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# Transfer of Soy Isoflavone into the Egg Yolk of Chickens

Sanshiroh SAITOH, Toshiro SATO, Hiroshi HARADA,*,† and Toshichika TAKITA*

*Food Research & Development Laboratories, Honen Corporation, Shinminato 2, Shimizu, Shizuoka 424-0824, Japan*

**Department of Nutritional Science, Faculty of Applied Biological Science, Tokyo University of Agriculture, Tokyo 156-8502, Japan*

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**A diet containing a high concentration of soy isoflavone was administered to laying hens and the contents of the isoflavones transferred to the plasma and egg yolk were measured. A method for quantitatively measuring the concentration of isoflavone in the yolk was first established, before a high concentration of soy isoflavone was administered to the laying hens over an 18-day period. The concentrations of isoflavone in the plasma and egg yolk reached their highest on the 12th day of the feeding period, the values being 3,167 nmol/l and 65.29 μg/100 g, respectively. The concentration of cholesterol in the yolk was slightly affected during the early stages of the feeding period. These findings clearly demonstrate that soy isoflavone was transferred into the yolk from the feed and that the cholesterol concentration in the yolk was affected by administering the soy isoflavone-enriched feed.**

**Key words:** isoflavone; egg; cholesterol; genistein; hypocotyl

Such physiological effects of soybean isoflavone as anti-cancer,[1] anti-oxidation,[2] cholesterol-lowering,[3,4] and anti-bacterial[5] have been reported. The structure of the isoflavone is similar to that of estrogen, a female sex hormone, so that the isoflavone acts as an estrogen-like substrate. It has been suggested that the intake of the isoflavone was useful for preventing osteoporosis and climacteric disturbance, because these are caused by estrogen shortage in the climacterium.[6] Soy isoflavone would therefore be an attractive ingredient as a nutrient supplement.

It is known that the isoflavone exists in such leguminous plants as soybean, arrowroot, and red clover. In the case of soybean, isoflavone is densely contained in the hypocotyls. Soybean is used as both a human food source and as feedstuff for domestic animals. Little information is available concerning the concentration of isoflavone in hen feces,[7] but it is thought that soy isoflavone is not present in stock farm products such as meats and eggs. To the best of our knowledge, there is no study describing the presence of soy isoflavone in hens' eggs.

In addition, isoflavone also has a blood cholesterol-lowering effect, which is caused by the reduction of cholesterol absorption,[8,9] and an effect for reducing the oxidation susceptibility of low-density lipoprotein,[10] so it has been suggested that eggs containing the isoflavone might be an effective method for preventing geriatric diseases such as hypercholesterolemia. It has been reported that fatty acids, fat-soluble vitamins, and so on can be transferred from the feed to the egg yolk.[11] Nutritionally enriched eggs, such as high vitamin E-containing eggs, have been developed by using this phenomenon.[12,13] However, due to its insolubility in both water and oil, the transfer of isoflavone from the feed to the egg is thought to be extremely difficult. Indeed, until now, there has been no study on the development of isoflavone-enriched eggs.

In the present study, a diet containing a high concentration of soy isoflavone extracted from soybean hypocotyls was administered to laying hens. The concentration of the isoflavone in the blood was measured during the period of the examination. The concentration of cholesterol and the concentration of the isoflavone in the egg yolk were both measured. This is the first study demonstrating that the soybean isoflavone can be transferred to the egg by feeding a diet with a high content of the isoflavone.

## Materials and Methods

*Reagents and feed.* Daidzein, genistein and glycitein standards were purchased from Nacalai Tesque (Kyoto, Japan). β-Glucuronidase from *Helix pomatia* was purchased from Wako (Osaka, Japan). Sep-pak C18 cartridges were purchased from Waters (Milford, MA, USA) and DEAE-Toyopearl was from Tosoh (Tokyo, Japan). Isoflavones for the feed were prepared according to the method of Izumi *et al.*[14] The concentrations of isoflavones in the diets

† To whom correspondence should be addressed. Tel: +81-3-5477-2458; Fax: +81-3-5477-2626

were measured by the HPLC method of Saitoh *et al.*[15] with an ODS column (YMC-Pack ODS-AM-303, YMC Co., Kyoto, Japan).

*Conditions for the feeding trial.* This study was conducted in accordance with current legislation on animal experimentation in Japan. A high concentration of isoflavones was added to basal diet, which is presented in Table 1. Twenty 644 day-old White Leghorn chickens were fed on the basal diet for two weeks before the experiment. Twenty chickens were divided into 4 groups and were fed 100 g of the experimental diets per day for 18 days, five chickens per group with two replicates being fed on the basal diet and on the isoflavone-enriched diet. The chickens were given free access to the experimental diets and to water. Eggs were collected every morning, and 1 ml of plasma was collected at the start and 1, 3, 6, 12 and 18 days from the start of the experiment.

**Table 1.** Components of the Basal Diet

| Ingredient | Composition (%) |
|---|---|
| Yellow corn | 69.4 |
| Soybean meal | 16.0 |
| Fish meal | 3.0 |
| Alfalfa meal | 2.0 |
| Calcium carbonate | 6.5 |
| Calcium phosphate dibasic | 2.0 |
| DL-Methionine | 0.1 |
| L-Lysine monohydrochloride | 0.1 |
| Sodium chloride | 0.3 |
| Vitamin B premix[a] | 0.2 |
| Vitamin A, D, E premix[b] | 0.2 |
| Mineral premix[c] | 0.2 |

[a] Thiamine nitrate, 2.0 g; riboflavin, 10.0 g; pyridoxine hydrochloride, 2.0 g; nicotinamide, 2.0 g; D-calcium pantothenate, 4.35 g; choline chloride, 138.0 g; folic acid, 1.0 g (/kg).
[b] Vitamin A, 10,000 IU; vitamin $D_3$, 2,000 IU; DL-$\alpha$-tocopherol acetate, 10 mg (/g).
[c] Mn, 80 g; Fe, 6 g; Cu, 0.6 g; Zn, 50 g; I, 1 g (/kg).

*Isolation of isoflavones from egg yolk.* Five grams of the egg yolk sample was dispensed into a silanized test tube (50 ml), and 10 ml of a 0.1 M sodium acetate buffer (pH 5.0) was added. $\beta$-Glucuronidase (*Helix pomatia*, 7,000 Fishman Units) was then added, and the mixture incubated overnight at 37°C. After the enzyme reaction, 29.2 ml of ethanol was added, and the mixture centrifuged at 2,000 × *g* for 10 min. The supernatant was collected, and the precipitate was resuspended in 10 ml of 70% ethanol and then centrifuged at 2,000 × *g* for 10 min. This second supernatant was mixed with the first supernatant, and a half volume of water was added. The lipids were then extracted with 2 × 15 ml of hexane. The ethanol/water layer was collected and then extracted three times with diethyl ether (69 ml, 20 ml and 15 ml). The diethyl ether layer was purified by DEAE-Toyopearl chromatography which was prepared in a silanized pasteur pipette. The column was prewashed with 5 ml of 2 N acetic acid in methanol and then with 5 ml of methanol. The diethyl ether solution was evaporated, and the residue dissolved in 1 ml of methanol and applied to the column. The column was washed with 1 × 4 ml of methanol, before the isoflavones were eluted with 1 × 7 ml of 0.5 N acetic acid in methanol. A suitable amount of cholesteryl acetate, as the internal standard, was added to this eluate which was then evaporated to dryness as a sample for gas chromatography-mass spectrometry.

*Isolation of isoflavones from laying hen plasma.* Isolation and determination of the isoflavone contents were according to the methods of Adlercreutz *et al.*[16,17] and Morton *et al.*[18] Namely, 1 ml of each plasma sample was dispensed into a silanized test tube (10 ml). An equal volume of a 0.1 mol/l sodium acetate buffer (pH 5.0) and then $\beta$-glucuronidase (*Helix pomatia*, 1,000 Fishman Units) were added and the mixture incubated at 37°C overnight. The hydrolysate was passed through a PVDF filter (0.2 $\mu$m pore size; Whatman, Clifton, NJ, USA). A Sep-Pak C18 cartridge was successively washed with 5 ml of methanol and 10 ml of water, and the filtered hydrolysate was applied to the cartridge. The cartridge was first washed with 2 ml of a 0.1 mol/l sodium acetate buffer (pH 5.0) and then eluted with 10 ml of methanol. The eluate was evaporated to about 1 ml, before being applied to DEAE-Toyopearl as just described for the egg yolk. The subsequent operations followed the same procedure.

*Gas chromatography-mass spectrometry.* The isolated samples were converted into trymethylsilyl ether (TMS) derivatives by adding *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA):pyridine = 4:1 (250 $\mu$l, v/v) while heating overnight at 80°C. Gas chromatography-mass spectrometry (GC-MS) was performed by a Shimadzu GC17A gas chromatograph coupled to a QP-5000 mass spectrometer, and Shimadzu Class-5000 processed the measurements. Two microliters of a TMS sample was applied to the capillary column (30 m × 0.32 mm, 0.25 $\mu$m, DB-1) which was programmed from 100°C to 300°C in 10°C/min increments. The other operating conditions were as follows: transfer line temperature, 315°C; ion source temperature, 320°C; ionization energy, 70 eV; accelerating voltage, 2 kV. The monitored ions and retention times of all the TMS derivatives are listed in Table 2.

*Analysis of cholesterol in egg yolk.* The total cholesterol in the egg yolks was measured enzymatically by a Cholesterol CII-test kit (Wako, Osaka, Japan).

*Statistical analysis.* The measurements were

**Table 2.** Retention Times and Ions Used for Selected Ion Monitoring

| Compound | Retention time (min) | Ion monitored[a] (a.m.u) | m/z[b] (a.m.u) |
|---|---|---|---|
| Daidzein | 21.07 | $M^+$ | 398 |
| Genistein | 21.31 | $M^+$-15 | 471 |
| Glycitein | 22.50 | $M^+$ | 428 |
| IS[c] | 24.07 | $M^+$ | 368 |

[a] $M^+$ represents the molecular ion of the analyte, and $M^+$-15 the ion produced by a loss of 15 atomic mass units (a.m.u).
[b] $m/z$ represents the mass ($m$) to charge ($z$) ratio.
[c] IS represents the internal standard (cholesteryl acetate).

analyzed by the $t$-test with the Excel 97 package (Microsoft, Redmond, WA, USA). Significance is based on a value of $p<0.05$.

## Results

*Isoflavone feeding trial*

Table 3 shows the concentrations of the isoflavone in the diets for the feeding experiment. The basal diet contained isoflavones at 36.87 mg/100 g, because it contained 16% of the soybean meal. On the other hand, the isoflavone-enriched diet contained isoflavones at 124.01 mg/100 g. No abnormality was observed in the health status of the laying hens, either in the test or control group, during the period of the feeding experiment. The egg production rate of the control and isoflavone-enriched diet groups were 72.4 ±14.1% and 81.0±14.7%, respectively.

*Establishment of the method for measuring isoflavone in the egg yolk*

The method for measuring the concentration of the isoflavones in the yolk has not previously been described. We modified Hartmann's method[19] for the enzyme reaction by β-glucuronidase to measure the steroid hormones in eggs. A major problem with this method was that the high viscosity of the enzyme and yolk cocktail made the filtration and column chromatography steps difficult. To solve this problem a step for separating the isoflavones, including extraction with 70% ethanol and liquid distribution with hexane and ether, was carried out. Preliminary experiments with the HPLC method[15] showed a high distribution efficiency of the isoflavones from the 70% ethanol solution in each of the steps as shown in Table 4. Throughout the operations, including extraction with hexane and ether, the recovery percentages of the isoflavones were 98% or greater. Throughout the extraction of the isoflavone from the plasma, the recovery percentage from column chromatography, both of Sep-Pak C18 and DEAE-Toyopearl, was 97% or greater.

*Concentration of isoflavone in the plasma*

Figure 1 shows the concentration of isoflavone in

**Table 3.** Contents of Isoflavones in the Experimental Diets (mg/100 g)[a]

| Isoflavone | Control diet | Isoflavone-rich diet |
|---|---|---|
| Daidzin | 7.38±0.08 | 9.42±0.01 |
| Glycitin | 2.03±0.02 | 2.33±0.00 |
| Genistin | 10.31±0.10 | 10.86±0.12 |
| M-Daidzin[b] | 4.27±0.11 | 4.63±0.13 |
| M-Glycitin[b] | 0.92±0.01 | 1.23±0.05 |
| M-Genistin[b] | 6.34±0.14 | 6.38±0.05 |
| A-Daidzin[c] | 1.00±0.04 | 1.02±0.01 |
| A-Glycitin[c] | 0.74±0.01 | 0.74±0.00 |
| A-Genistin[c] | 2.04±0.04 | 2.13±0.02 |
| Daidzein | 0.80±0.00 | 35.30±0.77 |
| Glycitein | 0.05±0.02 | 2.62±0.06 |
| Genistein | 1.00±0.01 | 47.69±0.99 |
| Total | 36.87±0.56 | 124.01±0.18 |

[a] Each value is the mean±standard deviation (n=3).
[b] M-Daidzin, 6″-*O*-malonyldaidzin; M-Glycitin, 6″-*O*-malonylglycitin; M-Genistin, 6″-*O*-malonylgenistin.
[c] A-Daidzin, 6″-*O*-acetyldaidzin; A-glycitin, 6″-*O*-acetylglycitin; A-Genistin, 6″-*O*-acetylgenistin.

**Table 4.** Recovery Rate of the Isoflavones by Each Extraction (%)[a]

| | Daidzein | Genistein | Glycitein |
|---|---|---|---|
| Hexane 1st | 99.67 | 99.68 | 99.66 |
| Hexane 2nd | 99.87 | 99.87 | 99.87 |
| Diethyl ether 1st | 92.25 | 87.01 | 93.74 |
| Diethyl ether 2nd | 6.55 | 9.44 | 3.53 |
| Diethyl ether 3rd | 0.85 | 1.61 | 0.39 |
| Diethyl ether total | 99.65 | 98.06 | 99.66 |
| Total recovery rate | 99.19 | 97.62 | 99.19 |

[a] The quantity of each isoflavone was determined by the HPLC method.

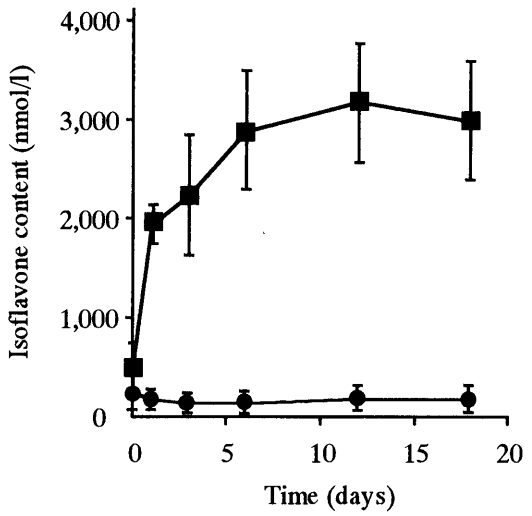


**Fig. 1.** Changes in the Concentration of Isoflavones in Laying Hen Plasma.

The isoflavone contents are the total of daidzein, genistein and glycitein which were measured by the GC-MS method described in the Materials and Methods section. ■ isoflavone-rich diet; ● control diet. Each value is expressed as the mean and standard deviation (n=3).

the plasma of laying hens. The concentration of isoflavone in the test group increased dramatically on the third day of the feeding experiment, reached 3,167 nmol/l on the 12th day, and maintained that level thereafter. The concentration of total isoflavone in the control group was 80–200 nmol/l throughout the experimental period, and no significant change was apparent.

*Concentration of isoflavone in the egg yolk*

Figure 2 shows the concentration of isoflavone measured in the yolk. The concentration of isoflavone slightly increased up to the third day of the feeding experiment, and then increased rapidly. On the sixth day, the concentration of isoflavone reached 33.26 μg/100 g, and thereafter it continued to increase gradually. On the 12th day of the experiment, the concentration of isoflavone was 65.29 μg/100 g, and this value was substantially maintained throughout the remainder of the experiment, although it appeared to slightly decrease. However, in the control group, the concentration of isoflavone was around 3 μg/100 g, and did not change during the 18-day period. To estimate the form of isoflavones in the yolk, the contents were measured without hydrolyzing with β-glucuronidase; there was 70% less in quantity without hydrolysis than with hydrolysis.

*Change in the cholesterol concentration of the yolk*

To clarify the effect of the isoflavone on the cholesterol metabolism of the laying hen, the concentration of total cholesterol in the yolk was measured. As shown in Fig. 3, the concentration of cholesterol in the yolk decreased significantly on the third day of the experiment. After that, the cholesterol concentration gradually returned to its normal level, such that, on the 6th day, no significant difference was apparent in the concentration of isoflavone in the yolk when compared with that on the first day.

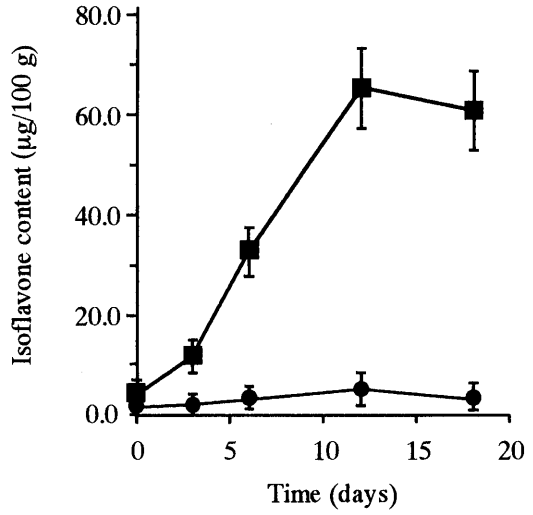


**Fig. 2.** Changes in the Concentration of Isoflavones in Egg Yolk. The isoflavone contents are the total of daidzein, genistein and glycitein which were measured by the GC-MS method described in the Materials and Methods section. ■ isoflavone-rich diet; ● control diet. Each value is expressed as the mean and standard deviation (n=3).

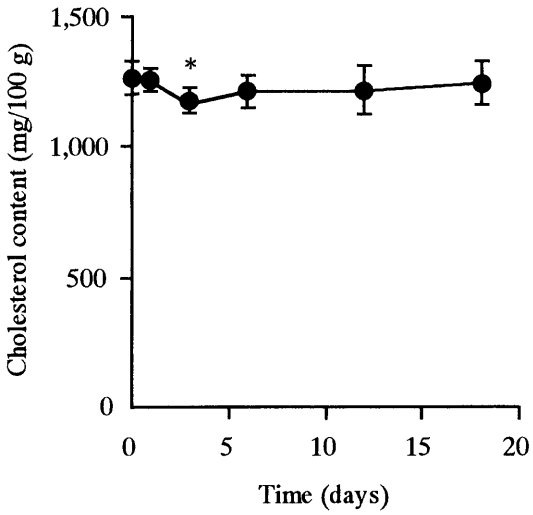


**Fig. 3.** Change in the Cholesterol Content of Egg Yolk. Measurements were carried out with the Cholesterol CII-test kit (Wako, Osaka, Japan). Each value is expressed as the mean and standard deviation of 5 samples. Differences are significant between day 0 and day 3 ($p<0.05$). No significant difference is apparent among days 0, 1, 6, 12 and 18 ($p>0.05$).

## Discussion

The quantitative determination of isoflavones in human plasma and urine has been widely investigated.[16-18] Before the measurements, the analytes were generally treated by β-glucuronidase to hydrolyze the isoflavones' glucuronide and sulfate forms. Although it has been reported that β-glucuronidase derived from a *Helix pomatia* extract was contaminated with daidzein at an approximate concentration of 4 ng/100 ml,[16] this was not an obstacle to isoflavone measurement in the present study, because it was confirmed that no contamination by isoflavones had been found in the enzyme reagent.

As has been shown in several studies, specific substances in feed can be transferred into the yolk. Murty and Reiser,[20] Navarro *et al.*,[21] and Couch and Saloma[22] have reported that the fatty acid composition of the yolk was changed when fish oil had been added to the chickens' feed. Van[23] has also reported that eicosapentaenoic acid and docosahexaenoic acid could be transferred into the yolk. Therefore, the transfer of fat-soluble substances into the yolk *via* the diet was not thought to be difficult. However, as isoflavone is scarcely soluble in either water or oil, the transfer of isoflavone into the yolk *via* food would seem to be almost impossible.

On the other hand, clinical and animal studies have demonstrated that isoflavone was absorbed from the

intestinal canal, and its structure changed into a glucuronide or sulfide conjugate in the liver. Thereafter, the various isoflavones were transferred into the blood stream, then into the peripheral tissue, and finally excreted in the feces and urine. In fact, Adlercreutz[16] has reported that 74-90% of daidzein and 91-96% of genistein existed as glucuronides by a quantitative determination of the isoflavonoids in plasma from omnivorous and vegetarian women. It had thus already been shown that isoflavone could be transferred into the yolk. To clarify the form of isoflavone in the yolk, as an aglycon or conjugate, the isoflavone contents were analyzed without the $\beta$-glucuronidase treatment. According to the present findings, isoflavone was reduced to about 30%, suggesting that 70% of the isoflavones existed in the conjugated form in egg yolk. The findings suggest that the isoflavone form changed to a conjugate, the soluble form of isoflavone, this making it easier for the transfer of soy isoflavones into the egg yolk. To fully understand the effect of the metabolism of isoflavone in the chicken, future studies will be needed.

It has been said that isoflavone acted as an estrogen-like substrate, and that estrogen played a key role in the biosynthesis of the egg.[24] The results of this study show that feeding with a high concentration of isoflavone could enhance the egg production rate, although the difference was not significant.

Moreover, isoflavone has such physiological activities as lowering the blood cholesterol level[3,4] and the ratio of LDL to HDL[25,26] in animals and humans. It is suggested that the consumption of isoflavone has some influence on the cholesterol metabolism *in vivo*. The results of the analyses demonstrate an effect on the cholesterol metabolism of laying hens such as decreasing the cholesterol concentration in the yolk during the early stage of the feeding period. However, to thoroughly clarify the effects of isoflavone on the laying hen, further studies are necessary.

In summary, the concentration of isoflavone in the blood began to increase in the laying hen 24 hrs after the start of feeding with a high-concentration isoflavone diet. This concentration peaked on the 12th day of the examination, and the high-concentration status was maintained throughout the rest of the study period. The concentration of isoflavone in the yolk slightly increased by the third day of the feeding experiment, and then rapidly increased. On the 12th day, the concentration of isoflavone reached 65.29 $\mu$g/100 g, and this value remained constant throughout the rest of the experiment. Feeding a high concentration of isoflavone to laying hens affected the cholesterol concentration in the yolk during the early stages of the feeding period, but this returned to the basal level for the remainder of the study period.

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