Daidzein and genistein but not their glucosides are absorbed from the rat stomach

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Abstract Absorption of isoflavone aglycones and glucosides was compared in rats. Daidzein, genistein, daidzin and genistin were orally administered at a dose of 7.9 μmol/kg in 25 mM Na_2CO_3 and next their metabolite concentration in blood plasma was monitored for 30 min. After isoflavone glucosides administration, their metabolites appeared in plasma with a few minutes delay as compared to aglycones, which suggested that aglycones, but not glucosides, were absorbed already in the rat stomach. This observation was confirmed when absorption site was restricted solely to the stomach and absorption was shown to be independent of the vehicle pH used for administration.

Key words: Daidzein; Genistein; Isoflavonoid absorption; Plasma; Stomach; Rat

1. Introduction

Isoflavones are an inherent diet component in populations consuming soy and soy products. The interest in isoflavones increased especially after low incidence of breast, colorectal and prostate cancers had been linked with consumption of diet rich in soy products [1]. Several studies demonstrated beneficial effects of isoflavones which were shown to influence steroid hormone metabolism [2], inhibit cell proliferation and in vitro angiogenesis [3], exert antioxidative activity [4] and be potent inhibitors of tyrosine protein kinases [5]. Nevertheless, some potential adverse effects of soybean isoflavonoids were demonstrated too [6].

Despite isoflavones abundance in soy derived foods and an increasing number of papers published on this topic, their absorption and metabolism have not been well recognized yet. Main soy isoflavones, daidzein and genistein appear in the form of their glucosides, daidzin and genistin, respectively. Absorption of flavonoid glucosides in the human small intestine has already been reported [7], but the question if they are hydrolyzed to their respective aglycones prior to absorption, or absorbed in the intact form still remains open. Recently published papers showed a β-glucosidase active towards flavonoid and isoflavonoid glucosides in the rat [8] and human [9] small intestine. This enzyme exhibits properties similar to those of cytosolic broad-specific mammalian β-glucosidase.

Bearing this in mind, we compared the absorption of daidzein and genistein with the absorption of their respective glucosides, daidzin and genistin, in rats. Since this absorption probably takes place at least in the small intestine, we focused on the first 30 min following oral administration of the compounds studied. We demonstrated that both aglycones and their glucosides are absorbed very fast and by restricting the site of absorption, that daidzein and genistein, but not their glucosides, are absorbed already from the rat stomach.

2. Materials and methods

2.1. Chemicals

Daidzein (> 99%) and genistein (> 97%) were purchased from Fujicco (Kobe, Japan), daidzin (> 99%) and genistin (> 99%) were the generous gifts from the Kikkoman Corp. R and D (Noda, Japan) and sulfatase type H-5 was purchased from Sigma Chemical Company (St. Louis, MO, USA). Other chemicals were of analytical or HPLC grade.

2.2. Animals, isoflavones administration and samples collection

Thirty two Wistar male rats weighing 300–320 g were used; two rats for isolation and identification of isoflavone in blood plasma, six rats for comparison of isoflavone aglycones and glucosides absorption and 24 rats for determination of isoflavones stomach absorption. Animals were supplied by Charles River Japan Inc. (Atsugi, Japan) and kept in a temperature (23 ± 1°C) and light (7.00–19.00 h) controlled room in the institute animal facility for about 1 week before experiments, with free access to tap water and artificial phenolics-free powder diet [10] (Oriental Bioservice Co., Chiba, Japan). Animals to be surgically treated were denied access to food at 9.00, i.e. 24 h before experiment.

The comparison of isoflavone aglycones and glucosides absorption was made on two groups of three rats, of which one received aglycones, the other glucosides. All compounds were administered at the same dose of 7.9 μmol/kg in 25 mM Na_2CO_3 [18]. Following isoflavones administration, blood samples (about 500 μl each) were collected from the tail vein into heparinized tubes at 3, 5, 10 and 30 min, and plasma was prepared by centrifugation for 20 min at 4°C and $1000 \times g$.

For the measurement of absorption restricted to the stomach only, 20 animals were randomly divided into four groups of five rats. Rats were intraperitoneally anesthetized with sodium pentobarbital (40 mg/kg) and remained unconscious throughout the experiment. An incision was made in the abdominal wall, the pylorus was ligated with surgical silk and the abdominal wall was sutured. Daidzein and genistein or daidzin and genistin were dissolved in DMSO on the day of experiment at a concentration of 31.6 mM each. Ten min before administration of isoflavones, aglycones or glucosides, DMSO solutions were mixed with 25 mM Na_2CO_3 (pH 10.6) or water (pH 2 adjusted with HCl) at the rate of 5/95, v/v. Each group received either aglycones or glucosides administered in 25 mM Na_2CO_3 (pH 10.6) or water (pH 2 adjusted with HCl) containing 5% DMSO after control blood samples had been taken.

Blood samples (about 500 μl) were collected from the jugular vein into heparinized tubes at 3, 5, 10 and 30 min following aglycones or glucosides stomach administration and plasma was prepared. At the end of each experiment the animal abdomen was opened again, stomach contents were collected, centrifuged for 5 min at 4°C and $5000 \times g$ and pH of supernatant was measured.

To obtain plasma for verification of the isoflavones identity, two 24 h fasted animals were anesthetized and orally administered daidzein

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and genistein in 2 ml of 25 mM Na_2CO_3 at a dose 10 mg/kg each. Ten min following administration, the abdominal wall was opened, blood was collected from the abdominal aorta into heparinized tubes, and plasma was prepared.

During all experiments, rats received humane care consistent with institutional guidelines.

2.3. Determination of isoflavones in rat plasma

Plasma concentration of isoflavones was measured with the method described in detail elsewhere [11,12]. Briefly, after enzymatic hydrolysis of isoflavone conjugates with H-5 sulfatase, resulted aglycones were extracted with methanol/acetic acid, centrifuged and supernatant was diluted with water and analyzed. Changes introduced in the determination procedure here were dilution of supernatant prior to injection onto HPLC column (TSKgel ODS-80TS, 5 μm, 150×4.6 mm, TOSOH, Japan) with 100 mM lithium acetate (1:1, v/v), composition of mobile phase for HPLC analysis (water/methanol/acetic acid (58/40/2; v/v/v) containing 50 mM lithium acetate) and monitoring of effluent with amperometric electrochemical detector (ICA-3060, TOA, Japan) set at +950 mV.

2.4. Isolation and identification of isoflavones in blood plasma

Isoflavone metabolites present in 4 ml plasma were hydrolyzed with H-5 sulfatase and released aglycones were extracted three times in the final methanol volume of 80 ml. Methanolic extracts were combined and centrifuged for 10 min at 4°C and 5000×*g*. Next, methanol concentration in supernatant was adjusted with water to about 60% and solution was extracted three times with the final methylene chloride volume of 20 ml. Collected methanol/water layers were evaporated at 45°C on a rotary evaporator under partial vacuum. Residue was sonicated with 30 ml of methanol and centrifuged for 10 min at 4°C and 5000×*g*. Next, supernatant was evaporated as above, sonicated with water and applied on water preconditioned Waters Sep-Pak C18 Vac (6 cc) cartridge (Millipore, Milford, MA, USA). The cartridge was washed with 30 ml of water and isoflavones were eluted with 8 ml of 90% methanol. Methanol was evaporated as above, residue redissolved in methanol and subjected to HPLC analysis in a system with diode array detector (SPD-M10A VP, Shimadzu, Japan), TSKgel ODS-80TS column (5 μm, 150×4.6 mm, TOSOH, Japan) and mobile phase of 50% methanol in water (v/v) at 0.8 ml/min flow rate. As a reference, authentic standards of daidzein and genistein methanolic solution was used.

2.5. Isoflavones solubility tests

Isoflavone aglycones and glucosides, at the doses used in this experiment, were solubilized completely at pH 10.6. However, they rapidly precipitated during preparation of the dose at pH 2. To determine the amount of daidzein, daidzin, genistein and genistin solubilized in the vehicles used for administration and additionally in water of pH 7.5 (adjusted with NaOH), appropriate mixtures were prepared as described in Section 2.2. Ten min following preparation, mixtures were filtered through 0.2 μm Puradisc syringe filter (Arbor Technologies, Inc., Ann Arbor, MI, USA). Isoflavones present in filtrates were regarded as solubilized and their content was measured as described in Section 2.3, of aglycones directly and of glucosides after enzymatic release of aglycones.

3. Results and discussion

Concentration of isoflavones in rat plasma was determined with the method previously used in absorption experiments with (−)-epicatechin and quercetin [11,12]. Isoflavones, similarly to other flavonoids, are extensively metabolized after absorption forming glucuronide and/or sulfate conjugates [13,14]. One of the ways to determine the total plasma concentration of absorbed flavonoids is enzymatic hydrolysis of their conjugates and measurement of released aglycones. For that purpose we used H-5 sulfatase, an enzymatic preparation from *Helix pomatia*. Since it has been shown that *Helix pomatia* digestive juice exhibits β-glucosidase activity [15] we confirmed that apart from sulfatase and β-glucuronidase activity, H-5 sulfatase is also a very efficient preparation in deglucosylation of daidzin and genistin. Therefore, we could expand application of this method towards experiments on glucosides absorption despite the controversy [16,17] whether

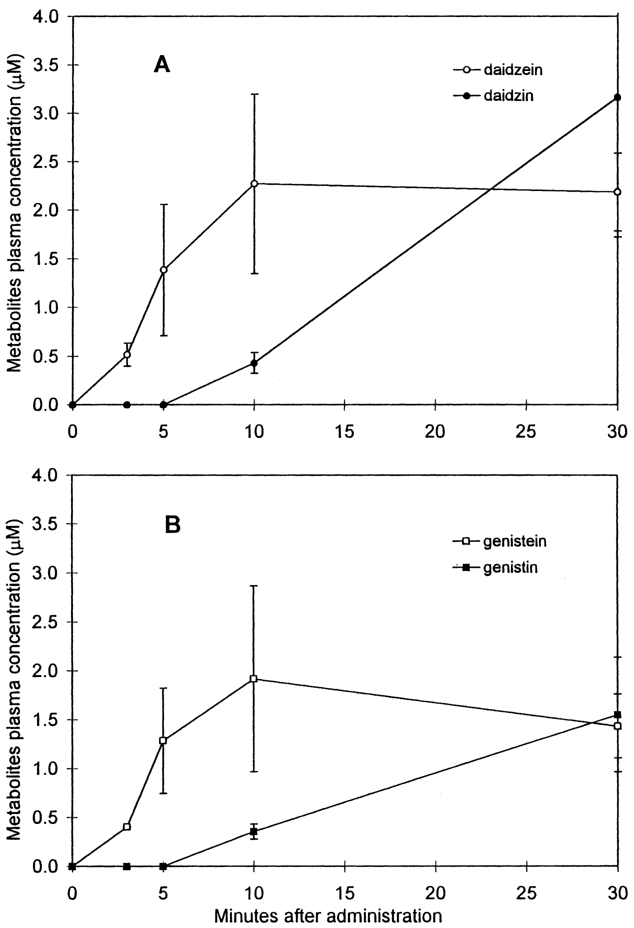

Fig. 1. Isoflavone metabolites concentration in rat plasma after oral administration of 7.9 μmol isoflavone per kg of rat body weight in ~1.5 ml of 25 mM Na_2CO_3; panel A: after administration of daidzein and daidzin, and panel B: after administration of genistein and genistin. Values are expressed as mean ± standard deviation, *n* = 3.

Table 1
Relative solubility of isoflavones at various pH; in 25 mM Na_2CO_3 (pH 10.6), H_2O (pH 7.5) and H_2O (pH 2)

pH	Daidzein	Genistein	Daidzin	Genistin
10.6	100%	100%	100%	100%
7.5	2.4 ± 0.2%	2.9 ± 0.1%	48.9 ± 2.9%	7.8 ± 2.5%
2	2.7 ± 0.4%	3.5 ± 0.6%	48.7 ± 3.8%	5.7 ± 1.4%

Solubility expressed as the percentage of 7.9 μmol isoflavone which passed through 0.2 μm filter from its mixture with 5 ml of test solutions containing 5% DMSO (v/v). Values are the mean ± standard deviation; aglycones *n* = 5, glucosides *n* = 7.

following absorption flavonoid glucosides can be present in plasma in their intact form.

Daidzein and genistein in the blood plasma of isoflavones administered rats were identified based on the appearance of two new peaks in the HPLC chromatographic profile of plasma hydrolyzed with H-5 sulfatase with simultaneous lack of these peaks on the chromatographic profile of H-5 hydrolyzed control plasma, the phenomenon illustrated previously [11,12]. Moreover, retention times of the new peaks and their UV spectra in diode array detection were identical with those of authentic standards of daidzein and genistein.

The simplicity and potential increase in sensitivity of this method must be also stressed, since after one step extraction, without any subsequent purification or concentration steps, the detection limit in plasma was about 0.05 μM and 0.03 μM for daidzein and genistein, respectively, while the actual concentration of these compounds in the sample injected onto the HPLC column was 40 times lower.

Keeping animals on standard chow containing some soy components resulted in constant concentration of isoflavones in blood plasma of about 0.8 μM so it was necessary to switch into artificial phenolics-free diet for about 1 week before the experiments to allow elimination of isoflavones from plasma below the detection limit.

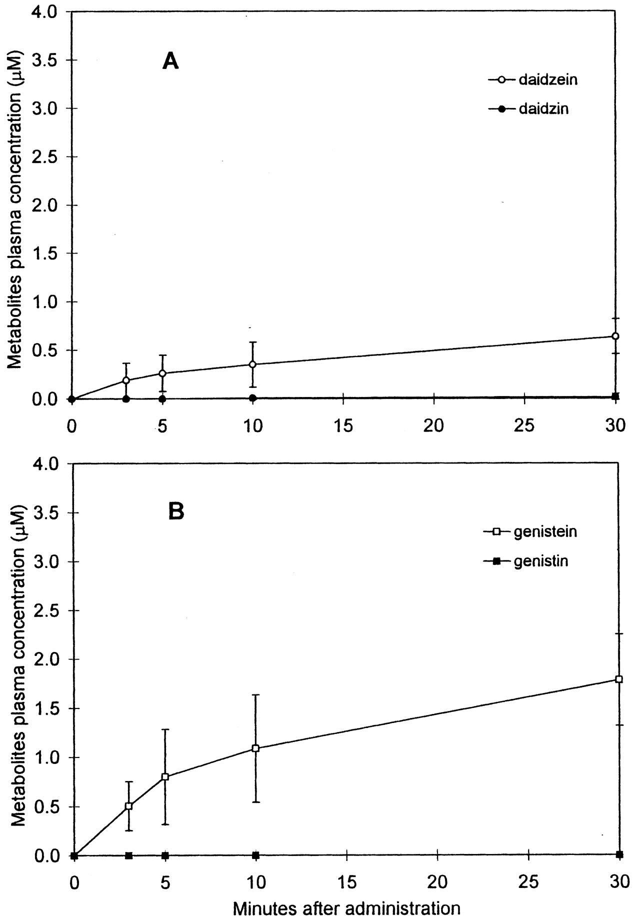

Fig. 2. Isoflavone metabolites concentration in rat plasma after oral administration of 7.9 μmol isoflavone per kg of rat body weight in ~1.5 ml H_2O (pH 2) for absorption site restricted to the stomach; panel A: after administration of daidzein and daidzin, and panel B: after administration of genistein and genistin. Values are expressed as mean ± standard deviation, $n = 5$.

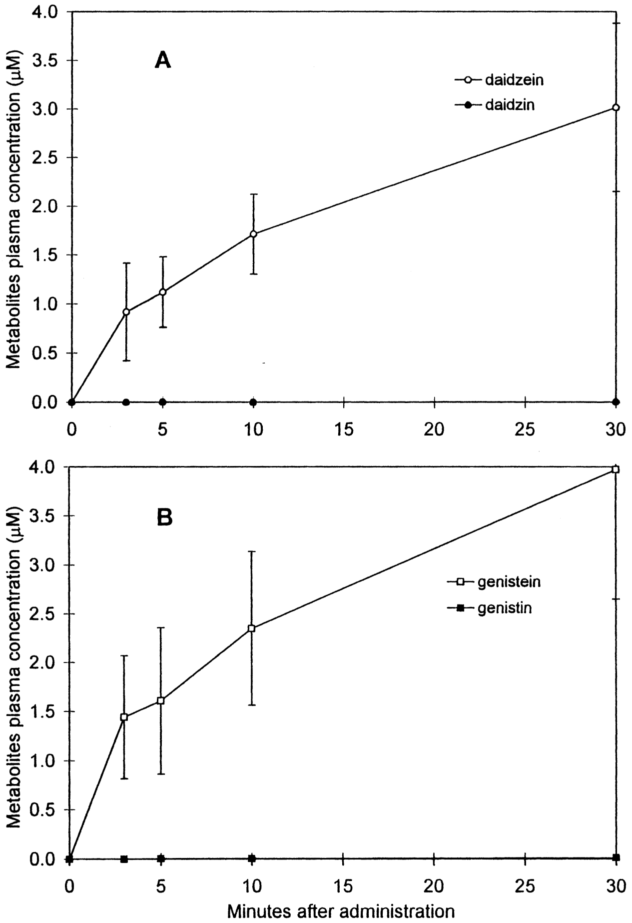

Fig. 3. Isoflavone metabolites concentration in rat plasma after oral administration of 7.9 μmol of isoflavone per kg of rat body weight in ~1.5 ml 25 mM Na_2CO_3 (pH 10.6) for absorption site restricted to the stomach; panel A: after administration of daidzein and daidzin, and panel B: after administration of genistein and genistin. Values are expressed as mean ± standard deviation, $n = 5$.

For the comparison of isoflavone aglycones and glucosides absorption in rats, the compounds were administered in 25 mM Na_2CO_3 as in the other study [18], where genistein and genistin absorption was compared in the rodent model. This time, the dose administered was 10 times lower and isoflavone content in plasma was monitored for the first 30 min following administration (Fig. 1). Rats rapidly absorbed aglycones, and isoflavone metabolites were found in the rat blood plasma already 3 min after administration. However, in the case of glucosides administration, metabolites were found in plasma with a few minutes delay as compared to aglycones. This delay was attributed to the time required by the administered glucosides dose to pass through the stomach contents before it reached the duodenum where glucosides were absorbed, while the aglycones were absorbed already from the stomach. Recently published data on β-glucosidase active towards flavonoid glucosides [8,9] and the profile of glucosides initial absorption in this study suggest that their hydrolysis is necessary prior to absorption and that this glucosidase activity is not present in the rat stomach. However, some hydrolytic potential of the 15 day old rat stomach towards amygdalin β-glucoside was shown previously [19].

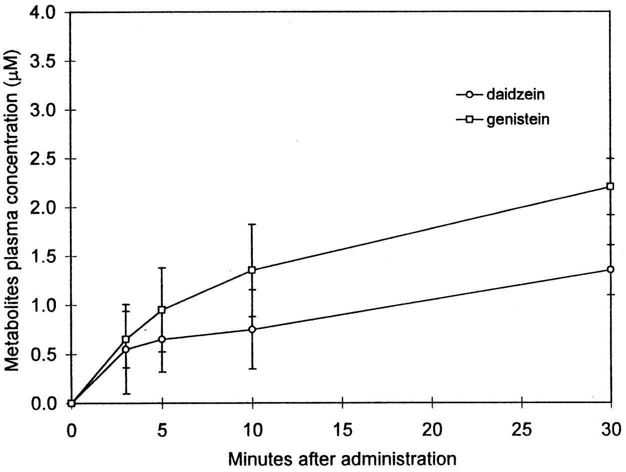

Fig. 4. Isoflavone metabolites concentration in rat plasma after oral administration of 7.9 μmol of isoflavone aglycones per kg of rat body weight when absorption site was restricted to the stomach; 24 h before administration the dose of daidzein and genistein was solubilized in ~1.5 ml 25 mM Na_2CO_3 and acidified to pH 6.2. Values are expressed as mean ± standard deviation, $n = 4$.

The possibility of aglycones but not glucosides stomach absorption was studied when isoflavones were administered to surgically treated rats in which absorption site was restricted to the stomach only. Since isoflavone aglycones and glucosides are soluble in weak alkaline conditions, in a preliminary experiment on animals with ligated pylorus the compounds were administered in 25 mM Na_2CO_3, as in the experiment comparing isoflavone aglycones and glucosides absorption in non-operated rats. Already this preliminary trial showed that aglycones, but not glucosides, were absorbed from the rat stomach (data not shown). Absorption of some drugs from the stomach is known and depends on specific physicochemical characteristics of the compound, its solubility or adsorption to food particles [20,21]. Hence, in order to find the influence of pH on stomach absorption, isoflavones were administered in vehicles at pH 10.6 and pH 2. Since at pH 10.6 isoflavones stomach absorption was DMSO independent, preliminary solubilization of isoflavones in DMSO was introduced. Thus, as described in Section 2.2, introduction of 5% DMSO to the vehicle significantly facilitated the doses preparation, especially at pH 2.

Since the stomach produces acidic secreta, the pH of the stomach contents was measured at the end of each experiment. In the groups administered with isoflavones at pH 10.6, the final stomach contents pH was about 7.5, while in those which received compounds at pH 2 this value remained almost unchanged. To measure the influence of pH on the solubility of administered isoflavones, solubility tests were made at pH 2, 7.5 and 10.6 (Table 1).

The absorption profiles of isoflavones administered to operated rats at pH 10.6 and pH 2 are shown in Figs. 2 and 3, respectively. It is clear from them that daidzein and genistein were absorbed from the stomach while their respective glucosides were not. Moreover, this phenomenon did not depend on the solubility of the administered compounds or the vehicle pH, since glucosides were better solubilized than their aglycones (Table 1). Particularly at pH 2 daidzin was found to be solubilized significantly better than daidzein and yet not absorbed. Results of solubility tests for aglycones at pH 7.5 and pH 2 were not significantly different (Table 1), and considering them versus absorption it is difficult to distinguish whether better absorption in animals given the compounds at pH 10.6 was related to their 100% solubility at the moment of administration, stomach contents pH during absorption, or both, especially when the initial differences in aglycone solubility at pH 10.6 and pH 2 were not directly reflected in the extent of their absorption.

As isoflavones were administered in two extreme pH conditions resulting in total and low solubilization, as well in the presence of DMSO which might be an absorption facilitating factor in the case of low solubilized isoflavones dose, an additional experiment on rats with ligated pylorus was carried out at pH 6.2. This additional trial was limited to daidzein and genistein only since their respective glucosides were not absorbed in any of the previously described conditions. Prior to administration of the same dose as above, the compounds were solubilized in 25 mM Na_2CO_3, solution was acidified to pH 6.2 with HCl and left for 24 h. Again, after administration in this similar to water condition, isoflavone aglycones were absorbed from the rat stomach (Fig. 4) demonstrating that this process was DMSO independent.

Another observation coming from this study is that at pH 2 and pH 6.2 genistein was better absorbed from the stomach than daidzein although high individual variations made this difference significant only after 30 min following administration. Yet, when considering each animal separately, genistein was better absorbed than daidzein at every sampling point.

The stomach absorption demonstrated here for daidzein and genistein may also be true for other flavonoid aglycones and it seems to make a substantial difference between aglycones and glucosides absorption.

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