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Effects of Fructose-Isoflavone Diet on Plasma Isoflavonoids and Cecal Enzyme Activity in Mice

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This paper studies the effects of fructose diet on plasma isoflavonoids and floral enzyme activities in adult mice. Male 6-week-old mice were fed a fructose-isoflavone (FI) diet or a starch-isoflavone (SI) diet for four weeks. Plasma daidzein and equol were analyzed by HPLC, and cecal bacterial β-glucosidase and β-glucuronidase activities were also measured. The plasma equol concentration was significantly higher in the FI diet group as compared to the SI diet group. No significant difference was observed in the plasma daidzein concentration between the two dietary groups. There were no significant differences in cecal β-glucosidase activities between the FI diet group and the SI diet group. However, the ratio of cecal β-glucuronidase activity to β-glucosidase activity was significantly higher in the FI diet group. The present study suggests that a high content of fructose in the diet may affect the biological activity of dietary isoflavones on the host.

Keywords: fructose, starch, daidzein, equol, cecal enzyme activity

Daidzin is a major component of soy isoflavones. It is also reported that isoflavones might be partially responsible for the risk-lowering effects of soy as related to cardiovascular disease (Anthony *et al.*, 1998). Isoflavones belong to the group phytoestrogens which are estrogenic compounds found in plants. The classical definition of phytoestrogens refers to compounds that exert estrogenic effects on the central nervous system, induce estrus, and stimulate growth of the genital tract of female animals (Lieberman *et al.*, 1996). Equol is a metabolic product of daidzein produced by intestinal flora (Bowey *et al.*, 2003), and is considerably more estrogenic than daidzein (Shutt & Cox, 1972). The structure of daidzein and equol are shown in Fig. 1. Intestinal flora play a key role in the metabolism and bioavailability of isoflavones (Setchell *et al.*, 1984). King & Bursill demonstrated that genistein (aglycone of genistin) and daidzein (aglycone of daidzin) are absorbed by humans (King & Bursill, 1998). Human metabolism and excretion of isoflavones following the consumption of soy products exhibit considerable variation (Kelly *et al.*, 1995; Xu *et al.*, 1995). Setchell *et al.* demonstrated that isoflavone glycosides were not intact when absorbed across the enterocyte of healthy adults, and their bioavailability required initial hydrolysis of the sugar moiety by intestinal β-glucosidases prior to uptake (Setchell *et al.*, 2002). Thus, bioavailability of isoflavones seems to differ based on the presence of their sugar moiety. Rowland *et al.* (2000) demonstrated that humans who excrete a high concentration of equol consumed less fat and more carbohydrate as a percentage of energy than those who excrete a low concentration of equol. The bioavailability of isoflavonoids seems to be affected by the composition of the diet. However, there are few reports of the effects of carbohydrates on the plasma isoflavonoids, or on cecal enzyme activity. The present study was undertaken to investigate the effects of a fructose diet containing isoflavones on plasma isoflavonoids and cecal enzyme activity in mice.

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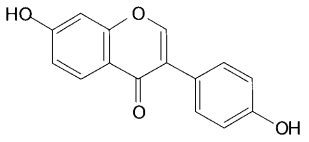
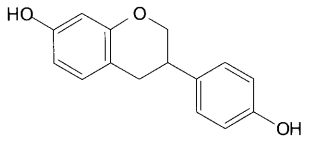
Materials and Methods

Materials A soybean extract with high isoflavone-glucoside content prepared from soybean was provided by Fuji Oil Co., Ltd. (Osaka, Japan). The soybean extract contained 31.1% (w/w) daidzin, 9.6% (w/w) genistin and 38.5% (w/w) gycitin. Daidzein, which was used as a standard for HPLC analysis, was purchased from Fujicco (Kobe, Japan). Equol was purchased from Extrasynthese (Genay, France). β-Glucuronidase type H-5, *p*-nitrophenyl-β-D-glucopyranoside, and p-nitrophenyl-β-D-glucuronide were purchased from Sigma (St. Louis, MO, USA).

Treatment of animals Male Crj: CD-1 (ICR) mice (5-week-old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The mice were randomly divided into two groups of six animals each. The animals were housed in suspended stainless-steel cages with wire mesh bottoms, in a room kept at 24±0.5°C and a relative humidity of 65%, with 12-h periods of light and dark. They were fed a starch-based diet for 1 week. After one week, the diet was replaced with a fructose-isoflavone diet (FI) or starch-isoflavone (SI) diet, which the mice received for four weeks. The composition of each diet is shown in Table 1. The SI diet contained 59.8% starch and a 0.4% isoflavone mixture, while the FI diet contained 20% fructose in place of starch. Body weight and food consumption were measured during the experiment. After the diet feeding period, the mice were sacrificed and blood and cecal contents were collected. The blood samples were then centrifuged, and the plasma was stored at −80°C until HPLC analysis for isofla-



Daidzein



Equol

Fig. 1. The structure of daidzein and equol.

Table 1. Composition of the experimental diet.

Ingredient	Starch diet (%)	Starch-isoflavone (SI) diet (%)	Fructose-isoflavone (FI) diet (%)
Casein	20	20	20
L-Methionine	0.3	0.3	0.3
Corn starch	60.2	59.8	39.8
Sucrose	4.8	4.8	4.8
Fructose	—	—	20
Corn oil	5	5	5
Cellulose powder	5	5	5
Mineral mixture[a)]	3.5	3.5	3.5
Vitamin mixture[b)]	1	1	1
Choline bitartrate	0.2	0.2	0.2
Isoflavone mixture	—	0.4	0.4

[a)] Mineral mixture was prepared according to the AIN-76 formulation. [b)] Vitamin mixture was prepared according to the AIN-76 formulation.

vonoids. Cecal contents were stored at −80°C until measurement of cecal enzyme activities.

This study was carried out in accordance with the "Guidelines for Animal Care and Experimentation" of the National Food Research Institute.

Analysis of plasma isoflavonoids For analysis of plasma isoflavones, 200 μl of plasma was added to 200 μl of β-glucuronidase type H-5 solution (35 mg/ml, Sigma, MO, USA) in 0.2 M sodium acetate buffer (pH 5.0). Next, the mixture was incubated at 37°C in a shaking water bath for 2 h, then treated with 3,600 μl of methanol/acetic acid (100/5, v/v), vortexed for 30 s, sonicated for 30 s, vortexed again for 30 s, and centrifuged for 10 min at 4°C and 5000×*g*. The supernatants were transferred to an eggplant-type flask and evaporated completely by a rotary evaporator. The sample was then dissolved with the mobile phase of the HPLC system at the same volume of plasma and filtered by a 0.2 μm filter. For HPLC analysis, we injected 20 μl of each preparation into a 250×4.6 mm Capcell Pak C18-5μ column (Shiseido, Tokyo, Japan). To detect isoflavonoids, we used ECD with a guard cell (model 5020), and an analytical cell (model 5010) (Coulochem II; ESA Inc., Bedford, MA, USA). The mobile phase consisted of methanol/acetic acid/water (28:5:67, v/v/v). The running conditions of HPLC were as follows: column temperature, 40°C; flow rate, 1 ml/min; guard cell, +850 mV; and analytical cell, +300 mV for electrode 1 and +800 mV for electrode 2. Electrochemical data from electrode 2 were collected. These conditions allowed a determination of daidzein (retention time=28.8 min) and equol (retention time=41.8 min).

Measurement of enzyme activity We measured enzyme activities as previously described (Rowland et *al.*, 1983). A 1 : 100 cecal suspension was prepared in 0.1 M phosphate buffer (pH 7.0), and nonbacterial debris was removed by centrifugation at 700×*g* for 2 min. The supernatant fluid was used immediately for the β-glucosidase and β-glucuronidase assays. β-Glucosidase activity was measured with *p*-nitrophenyl-β-D-glucopyranoside as the substrate. β-Glucuronidase activity was measured with *p*-nitrophenyl-β-D-glucuronide as the substrate. β-Glucosidase and β-glucuronidase activities were expressed as μmol *p*-nitrophenol that liberated/60 min/g wet weight of cecal contents.

Statistics The data were expressed as the mean±SE. The Student's *t*-test was used to determine the statistical significance of the differences between mean values.

Results

Body weight, food consumption, and cecal contents No significant differences were observed in body weight (g) or food consumption (g/day) between the mice fed the FI and SI diets. There were no significant differences in the wet weight (g) of cecal contents between the FI diet group and SI diet group.

Plasma isoflavones and cecal isoflavone aglycones The plasma equol concentration was significantly higher in the FI diet group as compared to the SI diet group (Fig. 2). Though the plasma daidzein concentration tended to be high in the FI diet group, no significant difference was observed in the plasma daidzein concentration between the two dietary groups.

Cecal enzyme activity Cecal β-glucuronidase activities tended to be high in the FI diet group though no significant difference between the groups was observed in these activities (Fig. 3). Nor were there any significant differences in cecal β-glucosidase activities between the two groups. However, the ratio of cecal β-glucuronidase activity to β-glucosidase activity was significantly higher in the FI diet group (Fig. 3).

Discussion

The plasma daidzein concentration tended to be high in the FI diet group, and in this group the plasma equol concentration was also significantly higher. It has been reported that daidzein

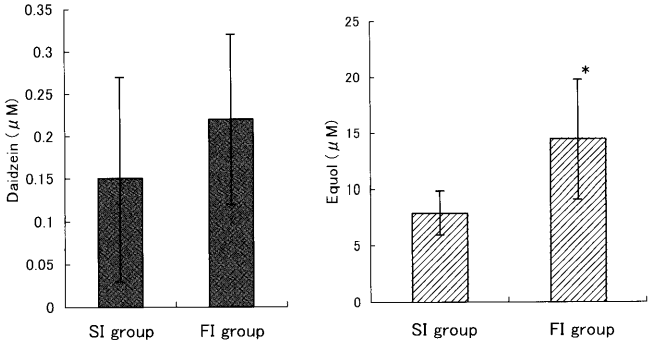


Fig. 2. Plasma daidzein and equol (aglycones+metabolites) concentrations of the mice fed the FI diet or the SI diet for four weeks. Values are means±SD. *Significantly different ($p<0.05$) from the SI diet group.

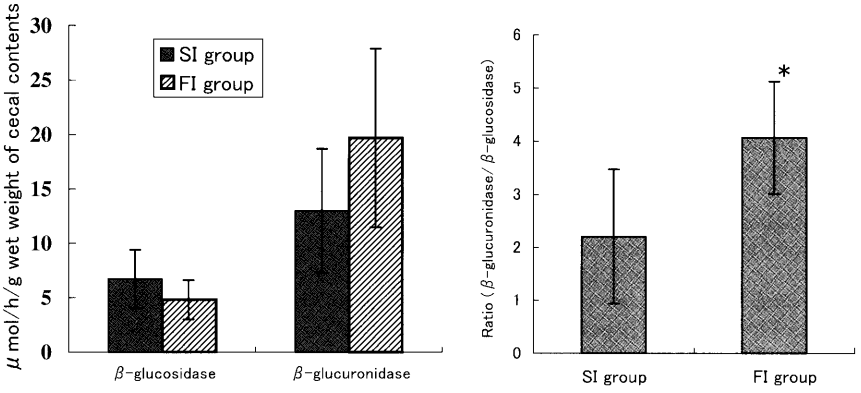


Fig. 3. Cecal β-glucosidase and β-glucuronidase activities and ratio of β-glucosidase activity/β-glucuronidase activity of the mice fed the FI diet or the SI diet for four weeks. Values are means±SD. *Significantly different ($p<0.05$) from the SI diet group.

absorbed from the intestines is partially excreted into bile as daidzein conjugates such as glucuronide, sulfate, and sulfate/glucuronide forms (Yasuda *et al.*, 1994). It has been demonstrated that daidzein conjugates excreted into bile undergo enterohepatic circulation (Watanabe *et al.*, 1998). In our study, cecal β-glucuronidase activities tended to be high in the FI diet group. These activities may partially contribute to the hydrolysis of daidzein-glucuronide and the reabsorption of daidzein in the lower intestine.

The isoflavones used in the diet were mainly glucoside forms. β-Glucosidase in both the small intestine and intestinal flora seems to be important for hydrolyzing isoflavone-glucoside. However, no significant differences were observed in the cecal β-glucosidase activities between the two dietary groups. It has been demonstrated that lactase/phlorizin hydrolase (LPH) in the small intestine hydrolyzes flavonoid-glucoside (Nemeth *et al.*, 2003) and seems to be important for the bioavailability of flavonoid-glucoside. It has been reported that fructose elicited an increase in transcription of the LPH gene, and that the transcription of LPH was influenced only slightly, if at all, by glucose intake (Tanaka *et al.*, 1998). The relationship between plasma daidzein concentrations and LPH activity should be studied.

Plasma equol concentration was significantly higher in the FI diet group than in the SI diet group. Equol is a bacterial metabolite from daidzein (Bowey *et al.*, 2003). A sucrose-based diet that contains fructose as a constituent might affect the intestinal flora differently when compared to a starch-based diet (Cresci *et al.*, 1999). It has been demonstrated that pattern and degree of β-glucosidase and β-glucuronidase activities are different in each intestinal bacteria (Tamura *et al.*, 1996). So, the ratio of cecal β-glucuronidase activity to β-glucosidase activity seems to be one of the good indicators of floral metabolic activity. In fact, in our results, the ratio of cecal β-glucuronidase activity to β-glucosidase activity was significantly higher in the FI diet group than in the SI diet group. This result means that the FI diet affected floral metabolic activity differently from the SI diet. Rowland *et al.* (2000) demonstrated that humans who excrete a high concentration of equol consume less fat and more carbohydrate as a percentage of energy than humans who excrete a low concentration of equol. These researchers also suggested that dietary fat intake decreases the capacity of intestinal flora to synthesize equol (Rowland e*t al.*, 2000). This report suggests that dietary composition plays an important role in the conversion of daidzein to equol. Tamura *et al.* (2002) demonstrated that plasma equol concentrations were significantly higher in a potato starch-isoflavone diet group than in a rice starch-isoflavone diet group, the number of bifidobacteria in the former group was significantly higher than that in the latter. These results suggest that not only a higher percentage of carbohydrate in the diet but also the type of carbohydrate might influence the rate of conversion of daidzein to equol. In our study, plasma equol concentration was significantly higher in the FI diet group than in the SI diet group. Equol production from daidzein by intestinal flora might be higher in the FI diet because of different floral metabolic activity in the two groups. However, it has also been reported in chicks that feeding fructose resulted in heavier weights of lower intestine than feeding glucose (Muramatsu *et al.*, 1993). A fructose diet might affect the function of the lower intestine by itself, through the change of floral metabolism, or both. The bioavailability of daidzein in the FI diet might be different from that in the SI diet. In summary, a high content of fructose in the diet may affect the biological activity of dietary isoflavones on the host.

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