

Biotransformation of soy isoflavone-glycosides in laying hens: intestinal absorption and preferential accumulation into egg yolk of equol, a more estrogenic metabolite of daidzein

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Received 6 February 2004; received in revised form 8 June 2004; accepted 11 June 2004

Available online 4 July 2004

Abstract

Dietary soy-isoflavones have recently been noted as phytoestrogens with potentially beneficial effects on human health, and they are biologically transformed in the intestinal tract into aglycones and further into several specific metabolites. Here we report that in laying hens daidzin, a soy isoflavone-glycoside, in the diet was transformed into equol, absorbed, transported in circulating peripheral blood, and preferentially accumulated into egg yolk in its conjugated form. Laying hens were fed experimental diets containing two levels of soy isoflavone-glycosides (177 or 528 mg per 100 g diet) for 21 or 42 days, and blood and eggs were collected at 1- to 9-day intervals. HPLC analyses revealed that most of the isoflavones (daidzein, glycitein, and genistein) and a metabolite, equol, were present in blood and egg yolk in conjugated form. The concentration of equol-conjugates in blood plasma and egg yolk was higher than any of the other three isoflavone-conjugates analyzed and, especially in egg yolk, the equol-conjugates comprised no less than 60% of the total isoflavone-conjugates. The isoflavones, including equol, distributed mostly (95%) in the high-density fraction of blood serum, and more (65%) in the granule fraction of egg yolk. These results raise the possibility that feeding domestic animals soy-based fodder produces animal-based foods rich in a more active form of phytoestrogens.

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Keywords: Soy isoflavone; Egg yolk; Phytoestrogen; Bioconversion; Equol

1. Introduction

Flavonoid is a generic name for the substances with two phenyls connected by three carbon atoms such as the pyran ring, and it is contained in a variety of plants species, especially as seeds. It has a high radical scavenging function in plants and often shows physiological activity in animals when it is ingested as food or feed [1]. Isoflavone is noted for its ability to prevent cancer, menopausal symptoms, cardiovascular disease, and osteoporosis [2–5], whereas some of the negative effects of phytoestrogens including thymic atrophy [6] and pathophysiological effects on reproduction [7] have also been reported.

Soy isoflavones exist naturally in the glycoside form [8], and some studies have shown the differences in pharmacokinetics between glycoside and aglycone isoflavones in human and animals [9–12]. Dietary isoflavones are hydrolyzed by intestinal glucosidases, which release the aglycones, daidzein, genistein, and glycitein [13,14]. These are absorbed or further metabolized to many specific metabolites, including equol, probably by intestinal fermentation. This metabolic pathway by intestinal microorganisms may clinically be relevant to the efficacy of soybean isoflavones, because the estrogenic potency of equol is an order of magnitude higher than its precursor, daidzein [15,16]. The binding affinity of equol for human estrogen receptors (ER α and ER β) was found to be similar to that of genistein, but equol induced transcription more strongly than any other isoflavone, especially with ER α [17]. Equol is thought to hold the clue to the mechanism of action and

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effectiveness of soy in studies of hormone-dependent diseases in several clinical studies [18]. However, about 30–50% of the adult population do not produce equol, even when challenged daily with soy foods. The reason is suggested to be due to the difference in intestinal bacterial flora [19,20]. Some data indicate the importance of taking equol status into consideration in several clinical studies on the effects of soy isoflavones on prevention of osteoporosis, relief for hot flashes associated with menopausal estrogen deficiency and regulation of plasma cholesterol [2–5]. These reports propose the importance of biotransformation of soy isoflavones to the equol as a more potent estrogenic isoflavone.

Defatted soybean meals, which contain isoflavones, have widely been used in animal feeds. Isoflavones ingested as feeds are believed to be biologically transformed to their metabolites and sometimes accumulated in animals. Indeed, equol has been identified in the urine of macaque monkeys [21], chimpanzees [22], rats [23], and domestic fowl [24], indicating the metabolic conversion of isoflavone to a more active form of phytoestrogen. Furthermore, we have reported on the transfer of dietary isoflavone aglycones into the hens' egg yolk [25]. From the view-points of food safety and food functionality, it is important to elucidate the absorption and dynamics of natural soy isoflavones administered as feed to domestic animals.

In this study, to obtain basic data on the biological transformation and transport of dietary isoflavones in birds, we administered natural soy isoflavones as feed to laying hens, and monitored the levels of isoflavones and their metabolites in blood and eggs by using HPLC and LC-MS analyses in combination with an enzymatic de-conjugation treatment. Equol, a metabolite of daidzein, was found to exist in hens' blood and to accumulate dominantly in egg yolk. Possible mechanisms of equol accumulation into egg yolk and the influence of daily intake of equol-enriched eggs on human health are discussed.

2. Materials and methods

2.1. Preparation of isoflavone-enriched diet

Control diet was mainly prepared from yellow corn, canola meal, fishmeal and corn gluten meal, free from soy based material as in the previous paper [25]. Isoflavones were extracted from soybean hypocotyls and purified at an industrial level (Honen Isoflavone-80, Honen, Tokyo, Japan). Experimental isoflavone-enriched diets were prepared by adding 400 $\mu\text{mol}/100\text{ g}$ (180 mg/100 g) as low dose (diet I), and 1200 $\mu\text{mol}/100\text{ g}$ (530 mg/100 g) as high dose (diet II) of the purified isoflavone, to the control diet. This isoflavone quantity in diet I is estimated to contain 10% soy hypocotyl or 40% of soybean meal.

2.2. Feeding of laying hens and collection of blood and eggs

All animal experiments described below were conducted in accordance with current legislation on animal experimentation in Japan. Eighteen White Leghorn hens (13-month old) were fed the control diet for 2 weeks before the experiment. The 18 hens were divided into three experimental groups (six hens per group), and fed 100 g of control diet, diet I (low dose), and diet II (high dose) per day, respectively. Three hens in each group were fed for 21 days for blood sample collection, while the other three were fed for 42 days for egg sample collection. Hens were given free access to experimental diets and tap water. Eggs were collected in the morning on days 0, 3, 6, 12, 18, and 42, and cracked to separate egg yolk from egg white. Plasma or serum was prepared as described previously [25] from blood collected on days 0, 1, 3, 6, 12, and 21 from the beginning of the experiment. All of the egg and blood samples were preserved at $-60\text{ }^{\circ}\text{C}$ until use.

2.3. Extraction and β -glucuronidase treatment of isoflavones from soybean hypocotyls

Isoflavones were extracted from 300 mg of soybean hypocotyls with 10 ml of sodium acetate buffer (pH 5.0) by stirring for 1 h, and the extractant was incubated with 1000 Fishman units of β -glucuronidase (*Helix pomatia*, Wako, Osaka, Japan) at $37\text{ }^{\circ}\text{C}$ for 2 h. After boiling for 5 min, these extracts were subjected directly to HPLC.

2.4. Extraction of isoflavones from diet, blood, and egg yolk

Isoflavone content of the diets was determined by the HPLC method after 70% aqueous ethanol extraction was performed as noted in a previous paper [25]. In the case of total isoflavone aglycones in egg yolks, the isoflavones were extracted with organic solvent after treatment with β -glucuronidase (*Helix pomatia*, Wako). Namely, 10 g of egg yolk was dissolved in 10 ml of 0.1 M sodium acetate buffer (pH 5.0) and incubated overnight at $37\text{ }^{\circ}\text{C}$ with 7000 Fishman units of β -glucuronidase. After the enzyme reaction, 35 ml of ethanol was added, and the mixture was centrifuged at $2000 \times g$ for 10 min. The supernatant was collected, and the precipitate was resuspended in 10 ml of 70% ethanol and centrifuged at $2000 \times g$ for 10 min. This second supernatant was mixed with the first supernatant, and distilled water (a half volume of the extract) was added. Lipids were then extracted twice with 5 ml of hexane. Then the ethanol/water layer was collected and extracted thrice with diethyl ether (69, 20 and 15 ml). The diethyl ether layer was evaporated to dryness as a sample for HPLC. In the case of those without β -glucuronidase treatment from egg yolk, isoflavones were extracted with 70% ethanol and the extracts were passed through a membrane (MICROCON YM-10, Millipore, Bedford, MA) to remove large size proteins, which disturb the

following evaporation process. The filtrates were evaporated and subjected to HPLC analysis.

The following method was used for the preparation of the hens' plasma and serum samples. One milliliter of 0.1 mol/l sodium acetate buffer (pH 5.0) and 1000 Fishman units of β -glucuronidase was added to 1 ml of plasma, and the mixture was incubated at 37 °C for 2 h. The isoflavone was collected from the hydrolysate by extracting it twice with 4.7 ml of ethanol and 1 ml of 70% ethanol. The extract was evaporated to dryness and dissolved in 1 ml of 70% aqueous ethanol before HPLC analysis.

2.5. HPLC analyses of isoflavone

Separation of isoflavones was achieved using a class Vp HPLC system (Shimadzu, Kyoto, Japan) equipped with a YMC-Pack ODS-AM-303 column (250 × 4.6 mm, YMC, Kyoto, Japan), by eluting with a linear gradient of distilled water/acetonitrile from 85:15 to 65:35 containing constant 0.1% (v/v) acetic acid for 50 min. The solvent flow rate was 1 ml/min and the eluted compounds were detected by monitoring absorbance at 254 nm using a photodiode array detector (SPD-M10Avp, Shimadzu). The isoflavone contents were estimated by comparison with the properly dissolved reagent grade of each isoflavones (Nacalai Tesque, Kyoto, Japan) as standards. For equol, the sample was eluted by a linear gradient of distilled water/acetonitrile from 85:15 to 72.5:27.5 in 0–5 min, then 72.5:27.5 to 67.5:32.5 in 5–40 min, containing constant 0.1% (v/v) acetic acid, and monitored by measuring absorbance at 282 nm. The equol contents were estimated by comparison with the properly dissolved reagent grade of equol (Wako) as a standard.

2.6. Liquid chromatography mass spectrometry (LC-MS) analyses

Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) system (Micromass, Altrincham, UK) was used for identification of isoflavone metabolites in hens' plasma. A plasma sample was applied to the YMC-Pack ODS-AM-303 column, eluted by a linear gradient of 0.1% aqueous acetic acid/acetonitrile 85:15 to 65:35 in 50 min at a flow rate of 1 ml/min. The desolvation temperature was 250 °C and the ion source temperature was 150 °C. For MS measurements, positive ions were acquired in full scan (m/z 100–1000 in 2-s cycle time) at a sampling cone-skimmer potential of 30 V.

2.7. Fractionation of blood serum and egg yolk

The serum (4 ml) gathered from hens that had been administered diet II for 10 days was used for the analyses. According to the method of Hatch and Lee [26], 2 ml of the sodium chloride solution ($d = 1.006$ g/ml) was layered over the serum, and centrifuged at $26,000 \times g$ for 30 min. Two milliliters of white turbid upper layer (low-density fraction)

and 4 ml of clear bottom layer (high-density fraction) were collected. For egg yolk fractionation, the eggs were collected on the tenth day from the diet II-group hens. Egg yolk (10 g) was mixed with 20 ml of PBS and separated into yolk-plasma and yolk-granule fractions by centrifugation at $4000 \times g$ for 15 min. All of the serum and yolk fractions were subjected to protein composition analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli's method [27] and isoflavone quantitative analysis.

2.8. Statistics

Data are presented as means \pm S.D. Statistical analyses were carried out with Excel for Windows 98 (Microsoft, Redmond, WA). The significance was tested using two-way repeated-measures ANOVA and Kruskal–Wallis test. Student's *t*-test was adopted to assess any differences among initial compound and the each time points of isoflavone contents. Differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. Determination of isoflavones in the experimental diet, hens' blood, and egg yolk

Isoflavone-glycosides as well as their aglycones extracted from soybean hypocotyl were analyzed successfully by HPLC. A typical elution profile is shown in Fig. 1A. Three kinds of *O*-malonylisoflavone glycosides (*O*-malonyldaizin, *O*-malonylglycitin, and *O*-malonylgenistin) were identified as the major peaks because of their higher UV absorbance at 254 nm, while isoflavone aglycones (daidzein, glycitein, and genistein) were the smaller peaks eluted later from the column. The isoflavone-glycosides (daidzin, glycitin, and genistin) were also identified as minor peaks eluted ahead of the other kinds of isoflavones. Thus, various forms of isoflavones could be analyzed simultaneously by a single chromatogram of this HPLC system. To determine isoflavone content of the experimental diet and background level of the control diet, these diet samples were subjected to the HPLC analysis for isoflavones. As summarized in Table 1, the total isoflavone contents of diet I and diet II were determined to be about 180 mg/100 g (400 μ mol/100 g) and 530 mg/100 g (1200 μ mol/100 g), respectively. These values as well as the composition of major isoflavone components were in good agreement with the theoretical values expected from the amount of purified isoflavones added to the diets.

Isoflavones administered to hens as diet were expected to be deglycosylated, metabolized, and conjugated completely or partially during digestion and absorption in the hens, resulting in the production of multiform isoflavones. Therefore, in the present study, all forms of isoflavones were

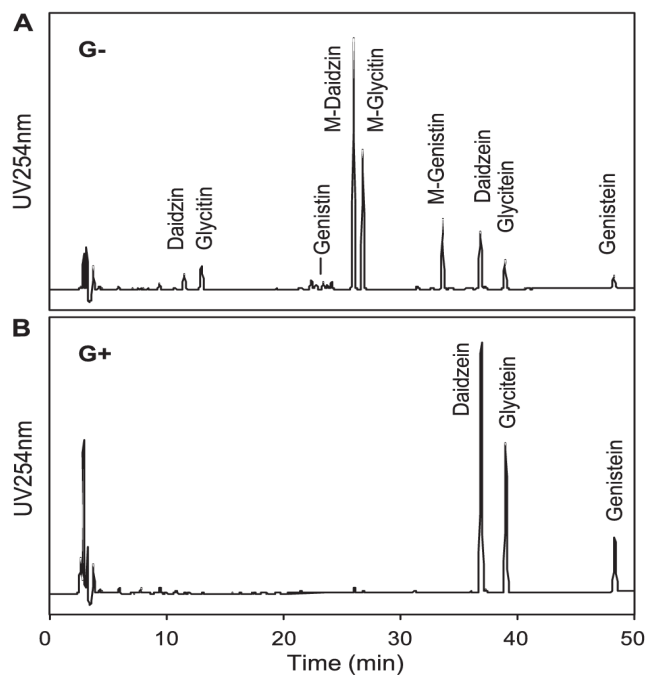


Fig. 1. Complete conversion of various isoflavone-glycosides into aglycone by β -glucuronidase treatment. HPLC chromatograms of extract from soybean hypocotyl by water (G $-$), and its hydrolyzate by β -glucuronidase (G $+$). The same sensitivity setting was used for each chromatogram. Abbreviations are as follows: M-Daidzin, 6''-O-malonyldaidzin; M-Glycitin, 6''-O-malonylglycitin; M-Genistin, 6''-O-malonylgenistin.

determined as aglycones after deglycosylation and deconjugation by the β -glucuronidase treatment. Firstly, to determine whether the β -glucuronidase treatment can convert isoflavone-glycosides to aglycones, the isoflavone-glycosides extracted from soybean hypocotyls were analyzed by HPLC after the enzymatic treatment. As shown in Fig. 1B, all peaks of the isoflavone-glycosides disappeared, whereas the aglycone peaks increased in a compensative manner, indicating that all forms of isoflavone-glycosides could be converted to aglycones even by the deconjugation enzyme, β -glucuronidase. Secondly, the isoflavones extracted from the blood serum and egg yolk samples from the isoflavone-enriched diet group (diet II) were analyzed with and without

Table 1
Content of the isoflavones in the experimental diets (mg/100 g)*

	Control diet	Diet I	Diet II
Daidzin	trace	37.5 \pm 0.8	111.6 \pm 2.5
Glycitin	1.0 \pm 0.0	109.4 \pm 2.0	325.6 \pm 6.8
Genistin	0.5 \pm 0.0	28.1 \pm 0.6	83.7 \pm 1.9
Other isoflavones ^a	0.7 \pm 0.0	2.2 \pm 0.0	7.2 \pm 0.0
Total	2.2 \pm 0.0	177.20 \pm 0.3	528.1 \pm 11.0

* Values are the mean \pm S.D. ($n=3$).

^a Other isoflavones include malonyl glycoside, acetyl glycoside and aglycone of daidzein, glycitein and genistein, respectively.

the β -glucuronidase treatment (Fig. 2). The extract from blood plasma with the β -glucuronidase treatment, but not that without the enzyme treatment, showed peaks corresponding to the aglycones of isoflavones and the daidzein metabolite, equol, in the HPLC chromatogram (Fig. 2A). Several unidentified minor peaks other than isoflavone aglycones appeared after the β -glucuronidase treatment. These peaks also appeared after the enzyme treatment in the chromatogram of the samples from the control diet group (data not shown), suggesting that these minor peaks were unknown conjugated compounds unrelated to isoflavones. Similar results were obtained in the HPLC analyses of the egg yolk samples, except that 3 isoflavone aglycones, daidzein, glycitein, and genistein, were detected even in the samples without the β -glucuronidase treatment.

To characterize these isoflavone-conjugates and/or glycosides detected in the hens' plasma, the LC/MS analysis was done without the β -glucuronidase treatment for the sample from the isoflavone-enriched diet group (Fig. 3). Seven major peaks in the LC elution profile (Fig. 3A) were identified as conjugated compounds containing either daidzein, glycitein, genistein, and equol as a constituent molecule (Fig. 3B) based on the mass spectrum of each peak. As a representative result, the mass spectrum data of peak 1 are shown in Fig. 3C, which gave major signals at m/z 255 and 431 corresponding to daidzein and its glucuronized form. Similarly, peaks 2 and 3 were identified to be the glucuronized form, whereas the other peaks (6–7) could not be identified by the mass data alone. The mass data indicate that none of these peaks corresponded to isoflavone-glycosides.

3.2. Isoflavone levels in blood and egg yolk from hens fed the isoflavone-enriched diets

The laying hens were fed the isoflavone-enriched diets for 21 or 42 days, and they were estimated to consume 180

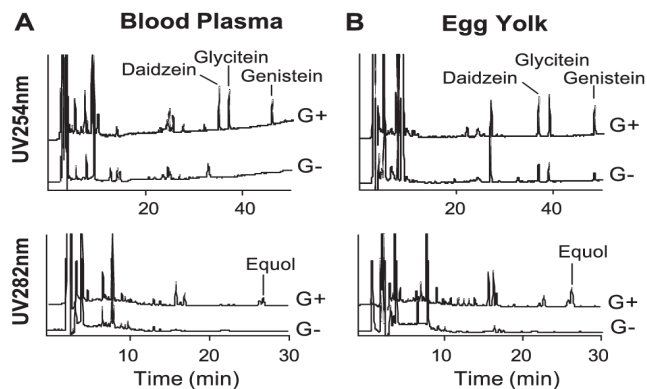


Fig. 2. HPLC analysis of diet-derived isoflavones as their aglycone form in hen's blood and egg yolk. Each chromatogram shows the comparison of β -glucuronidase hydrolysis before analysis. The same sensitivity setting was used in each chromatogram.

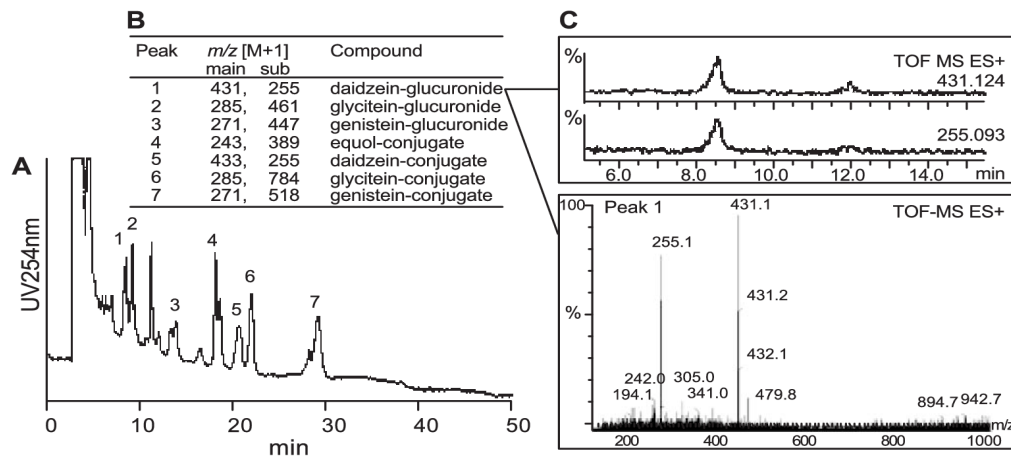


Fig. 3. Identification of the compound in hen plasma by LC-MS. LC chart of ultraviolet 254-nm detected ions ([M + 1]) that were judged as soy isoflavone metabolites from mass spectrum and their estimated compounds are described on the left side. Single ion chromatograms (top) and mass spectrum (bottom) of peak 1 are shown on the right side. Plasma sample had been processed in the same way as in Fig. 2A (G –). LC condition and MS detection parameters are noted in Materials and methods.

or 530 mg of dietary isoflavones per day during the experimental period. No obvious abnormality on hens and eggs was observed for the experimental groups. The total eggs laid by groups I, II, and control group in 6 weeks, were 120, 119, and 119, respectively.

Total isoflavones, including unidentified conjugates, in the hens' plasma and egg-yolk were determined by HPLC after the enzymatic conversion to each aglycone. Fig. 4 shows changes in the isoflavone concentrations of the plasma and

yolk samples collected during the experimental periods (21 or 42 days). In the blood plasma samples from the diet II (high dose) group, the concentrations of the three isoflavones (daidzein, glycitein, and genistein) increased within 24 h after the start of feeding and were then kept nearly constant at levels of about 0.5–1.0 nmol/ml. Equol was also detected as a major compound in the plasma, and changed in a similar manner to its parental molecule, daidzein, except that the equol concentration was slightly higher than that of daidzein.

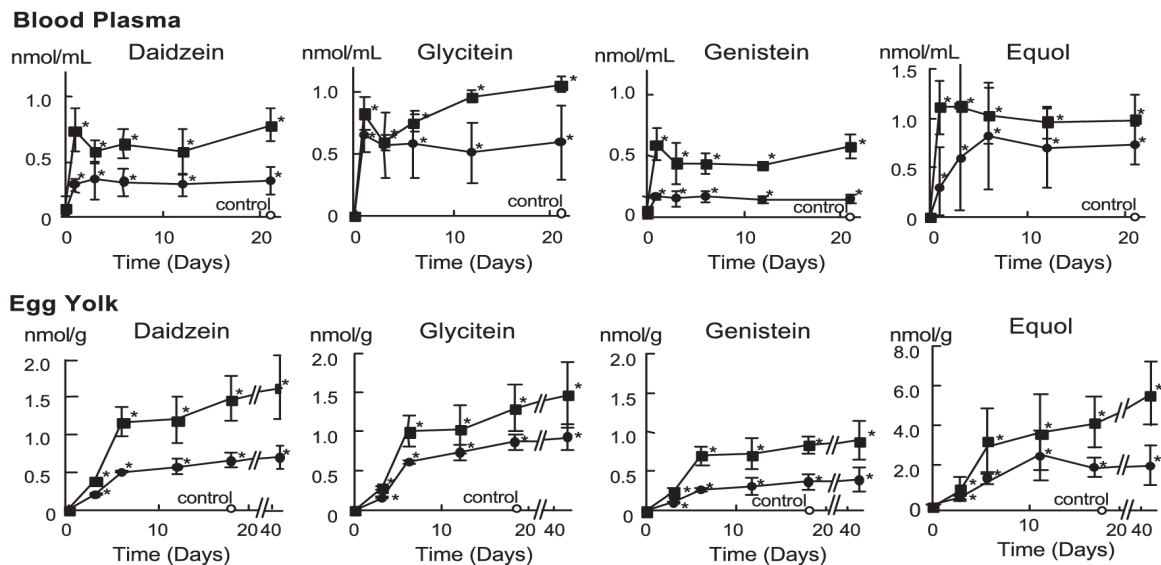


Fig. 4. Changes in the concentration of isoflavones in hen plasma (top) and egg yolk (bottom) during the feeding with experimental diet. The plot marks in graphs show (●) isoflavone diet I group, (■) isoflavone diet II group and (○) control group. Values are the means \pm S.D., $n=3$. Means with * are different from the initial compound at each time point, $P<0.05$. There were no significant differences on the two-way repeated-measures ANOVA and nonparametric Kruskal–Wallis test, $P>0.05$.

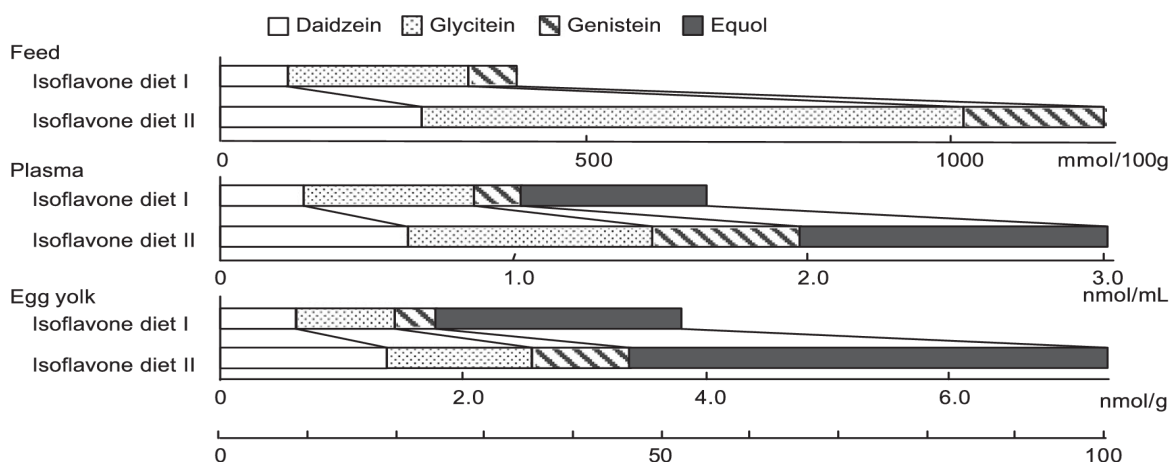


Fig. 5. The composition of the isoflavones in feeds, hen plasma, and egg yolks. The values in feeds were calculated from Table 1. The values in plasma were the average of 15 samples from day 1 to 21, and in egg yolks were the average of 12 samples from day 6 to 42 in Fig. 4.

The isoflavone and equol concentrations in the egg yolk began to increase after a few days' delay compared to those of the plasma, and reached a level of about 0.5–1.0 nmol/ml within 6 days. The equol value continued to increase slowly during the experimental period of 42 days. Interestingly, the equol concentration in egg yolk was much higher than the other isoflavone concentrations in yolk and the equol concentration in plasma. The isoflavone and equol concentrations of blood plasma and egg yolk samples from the diet I group (low dose) showed profiles similar to those of the diet II group described, except that the overall concentrations were at lower levels, but not necessarily one third of those of the diet II group.

The average isoflavone concentrations of the six plasma samples (day 1 to day 21 in Fig. 4) and the six yolk samples (day 6 to day 42 in Fig. 4) are summarized schematically as a relative ratio of three or four compounds (three isoflavones plus equol) in Fig. 5. The ratios of the three isoflavones (daidzein, glycitein, and genistein) in the plasma and yolk were not largely different in spite of the fact that glycitein consisted of no less than 60% of the isoflavones in the experimental diet, suggesting that glycitein was absorbed and/or conjugated less effectively than the other isoflavones. On the other hand, daidzein and equol were the dominant components in the plasma and yolk. Especially in yolk, equol consisted of more than a half of the total isoflavones analyzed. When the isoflavone concentrations were compared between the two groups (diet I and diet II), it was revealed that increasing isoflavones three times resulted in a 1.5- to 3-fold increase in the plasma and yolk isoflavone concentrations.

3.3. Distribution of isoflavones in plasma and yolk

The blood plasma was roughly separated into high and low density fractions, by centrifugation, while egg yolk was separated into granule and yolk plasma fractions for further analyses on isoflavone distribution in blood plasma

and egg yolk. The isoflavones and equol in each fraction were analyzed by HPLC as described above, and summarized in Fig. 6A. Protein components in these fractions were also analyzed by SDS-PAGE (Fig. 6B). The isoflavones and equol existed mostly (about 95%) in the high-density fraction, which contains mostly serum proteins, but only slightly in the lipoprotein-enriched fraction with low density. In the egg yolk, the isoflavones and equol were concentrated more in the yolk granule, in which the lipovitellin/phosvitin complex was selectively accumulated

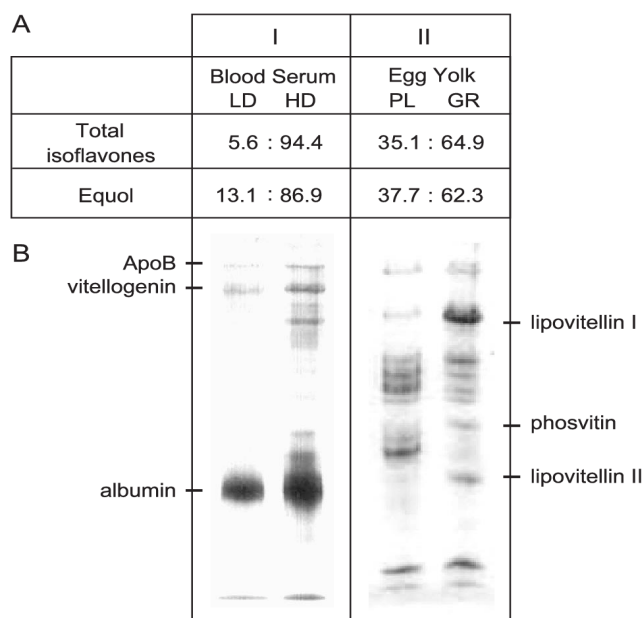


Fig. 6. Comparison of isoflavones and equol proportion in each fraction of centrifuged hens' serum (I) and egg yolk (II). Abbreviations are as follows: LD, low-density fraction; HD, high-density fraction; PL, plasma fraction; GR, granule fraction.

(Fig. 6A and B), though yolk plasma rich in LDL with apoB protein also contained about one third of the yolk isoflavones.

4. Discussion

4.1. Determination of isoflavone-glycosides, conjugates and aglycones by HPLC

Soybean isoflavones and their metabolites were successfully measured in their aglycone-forms by HPLC equipped with a photodiode-array UV detection system after treatment with β -glucuronidase (Figs. 1 and 2). In several previous studies on the blood plasma isoflavones, GC-MS was used more frequently than HPLC with UV, MS and amperometric electrochemical detection systems, probably because the detection sensitivity and separation ability of HPLC were not sufficient for the analyses of blood isoflavones. In the present study, however, the isoflavones could be analyzed quantitatively by UV detection due to the low interference peak from the hen samples and the photodiode-array system, which enabled monitoring of the absorbance at a wide range of wavelengths of eluted isoflavones at the same time.

β -Glucuronidase is a deconjugation enzyme that hydrolyses the β -glycoside bond of glucuronate-conjugated compounds, and has widely been used for the quantitative analyses of isoflavones as aglycones in biological samples such as blood and urine. In the present study, the β -glucuronidase converted not only the glucuronides but also glycosides and malonyl-glycosides to aglycons (Figs. 1 and 2). Previous reports also suggest that β -glucuronidase has arylsulfatase activity [28]. Therefore, we adopted the β -glucuronidase treatment prior to the HPLC analysis for the determination of isoflavones as a sum of various forms of isoflavones.

4.2. Fate of dietary isoflavone-glycosides in laying hens

In the blood plasma of hens fed the isoflavone-enriched diet, various conjugated forms, including glucuronate-conjugates, of isoflavones were detected as the major components, whereas their glycoside forms and aglycones were not detected. These results agree well with the previous reports on mammals such as rat [29] and human [10–12]. Furthermore, the results suggest that the dietary soybean isoflavone-glycosides were deglycosylated in the hen's intestinal tract, absorbed as aglycones, mostly metabolized and conjugated in intestinal epithelial cells, and released in the bloodstream mainly as conjugated forms. Recently, Fang et al. [30] identified 17 isoflavone metabolites in female rat urine by LC/MS/MS analysis, indicating that glucuronide conjugates were the major components in the urine. This agreed overall with the results on hen blood plasma, but the mass data of unknown conjugated forms of isoflavones

determined in the present study (peaks 6–7 in Fig. 3) did not coincide with those of any metabolites. Metabolic systems for isoflavone conjugation in the intestinal epithelial cells might be different between birds and mammals.

The changes in isoflavone concentrations in laying hens' blood and laid egg yolk suggest that isoflavone conjugates in laying hens' plasma were transported and accumulated into egg yolk of the growing oocytes. During the growth and maturation of hens' oocytes, especially before the final 7–11 days of ovulation, the majority of yolk components, such as yolk lipoproteins and some serum proteins, are transported specifically into the growing oocytes from the bloodstream [31]. This time course of yolk deposition in the oocytes appears to correlate with the increasing profiles of isoflavone concentration in the egg yolk (Fig. 4). Therefore, the isoflavone conjugates detected in the egg yolk might be transported from the blood into the growing oocyte possibly as complexes with yolk precursors in laying hens' blood. However, most of isoflavones in the blood plasma were present in the high-density fraction but not the low-density lipoprotein-rich fraction (Fig. 6). These results and the presence of isoflavones in more hydrophilic forms, such as conjugates, than aglycones would rule out the possibility that the dietary isoflavones form complexes directly with yolk precursor lipoproteins, such as vitellogenin, in the laying hens' blood and are co-transported as lipoprotein complexes into the oocyte.

The concentration of isoflavones was consistently higher in the egg yolk than in the blood plasma except at the beginning of the administration experiment (Fig. 4). These high concentrations of isoflavones in egg yolk indicate that the yolk isoflavones are not simply due to contamination with blood plasma components. Especially for equol, the egg-yolk/blood-plasma concentration ratio was about four- to fivefold, and that of yolk-granule/blood-plasma was about six- to eightfold. Such higher concentration of equol in egg yolk suggests that equol conjugates are selectively transported and accumulated from the bloodstream into egg yolk granules. The mechanisms explaining such selective transports of equol remain to be investigated. One hypothesis is that a part of isoflavone conjugates and aglycones are transported into liver, further metabolized into equol conjugates, and secreted again as complexes with unknown compound(s) which are committed to be transported into growing oocytes. The detection of daidzein aglycone but not the equol aglycone in the egg yolk suggests full conjugation of equol and might support this possibility.

4.3. Biological significance of the blood plasma and egg yolk isoflavones as phytoestrogens

The blood plasma from hens, which daily ingested soybean isoflavones, contained isoflavones mostly as conjugates at the level of 1–3 nmol/ml ($\sim 1 \mu\text{g/ml}$). This plasma concentration of total isoflavones is consistent with

those reported previously in experimental models using rat [32] and human [10]. Such levels of plasma isoflavone concentrations seem to have no acute effects, at least harmful ones, on laying hens, because the laying hens themselves and the laid eggs were apparently normal. Furthermore, the differences in the number of laid eggs among the experimental and control groups were not statistically significant. However, known estrogenicity of equol suggests that the increased equol concentration in soy-fed hens could potentially have action in the hen itself as a result of estrogenic action. Some experiments using old-aged hens with decreased estrogen synthesis might show some effect of dietary isoflavones on hens' physiological properties such as egg-laying ability.

The yolk of eggs from the hens fed isoflavone-enriched diets contained isoflavones at higher concentrations ranging at 5–10 nmol/g (2–5 µg/g) as total isoflavones. These included equol, a more estrogenic metabolite, and, furthermore, about 30% of daidzein and genistein existed as active aglycones (Fig. 2). Thus, potential estrogenic activity seems to be much higher in egg yolk than in blood plasma. The egg yolk isoflavones might affect embryonic development and hatching in cases of fertilized eggs. The neonatal chick is known to be very susceptible to estrogen treatment [33], and indeed much of what we know about the mechanism of action of steroids such as estrogen has been determined using the neonatal chick oviduct as a model system [34]. Thus, the estrogenic effect of equol in fertilized eggs and neonatal chicks could be important, though it remains to be established whether such effects could occur, and whether they would be beneficial or deleterious if they do occur.

It has been reported that plasma concentrations of isoflavones in adult human reached 50–800 ng/ml when they consumed soy foods containing ~ 50 mg per day [1]. These values on isoflavone intake and plasma concentration are similar to those of people in Japan consuming their traditional diet [35], and such levels of isoflavone intake are epidemiologically shown to be responsible for several beneficial effects on human health [1]. Defatted soybean meals, which contain 100–250 mg/100 g of isoflavones [36,37], are often used as a protein source for feeding domestic animals and, therefore, considerable amounts of isoflavones are expected to be contained in animal products such as eggs and milk. In fact, in the present study, egg yolk was shown to contain isoflavones in more active forms, and the occurrence of isoflavones, particularly equol, in regular cow's milk samples was recently reported in the concentration range 1–30 ng/ml [38]. No particular attention has been paid to foods other than soy-based ones, especially animal-based foods, on the issue of isoflavones as dietary phytoestrogens. However, isoflavones derived from animal-based foods might give a few percent of daily total-isoflavone intake in quantity and could have stronger biological because of their components with more estrogenic activity. Further studies on digestion, absorption, and dynamics of the conjugated forms of isoflavones and equol are needed to

clarify whether dietary phytoestrogens in animal-based foods have beneficial or harmful effects on human health.

Acknowledgements

This work was partly supported by a grant from the Japanese Research and Development Association for New Functional Foods (S.S.), and by the Grant-in-Aid for The 21st Century COE Program (14COEA12) from the Ministry of Education, Science, Sports and Culture of Japan (T.M.).

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