasaponin B metabolism varied significantly between human fecal specimens and its main metabolite was soyasapogenol B [22]. Furthermore, we identified soyasapogenol B and a new metabolite in six of the ten human fecal specimens. These results suggest that the pharmacological effect of soyasaponin B may be depen-

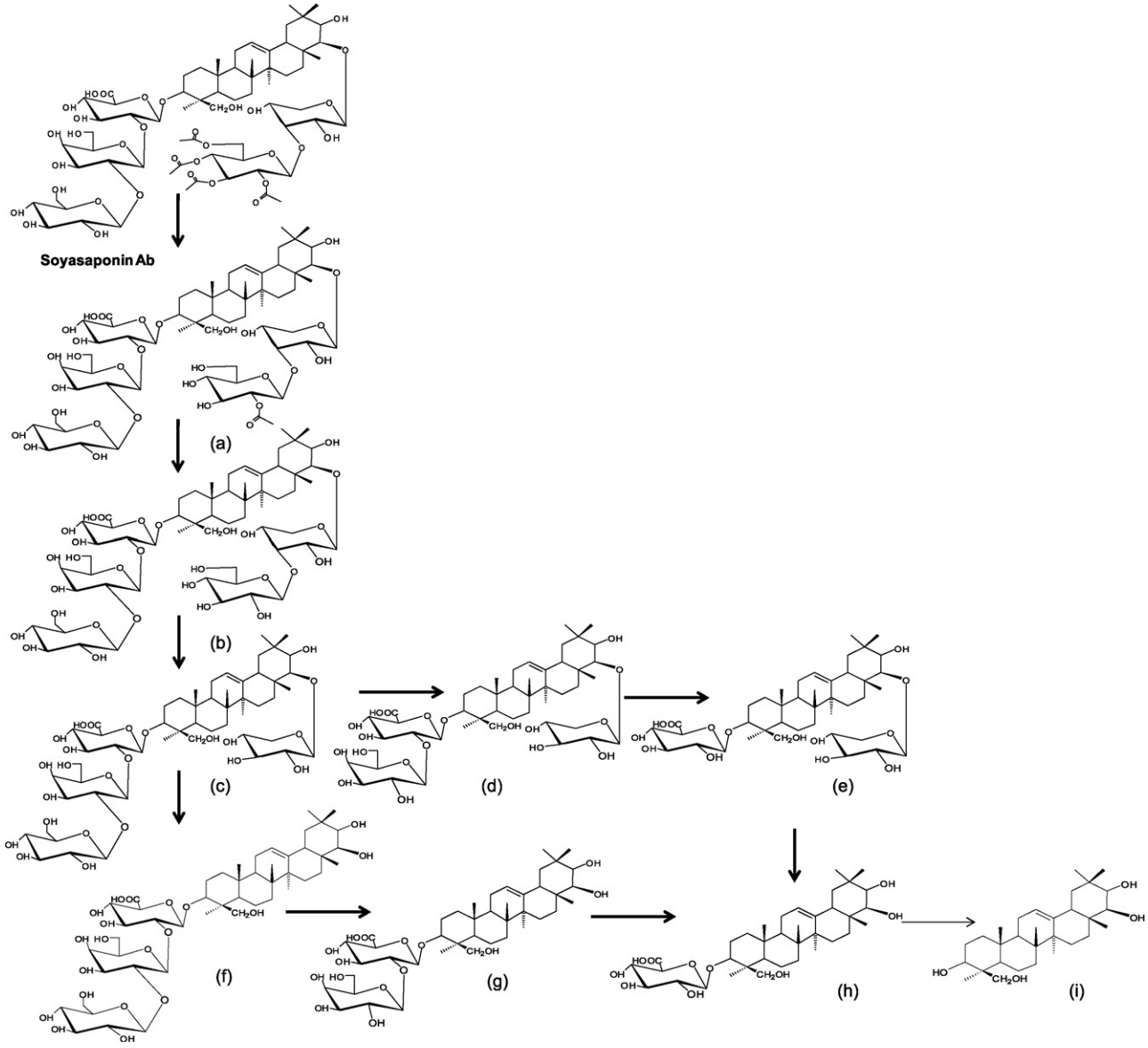

Fig. 4. Proposed metabolic pathway of soyasaponin Ab by human intestinal bacteria. Bold arrow, main pathway; normal arrow, minor pathway. p, *m*/*z* 1435.0; (a), *m*/*z* 1310.0; (b), *m*/*z* 1267.9; (c), *m*/*z* 1105.3; (d), *m*/*z* 973.2; (e), *m*/*z* 943.4; (f), *m*/*z* 811.1; (g), *m*/*z* 781.2; (h), *m*/*z* 649; (i), *m*/*z* 458.8.

dent on its intestinal bacterial metabolism to soyasapogenol B, which exhibits estrogenic and anti-tumor effects. However, the metabolism of group A soyasaponins by intestinal microflora has not been studied. Group A soyasaponins are also hydrophilic. Therefore, if soyasaponin Ab is orally administered, it is metabolized in intestine by intestinal bacteria and then its metabolite(s) may be absorbed because hydrophobic metabolites may be produced by intestinal bacteria.

In the present study, soyasaponin Ab was metabolized to soyasapogenol A 3-β-D-glucuronide and/or soyasapogenol A via hydrophilic metabolites a–i. The metabolic pathway of soyasaponin Ab was classified to two groups: the first group potently produced soyasapogenol A, and the second group accumulated soyasapogenol A 3-β-D-glucuronide. Nevertheless, soyasapogenol A 3-β-D-glucuronide produced by the second group may be metabolized to soyasapogenol A, due to its hydrophilicity. Actually, soyasapogenol A, a metabolite of soyasaponin Ab, by intestinal microflora may be absorbed from the intestine into the blood.

Based on these findings, orally administered soyasaponin Ab may be metabolized to soyasapogenol A and/or soyasapogenol A 3-β-D-glucuronide by intestinal microflora, even if acetyl ester bonds in soyasaponin Ab are degraded by stomach acid. These hydrophobic metabolites may be absorbed into the blood and the pharmacological effect of soyasaponin A may depend on its metabolism by intestinal microflora.

4. Conclusion

When soyasaponin Ab (m/z 1435 $[M-H]^-$ ion) was anaerobically incubated with fecal specimens from ten individuals, the metabolic activity varied significantly between individuals its main metabolites, soyasapogenol A and soyasapogenol A 3-β-D-glucuronide, were produced via m/z 1105.0 [M-Glc-4C_2H_2O-H]$^-$ ion, although the metabolic products and pathway differed between individuals.

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