Original Research Article

Gastrointestinal metabolism of isosaponarin derived from wasabi leaves

Abstract

Objective: The metabolism of isosaponarin was investigated using a Caco-2 intestinal epithelial model and animal experiment.

Background: Isosaponarin is a flavonoid in wasabi (*Wasabia japonica*) leaves and has unique structure, in which two glucose molecules bind to apigenin through *O*-glycosidic and *C*-glycosidic bonds.

Material and methods: The absorption and metabolism of isosaponarin was investigated by a Caco-2 intestinal epithelial model *in vitro* and a single oral administration to mice *in vivo*.

Results: These experiments showed that isosaponarin was hardly absorbed into the body. However, isosaponarin was metabolized to isovitexin (apigenin-6-*C*-glucoside) by hydrolysis of *O*-glycosidic bond. This hydrolysis was mainly caused at small intestine, and the gastric acid in the stomach might partially contribute to the hydrolysis. Both Caco-2 intestinal epithelial model and animal experiment indicated that isovitexin was also not absorbed into the body, and that a half of the administered isosaponarin was excreted as isovitexin in feces.

Conclusion: These results indicated that bioavailability of dietary isosaponarin and its major metabolite, isovitexin are low.

Keywords

Isosaponarin, Isovitexin, Wasabi, *Wasabia japonica*, Caco-2 cells, ICR mice

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1. **Introduction**

Wasabi (*Wasabia japonica*) is a Japanese indigenous plant belonging to the Brassicaceae, and the beneficial effects including the appetite improvement and the antimicrobial activity have been well known from ancient times as reviewed by Chadwick *et al* [1] and Hashimoto *et al* [2]. The whole plant of wasabi including rhizomes, lateral roots, stalks, leaves, and flowers can be used as foodstuffs in Japan, *e.g.* the grated fresh wasabi rhizome is used as a popular condiment in Japanese cuisine such as *sushi*, *sashimi*, and *soba*. The stalks and leaves are used as the ingredients of wasabi paste condiment, and the stalks, leaves and flowers are also used for *tsukemono* (Japanese pickles) and *tempura*. On the other hand, 6-Methylsulfinylhexyl isothiocyanate in wasabi has been reported to possess several health promoting activities related with cell cycle progression [3,4], and drug-metabolizing enzymes [5]. Furthermore, Nagai *et al* [6] reported that isosaponarin (apigenin-6-*C*-glucosyl-4'-*O*-glucoside) (Fig.1) derived from wasabi leaves promotes the production of type I collagen in human fibroblasts, and this compound is blended in cosmetics nowadays and further expected to use as functional food materials. However, there is no information on the absorption and metabolism of dietary isosaponarin.

In general, flavonoid glycosides, particularly the mono-glucosides are absorbed into the body through two major pathways of the small intestine [7]. The first pathway is mediated by sodium-dependent glucose transporter-1 (SGLT-1) on the intestinal cellular surface, and flavonoid glycosides are absorbed through SGLT-1 as their glycoside forms [8]. Following the absorption, aglycones are released from the glycosides by intracellular β-glucosidases [9]. In the second pathway, lactase phlorizin hydrolase (LPH) on the intestinal cellular membrane hydrolyzed flavonoid glycosides to the aglycone and sugar moiety followed by the absorption of aglycones into the intestinal cells by simple diffusion [10]. Most of intracellular flavonoid aglycones incorporated into intestine are conjugated with sulfate and/or glucuronic acid in the intestinal cells, followed by entering into the blood and/or lymph [11]. Many studies have been reported that absorption and metabolism of flavonoids depend on their chemical structures [12,13].

Thus, absorption and metabolism of flavonoid *O*-glycosides have been well studied. On the other hand, several flavonoid *C*-glycosides also naturally occur in plants [14-17]. It is likely that flavonoid *C*-glycosides is hardly metabolized and absorbed into the body from small intestine [18]. Isosaponarin have two glucose molecules; *i.e.* one is *O*-glycoside, and another is *C*-glycoside, as shown in Fig. 1. In the present study, the absorption and metabolism of isosaponarin was investigated by a Caco-2 intestinal epithelial model *in vitro* and a single oral administration to mice *in*

vivo, on the basis of the prospect that this compound would be hydrolyzed to isovitexin (apigenin-6-*C*-glucoside) and/or apigenin in the gastrointestine.

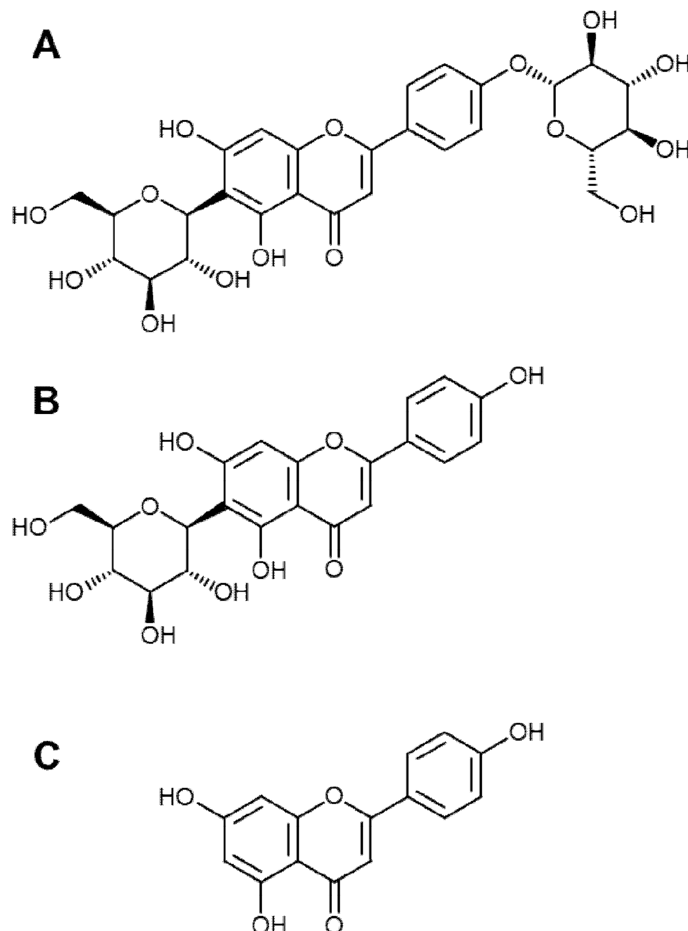

Fig. 1. Chemical structures of isosaponarin (A), isovitexin (B) and apigenin (C).

2. **Materials and Methods**

2.1. Materials

Isosaponarin was kindly provided from Kinjirushi (Aichi, Japan). Isovitexin was purchased from ChromaDex™ (Irvine, CA). Apigenin, quercetin and phloretic acid were purchased from Extrasynthese (Genay, France). Sulfatase (≥10,000 unit/g solid) type H-1 from *Helix pomatia* containing β-glucuronidase (≥300 unit/mg solid at pH 5.0), phloroglucinol acid, *p*-coumaric acid, quercetin 3-β-D-glucoside, and Dulbecco's Modified Eagle's Medium with 4,500 mg/ml glucose (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO). Hanks' balanced salt solution (HBSS)

and flavone were purchased from Nacalai Tesque (Kyoto, Japan). Lucifer yellow CH lithium salt was purchased from Invitrogen (Eugene, OR). Fetal bovine serum (FBS) was purchased from BioWest (Nuaille, France). All other reagents were of the highest quality commercially available.

2.2. Cell culture

Human colon carcinoma Caco-2 cells (passage number 45) were obtained from the Riken Cell Bank (Ibaraki, Japan). Cells were maintained in a complete DMEM supplemented 50,000 U/L penicillin G, 50,000 μg/L streptomycin and 10% FBS, in a humidified atmosphere of 5% CO_2-95% air at 37 °C. Cells were sub-cultured at 80-90% confluency.

2.3. Caco-2 intestinal epithelial model

Caco-2 cells at passage of 50-60 were used for an intestinal epithelial model, which was performed with the BD BioCoat™ HTS Caco-2 Assay System (BD Bioscience, Bedford, MA) according to the manufacture's protocol. Caco-2 monolayer was estimated by the transepithelial electrical resistance (TEER) values and the lucifer yellow permeability assay. The TEER value was routinely measured with the Millicell®-ERS system (Millipore) according to the method described previously [19]. When the TEER value of Caco-2 monolayer, which was calculated as [(the TEER value of an insert seeded cells) – (the TEER value of a blank insert without cells)] × growth area (0.3 cm^2), was more than 350 $\Omega \cdot cm^2$, the Caco-2 monolayer was judged to complete the formation of the intestinal epithelial model.

The lucifer yellow permeability assay was performed according to the protocol "Lucifer Yellow Permeability Assay Using BD Falcon™ HTS 96-Multiwell Insert Systems" provided by BD Bioscience with some modifications to adjust for 24-multiwell inserts. In brief, the HBSS or medium in the apical and basolateral compartments were removed, and the compartments were gently washed with HBSS. Lucifer yellow dissolved in distilled water at 100 mM was diluted in HBSS at 100 μM, and 500 μl of the solution was gently added to the apical compartment, while 1 mL of HBSS was immediately added to the basolateral compartment. The multiwell plate was placed on a reciprocal shaker at 70-90 rpm in a 37°C in an incubator for 1 h. The HBSS in the basolateral compartment was collected, and the lucifer yellow leaked across the Caco-2 monolayer was determined using a fluorescence spectrophotometer at 485 nm of excitation and 530 nm of emission wavelengths with a standard curve of lucifer yellow (1-100 μM). In a complete Caco-2 intestinal epithelial model, the amount of lucifer yellow in basolateral compartment was less than 1% of lucifer yellow added to the apical compartment.

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2.4. Permeability assay of isosaponarin and isovitexin in a Caco-2 intestinal epithelial model

The medium was removed from both apical and basolateral compartments, and then the Caco-2 monolayer in the insert were gently washed with HBSS (pH 7.4) for 30 min in an incubator. The inserts were set on an assay plate, the Enhanced Recovery Plate (BD Bioscience). The test compounds dissolved in DMSO at 10 mM were diluted in HBSS at 10 μM, and the solution (500 μl) was gently added to the apical compartment. The basolateral compartment was immediately added 1,000 μl of HBSS. After 0.5, 1, or 2 h of incubation at 37°C, HBSS in the apical and basolateral compartments were separately collected as apical and basolateral solutions, respectively. The apical and basolateral compartments were added 200 μL and 800 μL of methanol, respectively, and shaken for 30 min on a reciprocal shaker at 37°C. The sum of methanol was collected as a cellular extract. The apical and basolateral solutions and cellular extract were added 5 μl of 100 μM flavone as an internal standard. The basolateral solution and cellular extract were divided into two aliquots, and these aliquots and the apical solution were evaporated with a centrifugal concentrator. The dried residues were stored at -80°C until HPLC analysis. To determine the concentration of conjugates, one of the aliquots from the basorateral solution and cellular extract was dissolved in 50 μl of distilled water and incubated at 37°C for 45 min with 50 μl of 0.2 M acetate buffer (pH 5.0) containing 20 units of sulfatase. The mixture was added same volume (100 μL) of methanol and centrifuged at 11,000 × *g* for 10 min, and the supernatant was subjected to HPLC analysis. To determine the concentration of aglycones, the dried residues from another aliquot and the apical solution were dissolved in 100 μl of 50% methanol (v/v) filtered through a 0.2-μm membrane filter (Millex-LG, Millipore), and subjected to a HPLC analysis.

2.5. HPLC analysis

Isosaponarin, isovitexin and apigenin were quantitatively analyzed with the Hitachi HPLC system (Tokyo, Japan) equipped with a pump (L-7100), a column oven (L-7300), an UV-VIS detector (L-7420), and a D-7000 chromatography data station software. These flavonoids were monitored with a wavelength at 340nm, and the column used was a Capcell pak C18 UG120 column (250 mm × 4.6 mm i.d., Shiseido, Tokyo, Japan) maintained at 35°C and joined with a guard column (10 mm × 4.0 mm i.d., Shiseido). The mobile phase consisted of (A) 50 mM sodium phophate adjusted to pH 3.3 with phosphoric acid, methanol (9:1, v/v) and (B) sodium phophate (pH 3.3), methanol (3:7, v/v). The gradient program started at 1.0 mL/min at 30% B, 30-50% B in 10 min, 50-80% B in 10 min, 80-100% B in 20 min and then 100% B for 10 min. In

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all analyses, the column was re-equilibrated at 30% B for 8 min. The injection volume was 10 μl.

2.6. Animal experiments

The animal treatment was approved by the institutional Animal Care and Use Committee (permission number 20-05-11) and carried out according to the Guidelines on Animal Experimentation of Kobe University. Female ICR mice (6 weeks old; Japan SLC, Shizuoka, Japan) were maintained with standard diet Labdiet® 5L37 (Japan SLC) in a temperature-controlled room at 22-25°C with 12-h light/dark cycles, and acclimated for 1 week before animal experiments. Mice were fasted overnight but allowed free access to drinking water. Mice were then administered isosaponarin (50 mg/kg body weight) dissolved in distilled water by gavage. Twelve mice were housed in metabolic cages to collect the feces and sacrificed 8, 12, 24 and 48 h after the administration, while nine mice were housed in plastic cages and sacrificed 1, 2, and 4 h after the administration. Mice were physically normal without decrease in body weight and diarrhea throughout the experiment, and the food and water intake were not different from control mice. The mice were anesthetized with pentobarbital sodium, and the blood was collected from the heart with a heparinized syringe at corresponding time points. The stomach, small intestine, and large intestine were carefully removed, and the gastrointestinal remnants were separately collected by perfusion with 3 ml of ice-cold PBS and transferred to conical tubes. These tissues and remnants were immediately frozen by liquid nitrogen and stored at -80°C until HPLC analysis. The plasma was prepared from the blood by centrifugation at 450 × *g* for 15 min at 4°C. The 0-h control mice were administered nothing.

2.7. Extraction of isosaponarin, isovitexin and apigenin from plasma

Forty micro-liter of plasma was transferred into a microtube, and incubated at 37°C for 45 min with 40 μl of 0.1 M acetate buffer (pH 5.0) with or without 20 units of sulfatase. The mixture was added 5 μl of 100 μM flavone as an internal standard and 450 μl of methanol, and agitated with a vortex mixer. After centrifugation at 2,000 × *g* for 15 sec, the 400 μl of supernatant was transferred to a new microtube. The residue was added 400 μl of methanol again and centrifuged at 2,000 × *g* for 15 sec to obtain methanol extract. This extraction process was repeated twice, and the sum of supernatant was evaporated with a centrifugal concentrator. The dried residue was dissolved in 100 μl of 50% (v/v) methanol and filtered through a 0.2-μm membrane filter, and analyzed on HPLC.

2.8. Extraction of isosaponarin, isovitexin and apigenin from gastrointestinal tisuues,

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its remnants and feces

The extraction of isosaponarin, isovitexin and apigenin from the tissues, gastrointestinal remnants and feces was performed according to the method described previously [20] with some modifications. The tissues; stomach, small intestine, and large intestine, were minced with surgical scissors, added 4 ml of 50% methanol (v/v) and 5 μl of 100 μM flavone as an internal standard, and homogenized thrice at 2,500 rpm for 7 sec by a cell disruptor, Multi-beads shocker. On the other hand, the gastrointestinal remnants and feces were homogenized with 6 volumes of 50% methanol (v/v) by the same method. The homogenates were agitated with a vortex mixer, sonicated for 15 min and centrifuged at 1,780 × *g* for 10 min. The supernatants were collected, agitated with a vortex mixer and filtered through a 0.2-μm membrane filter. The filtrate was subjected to a HPLC analysis.

3. Results

3.1. Assembly of Caco-2 intestinal epithelial model by 5-day method

To investigate the intestinal absorption of isosaponarin, a Caco-2 intestinal epithelial model assembled by a 5-day method was used for isosaponarin permeability assay. The Caco-2 intestinal epithelial model was estimated by a TEER value and a lucifer yellow permeability assay. To compare a 5-day method used in the present study and a 21-day method used in the previous study [19], 3 nmol of quercetin or quercetin glucoside was subjected to the model assembled by a 5-day method in prior to the isosaponarin permeability assay. The amount of quercetin in the apical compartment was decreased in a time-dependent manner, while that of quercetin glucoside was hardly changed at 0.5, 1 and 2 h after the addition (Fig. 2). These results were consistent with the results of the previous report [19]. Therefore, the Caco-2 monolayer assembled by a 5-day method used in the present study was able to be used as an intestinal epithelial model for isosaponarin permeability assay as well as that by a 21-day method.

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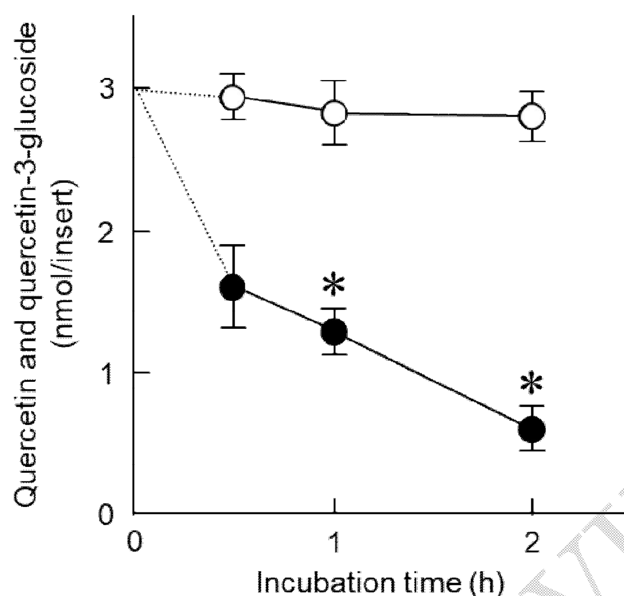

Fig. 2 Amounts of quercetin and quercetin glucoside in apical solutions. Quercetin or quercetin glucoside were added at 3 nmon/insert to the apical compartment. The amounts of quercetin (solid circle) and quercetin glucoside (open circle) in the apical compartment was determined at 0.5, 1, and 2 h incubation by HPLC as described in Materials and Methods. Data are expressed as means ± SD (*n*=6). $*p < 0.05$ compared with the corresponding values at 0.5 h.

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3.2. Isosaponarin permeability assay

Five hundred micro-liter of 10 μM isosaponarin (5 nmol) was added to the apical compartment of the Caco-2 intestinal epithelial model, and the amounts of isosaponarin and the prospected metabolites, isovitexin and apigenin, were measured by HPLC analysis in the apical solution, cellular extract and basolateral solution 0.5, 1 and 2 h after the addition (Table 1). The chromatogram with standards showed 5.8 min of retention time for isosaponarin, 13.6 min for isovitexin, 25.7 min for apigenin, and 32.5 min for flavones as an internal standard (Fig. 3). Isosaponarin in the apical compartment slightly decreased to 4.9 nmol at 0.5 and 1 h, and 4.8 nmol at 2 h, while isosaponarin in the cellular extract and isovitexin in the apical compartment slightly increased. However, it is unlikely that these metabolism and absorption was due to the aggressive capacity of cells, because these changes were very small. Apigenin was undetected in any fractions. Thus, isosaponarin was hardly received metabolism and absorption in the Caco-2 intestinal epithelial model. In addition to isosaponarin, isovitexin was also subjected to the permeability assay with the Caco-2 intestinal epithelial model. More than 95% of isovitexin added to the apical compartment

remained in the apical compartment 0.5 and 1 h after the addition. Apigenin were undetected in both apical and basolateral compartments (Table 2). These results indicated the isovitexin were also not received deglycosidation and absorption at the small intestine.

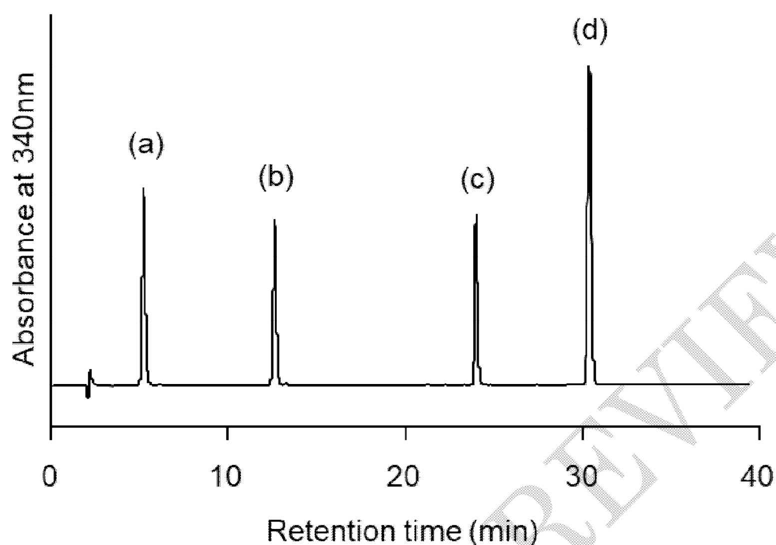

Fig. 3 Chromatogram for standards

(a) isosaponarin at a retention time 5.8 min, (b) isovitexin at 13.6 min, (c), apigenin at 25.7 min, (d) flavone at 32.5 min as an internal standard (I.S.).

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Table 1. The isosaponarin permeability assay with Caco-2 intestinal epithelial model*

Incubation time (h)	Fractions	Isosaponarin (nmol/insert) [1]	Isovitexin (nmol/insert)	Apigenin (nmol/insert)
0.5	Apical solution	4.92 ± 0.06 (98.4 ± 1.2) [2]	0.083 ± 0.002 (1.65 ± 0.06) [3]	N.D.
	Cellular extract	0.13 ± 0.00 (2.55 ± 0.04)	N.D.	N.D.
	Basolateral solution	N.D.	N.D.	N.D.
1	Apical solution	4.88 ± 0.04 (97.6 ± 0.8)	0.085 ± 0.001 (1.70 ± 0.02)	N.D.
	Cellular extract	0.15 ± 0.01 (2.95 ± 0.01)	N.D.	N.D.
	Basolateral solution	N.D.[4]	N.D.	N.D.
2	Apical solution	4.80 ± 0.07 (96.0 ± 1.4)	0.22 ± 0.02 (4.35 ± 0.40)	N.D.
	Cellular extract	0.17 ± 0.00 (2.95 ± 0.01)	N.D.	N.D.
	Basolateral solution	N.D.	N.D.	N.D.

*Isosaponarin (5 nmol/insert) was subjected to the Caco-2 intestinal epithelial model. Isosaponarin, isovitexin, and apigenin were measured by HPLC analysis as described in Materials and Methods. Data are

expressed as means±SD (n=6). N.D., not detected.

[1] The concentration of flavonoids without conjugation

[2] Parentheses show the recovery % of amounts of isosaponarin added to the insert (5 nmol/insert).

[3] Parentheses show the recovery % of equivalent amounts of isosaponarin added to the insert (5 nmol/insert).

Table 2. The isovitexin permeability assay with Caco-2 intestinal epithelial model*

Incubation time (h)	Fractions	Isovitexin (nmol/insert)	Apigenin (nmol/insert)
0.5	Apical solution	4.77± 0.07 [1] (95.3± 1.3) [2]	N.D.
	Cellular extract	N.D.	N.D.
	Basolateral solution	N.D.	N.D.
1	Apical solution	4.87± 0.11 (97.3± 2.1)	N.D.
	Cellular extract	N.D.	N.D.
	Basolateral solution	N.D.	N.D.

* Isovitexin (5.0 nmol/insert) was subjected to the Caco-2 intestinal epithelial model. Isovitexin and apigenin were measured by HPLC analysis as described in Materials and Methods. Data are expressed as means ± SD (*n*=6). N.D., not detected

[1] Concentration of flavonoids without conjugation

[2] Parentheses show the recovery % of equivalent amounts of isosaponarin added to the insert (5 nmol/insert).

3.3. Oral administration of isosaponarin in mice

To confirm the results from the Caco-2 intestinal epithelial model under the *in vivo* condition, mice were orally administered 2.5 μmol isosaponarin (approximately 50 mg/kg body weight) dissolved in distilled water, and the concentration of isosaponarin and the prospected metabolites, isovitexin and isosaponarin, in the plasma was determined by HPLC analysis. The recovery percentage of flavones, an internal standard, was more than 98.5%. The neither isosaponarin nor prospected metabolites were undetected in the intact plasma and sulfatase-treated plasma within 48 h under the HPLC condition used in the present study (Fig. 4). On the other hand, isovitexin was slightly detected in the homogenates of gastrointestinal tissues, *i.e.*, stomach (0.31% of equivalent amounts of isosaponarin administered mice), small intestine (4.9%), and large intestine (2.5%) (Table 3). Since these amounts were exceedingly small,

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isovitexin might be adsorbed on the surface of the tissues, *i.e.* isovitexin was not absorbed into the tissues. In addition, isosaponarin and apigenin were undetectable in these tissues (data not shown). These results indicate that isosaponarin and/or the prospected metabolites are not absorbed into the body *in vivo*, and are consistent with the results from the Caco-2 intestinal epithelial model.

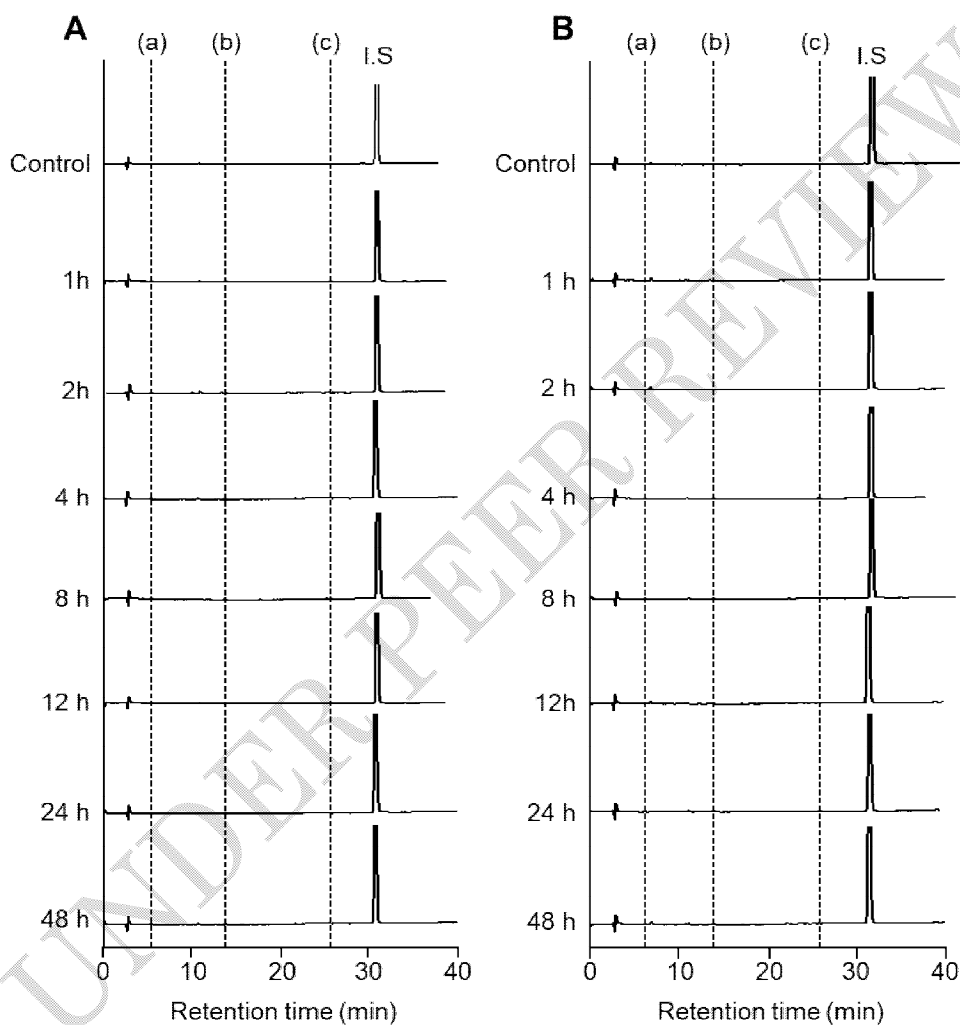

Fig. 4 Chromatogram of plasma after oral administration of isosaponarin
Mice were orally administered 2.5 μmol isosaponarin and sacrificed at the indicated time points. The intact plasma (*A*) and sulfatase/glucuronidase-treated-plasma (*B*) were analyzed on HPLC. As shown in Fig.3 (a), (b) and (c) indicated the retention times for isosaponarin (5.8 min), isovitexin (13.6 min), and apigenin (25.7 min), respectively. I.S., internal standard.

Table 3 Isovitexin in the gastrointestines of isosaponarin-administered mice*

Time (h)	Stomach (μmol)	Small intestine (μmol)	Large intestine (μmol)
1	0.0078 ± 0.0025 [1] (0.31 ± 0.10) [2]	0.12 ± 0.05 (4.9 ± 1.9)	0.061 ± 0.031 (2.5 ± 1.2)
2	0.0023 ± 0.0015 (0.09 ± 0.06)	0.012 ± 0.003 (0.47 ± 0.12)	0.059 ± 0.003 (2.4 ± 0.1)
4	0.0023 ± 0.0018 (0.09 ± 0.07)	0.0045 ± 0.0035 (0.18 ± 0.14)	0.013 ± 0.003 (0.50 ± 0.12)
8	0.0025 ± 0.0018 (0.10 ± 0.07)	N.D.	N.D.

* Mice were orally administered 2.5 μmol isosaponarin and sacrificed at the indicated time points. Isovitexin in the gastrointestinal tissues, stomach, small intestine, and large intestine, were measured by HPLC analysis as described in Materials and Methods. Data are expressed as means ± SD (n=3). N.D., not detected.

[1] Amounts of isovitexin without conjugation

[2] Parentheses show the recovery % of equivalent amounts of isosaponarin administered mice (2.5 μmol).

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3.4. Isosaponarin and the metabolites in the gastrointestinal remnants

Because isosaponarin and the prospected metabolites were unlikely to be absorbed into the body, the amounts in the gastrointestinal remnants and feces were determined by HPLC analysis. Thirty six percent of the original dose (2.5 μmol) remained in the gastric remnants 1 h after the administration, and isosaponarin gradually decreased to 1.7% at 12 h (Fig. 5A). Although isosaponarin was also detected in the small and large intestinal remnants, the maximum amounts were less than 1% of the original dose (Figs. 5B,C). Isovitexin equivalent to 8.6% of the administrated isosaponarin was detected in the gastric remnants 1 h after the administration, and then the amount was gradually decreased to less than 1% at 12 h (Fig. 5A). The amounts of isovitexin in the small and large intestinal remnants were equivalents to 13% (Fig. 5B) and 28% (Fig. 5C), respectively, 1 h after the administration. The amounts of isovitexin in the small and large intestinal remnants were obviously higher than that of isosaponarin detected at the same time points.

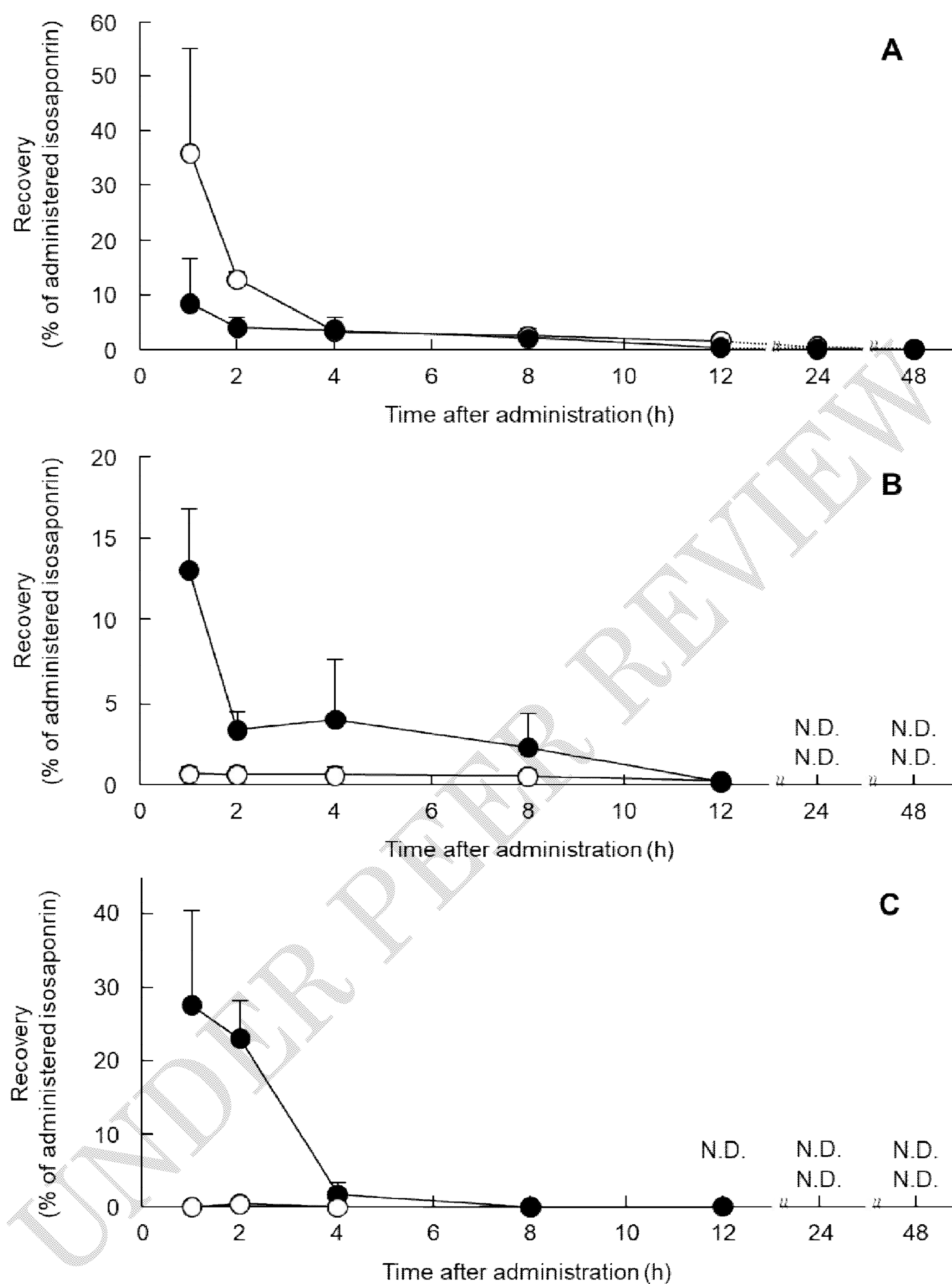

Fig. 5 The recovery of isosaponarin and isovitexin in the gastrointestinal remnants

Mice were orally administered 2.5 μmol isosaponarin. Isosaponarin (open circle) and isovitexin (solid circle) were extracted from the stomach (*A*), small intestine (*B*), and large intestine (*C*), and measured by HPLC as described in Materials and Methods. Data are expressed as means ± SD (n=3). N.D., not detected.

Furthermore, isovitexin was detected in the feces but isosaponarin was not. The isovitexin excreted into the feces for 8 h accounted for 51% equivalents of dosed isosaponarin (Fig.6), and did not increase thereafter. This result suggests that most of isovitexin was excreted into the feces within 8 h after the administration while a little isovitexin and isosaponarin remained in the gastrointestinal remnants. Apigenin were not detected in the gastrointestinal remnants and feces throughout the experiments. These results indicated that isosaponarin was mainly metabolized to isovitexin in the stomach or small intestine, and most isovitexin was not absorbed into body and was excreted with feces.

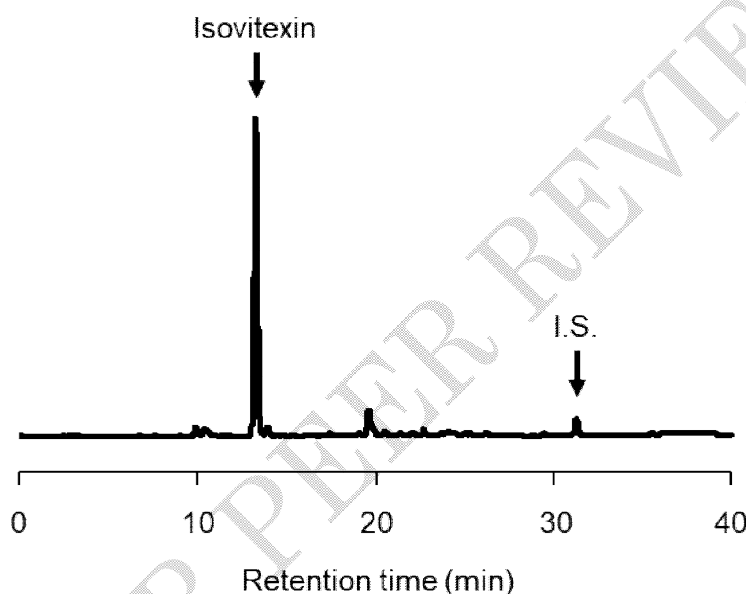

Fig. 6 Representative chromatogram of feces of mice
Mice were orally administered 2.5 μmol isosaponarin, and their feces were collected for 8, 12, 24, and 48 h. Isosaponarin and isovitexin were extracted from the feces, and measured by HPLC as described in Materials and Methods. Isovitexin and flavone as internal standard (I.S.) were detected at 13.6 min and 32.5 min, respectively.

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3.5. Hydrochloric acid hydrolysis of isosaponarin *in vitro*

To investigate whether isosaponarin is metabolized to isovitexin by gastric acid in the stomach, 0.15 μmol of isosaponarin was incubated with 2N HCl at 37°C for 30 min. Isosaponarin very slightly decreased to 0.148 μmol, and isovitexin was slightly detected at 0.009 nmol (Fig. 7). Therefore, the acidic hydrolysis was minor deglycosidation of isosaponarin to isovitexin. This result suggests that isosaponarin is metabolized to isovitexin mainly in small intestinal tract, although a small amount of

isosaponarin may be deglycosilated to isovitexin by gastric acid in stomach.

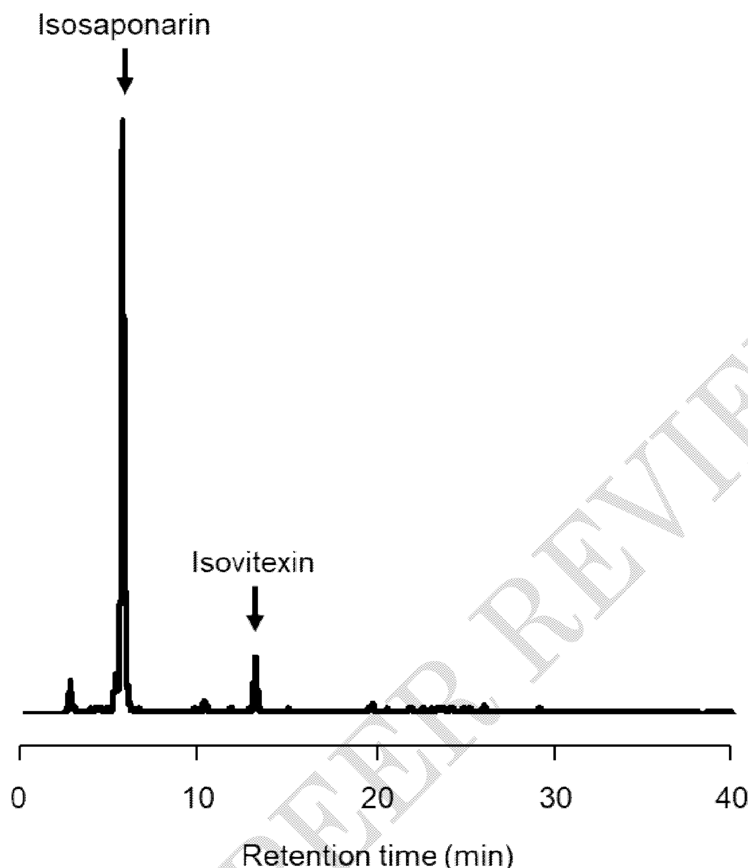

Fig.7 Chromatogram of HCl-hydorolyzed isosaponarin
Isosaponarin were incubated with HCl at 37ºC for 30 min. The solution was subjected to HPLC analysis as described in Materials and Methods. Isosaponarin and isovitexin were detected at 5.8 min and 13.6 min, respectively.

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4. Discussion

The aim of the present study was to investigate the absorption and metabolism of isosaponarin derived from wasabi (*Wasabia japonica*) leaves. The Caco-2 intestinal epithelial model and animal experiment indicated that isosaponarin and isovitexin were hardly absorbed into the body. The animal experiment suggested that isosaponarin was metabolized to isovitexin, and isovitexin equivalent to the approximately half of administered isosaponarin was excreted with feces within 8 h after the administration. Thus, the present study demonstrated that dietary isosaponarin was hardly absorbed into the body and preferably metabolized to isovitexin, and isovitexin was also hardly absorbed.

Isosaponarin (apigenin-6-*C*-glucosyl-4'-*O*-glucoside) have two glucose molecules in the structure; one is bound to the OH group at 4'-position on the B-ring of apigenin aglycone by *O*-glycosidic bond, and another one is bound to the carbon at 6-position on the A-ring by *C*-glycosidic bond. In the present study, apigenin was not detected in the any experiments; the Caco-2 intestinal epithelial model, animal experiment, and HCl hydrolysis experiment *in vitro*, although isovitexin (apigenin-6-*C*-glucoside) was detected as an intestinal metabolite. These results were indicated that deglycosylation of *O*-glucoside occurred in the gastrointestineal tract but the deglycosylation of *C*-glucoside was not. Quercetin-4'-*O*-glucoside was reported to be hydrolyzed by LPH [19], suggesting that *O*-glucoside at 4'-position on flavonoids is likely to be hydrolyzed by LPH. In the present study, the animal experiment demonstrated that isovitexin increased at small intestine immediately after the administration. Furthermore, the HCl hydrolysis experiment showed that most isosaponarin was hardly hydrolyzed by gastric acids though it was slightly influenced. These results suggested that isosaponarin was mainly metabolized to isovitexin by LPH, which hydrolyzed *O*-glucoside of isosaponarin at small intestine, while the acidic hydrolysis by gastric acid is also considered as the minor metabolic pathway of isosaponarin.

Zhang *et al* [18] demonstrated that flavone *C*-glucosides; orientin (luteolin-8-*C*-glucoside), homoorientin (luteolin-6-*C*-glucoside), vitexin (apigenin-8-*C*-glucoside) and isovitexin were poorly absorbed in the gastrointestinal tract, and 21% of *C*-glucosides were excreted in the feces at 24 h. Thus, most flavonoid *C*-glycosides are unlikely to be absorbed at gastrointestinal tracts and excreted in the original form. In the present study, isosaponarin and isovitexin were undetected in the plasma at any time points in this study, suggesting that isovitexin and isosaponarin was not absorbed in the original forms at the intestine. On the other hand, certain flavonoid *C*-glycosides were reported to be absorbed in the original form. Puerarin (daidzein-8-*C*-glucoside) was rapidly absorbed from the intestine without metabolism, and mainly excreted in the urine as the original form of puerarin though puerarin was partially hydrolyzed to the aglycone [21] and metabolized to dihydrodaidzein and equal [22]. Further study is needed to clarify the differences between the unabsorbed and absorbable flavonoid C-glycosides.

Flavonoid *C*-glycosides have been considered to contribute a diverse range of biological activities including the antimicrobial activity [23], and antioxidantive activity [24]. Isosaponarin have been reported to promote the biosynthesis of type I collagen in human fibroblasts *in vitro* [6], but there is no information on the biological activity of dietary isosaponarin *in vivo*. Since dietary isosaponarin is immediately metabolized to isovitexin as described in the present study, some health beneficial

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effects of isovitexin are expected rather than that of isosaponarin following the intake of isosaponarin. A recent study demonstrated that oral administration of isovitexin (15 mg/kg) has an anti-hyperglycemic action in rats [25]. Huang *et al* [26] demonstrated that isovitexin suppressed the release of tumor necrosis factor α (TNF-α), production of prostaglandin E2, and expression of cyclooxygenase-2 in lipopolysaccharide-activated RAW264.7 macrophages. It is well known that TNF-α production is increased under chronic hyperglycemia, and TNF-α has harmful effects on insulin sensitivity [27]. The suppressive effects of isovitexin on TNF-α production in intestinal macrophages [26] may contribute to the anti-diabetic activity *in vivo* [25]. In addition to isovitexin, daily oral administration of luteolin-6-*C*-glucoside (isoorientin) was reported to show subacute hypoglycaemic effect on streptozotocin-induced diabetic rats [28]. Oral administration of apigenin-*6-C*-(2"-*O*-α-L-rhamnopyranosyl)-β-L-fucopyranoside (50 mg/kg body weight) showed an acute effect on blood glucose lowering in diabetic rats and stimulated glucose-induced insulin secretion after oral treatment in hyperglycemic rats [29]. Oral administration of apigenin-6-*C*-β-L-fucopyranoside (50 mg/kg body weight) showed an acute effect on blood glucose lowering in hyperglycemic rats and stimulated glucose-induced insulin secretion [30]. Thus, the consumption of flavonoid *C*-glycosides showed anti-diabetic activity *in vivo*. Dietary isosaponarin may also contribute to the prevention and treatment of diabetes.

Although isovitexin was excreted with feces in the original form, isovitexin was also catabolized by intestinal microflora. Zhang et al [18] proposed the metabolic pathway of isovitexin degradation initiated by the intestinal microflora; *i.e.*, the C-glucoside of isovitexin was hydrolyzed by deglycosylation, and then phloroglucinol and phloretic acid are produced from the A-ring and B-ring of apigenin, respectively, by cleavage of the C-ring. In this study, recovery amounts in the feces were approximately half of the administered isosaponarin, indicating that the other half was considered to be catabolized to the small phenolic molecules such as phloroglucinol and phloretic acid by intestinal microflora. These phenolic compounds are reported to have a several biological activities; *e.g.*, the protective effects of phloroglucinol on ionizing radiation-induced cell damage through inhibition of oxidative stress *in vitro* and *in vivo* [31] the protective effect of phloroglucinol on myocardial ischaemia-reperfusion injury [32], and the antibacterial activity of phloretic acid [33]. It may be necessary to examine the pharmacokinetics of catabolites such as phloroglucinol and phloretic acid after ingestion of isosaponalin.

5. Conclusion

The present study demonstrated that dietary isosaponarin was hardly absorbed

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into the body, but metabolized to isovitexin in the gastrointestinal tracts. Although most isovitexin was excreted with feces, isovitexin would cause beneficial effects to human health, because several studies suggest the health promoting activity of the consumption of flavonoid *C*-glycosides. Since flavonoid *C*-glycosides have been reported to be hardly absorbed into the body, the mechanism of the activity is still unknown yet. Further study is needed to elucidate the health promoting activity of dietary isosaponarin and its metabolite isovitexin *in vivo*.

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