〈Brief Note〉

HPLC measurement of hydroxylated isoflavones in processed soybean foods

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Summary Soybean isoflavones have attracted considerable attention as functional ingredients, having demonstrated estrogen-like effects, antioxidant activity and osteoporosis prevention. Recent studies have shown that fermented soybean foods made using koji mold such as soybean miso and soy sauce, have strong antioxidant properties, and that soybeans contain hydroxylated isoflavones. In this study, we used HPLC to determine whether hydroxylated isoflavones were present in processed soybean foods eaten daily in Japan. Measurement samples included tofu, soy milk, and natto. A calibration curve was prepared from commercial standards of each hydroxylated isoflavone, and the content of hydroxylated daidzein (8-OHD), hydroxylated genistein (8-OHG), and hydroxylated glycitein (8-OHGL) was determined. Silken tofu contained 24.1 μg/g hydroxylated isoflavones, with 8-OHD, 8-OHG and 8-OHGL accounting for 9.4, 12.6 and 2.1 μg/g, respectively. Firm tofu contained 44.0 μg/g hydroxylated isoflavones (11.4, 8.0 and 24.6 μg/g, respectively) and unadjusted soymilk contained 16.3 μg/g (3.8, 4.5 and 8.0 μg/g, respectively). Functional soymilk contained 12.1 μg/g hydroxylated isoflavones, (5.3, 2.5 and 4.3 μg/g, respectively). In two types of natto, only 8-OHD was detected at amounts of 69.0 and 60.2 μg/g. Natto had the highest hydroxylated isoflavone content. The lowest hydroxylated isoflavone content was found in the soymilk samples, with functional soymilk having the lowest content.

Key words: HPLC, Soybean, Hydroxylated, Isoflavones

1. Introduction

Japan and Southeast Asian countries that consume large amounts of soybean have lower levels of breast cancer and prostate cancer than the West. This has recently generated strong interest in the link between soybean food intake and protection against disease, leading to a large number of studies on the relationship with isoflavones, which are found in abundance in soybeans[1–4.] Soybean isoflavones are structurally similar to female hormones, and exhibit estrogen-like effects. It is anticipated that these compounds could protect against breast and prostate cancer, as well as providing antioxidant effects, preventing osteoporosis and alleviating menopause symptoms[5–9]. The term 'isoflavone' encompasses several chemical compounds: daidzin, genistin, and glycitin are glycosides bound to a sugar. They take the form of acetylated glycosides when an acetyl group is

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attached to the glycoside, and malonylated glycosides when a malonyl group is attached to the glycoside. When the sugar is eliminated from the glycoside, these compounds become aglycones in the form of daidzein, genistein, and glycitein, and when a hydroxyl group is attached to the aglycone, these compounds are called hydroxylated derivatives, and exist as hydroxylated daidzein (8-OHD), hydroxylated genistein (8-OHG), and hydroxylated glycitein (8-OHGL)[10]. The Japanese have a high daily soybean intake, and they also have lower mortality due to heart disease, lower fracture rates due to osteoporosis and lower mortality due to breast and prostate cancer when compared to Westerners[11]. Studies have been published around the world on the health benefits of the Japanese diet, and particularly the effect of isoflavone intake in soybeans. Previous research has shown that hydroxylated isoflavone intake lowers the risk of glycation in the body and prevents skin aging. Hydroxylated isoflavones have been found to exert a stronger antioxidant effect than aglycones and glycosides[12]. This study therefore sought to identify which processed soybean foods are effective for disease prevention by measuring the hydroxylated isoflavone content in processed soybean foods frequently consumed in Japan.

2. Materials and Methods

Samples for determination of hydroxylated isoflavone content

One type of silken tofu, one type of firm tofu, one type of unadjusted soymilk, one type of functional soymilk, and two types of natto were used as samples of commercially available processed soybean foods.

Preparation of samples

Samples were prepared by adding 9 mL of 80% ethanol to 1 g of each commercial processed soybean food (silken tofu, firm tofu, unadjusted soymilk, functional soymilk, two types of natto), and performing shaking extraction for 2 h. Solid samples were crushed in a mortar before extraction by the same method. The supernatant was filtered through a membrane filter (0.25 μm) to prepare samples for chromatogram analysis.

Calculation of hydroxylated isoflavone content

Commercial standard solutions of each hydroxylated isoflavone were prepared at concentrations of 5, 25 and 50 μg/mL. Peak area values were obtained from the chromatograms of these solutions and calibration curves were prepared in order to determine the content of 8-OHD, 8-OHG and 8-OHGL. The concentrations of hydroxylated isoflavones in each processed soybean food were then calculated using these calibration curves.

Spike recovery test

Spike recovery test samples were prepared by adding 80% methanol to 1 g of each processed soybean food. To this was added 1.0 mL of a standard solution at a concentration of 50 μg/mL in order to obtain a total volume of 10 mL. The amount of each hydroxylated isoflavone was then measured by chromatography using the same procedure as described in 2) above.

Conditions of chromatographic analysis

Device: Fluorescence detector for high-performance liquid chromatography (Shimadzu Corporation, Tokyo, Japan)

Column: CAPCELL PAK C18 MG S5 2.0mm I.D × 250mm (Shiseido Corporation, Kyoto, Japan)

Flow rate: 700 μL/min

Column temperature: 40°C

Measured wavelength: 254 nm

Injection volume: 20 μL

Mobile phase: Phase A: 0.1% acetic acid; Phase B: acetonitrile 100%

Concentration gradient: Phase B: 15% (0 min) →35% (50 min), 15% after measurement (10 min)

3. Results

Standard solutions of each hydroxylated isoflavone were prepared at concentrations of 5, 25, and 50 μg/mL. Calibration curves of 8-OHD, 8-OHG and 8-OHGL were prepared from the measured peak areas (Fig. 1). The calibration curves of 8-OHD,

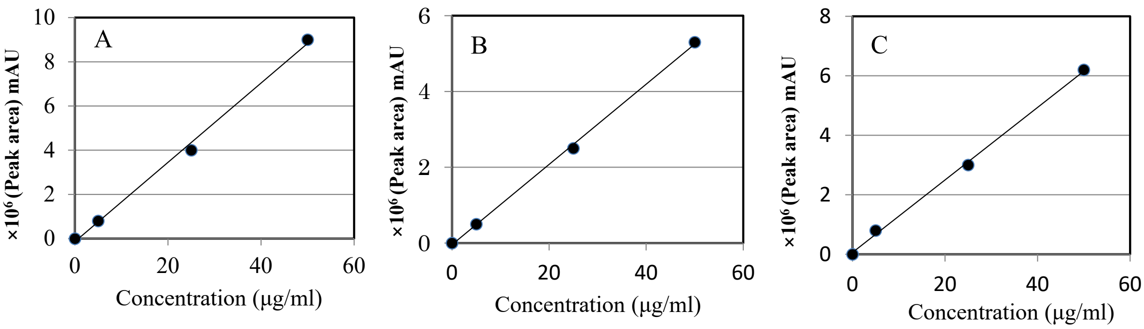

Fig. 1. Calibration curve of 8-OHD, 8-OHG and 8-OHGL. Calibration curves were created after preparing 8-OHD (A), 8-OHG (B) and 8-OHGL (C) standard solutions at 0, 5, 25, and 50 μg/mL, respectively. The calibration curves passed through the origin, and the correlation coefficients were R2 = 0.99.

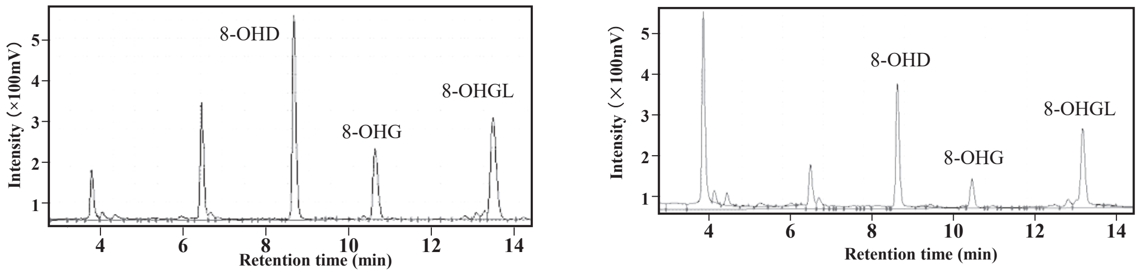

Fig. 2. Chromatogram of silken tofu (A) and firm tofu (B). The retention times of the hydroxylated isoflavones were 8.8 min for 8-OHD, 10.8 min for 8-OHG, and 13.6 min for 8-OHGL.

8-OHG and 8-OHGL all showed good linearity (R^2 = 0.99) in the range of 0–50 μg/mL and passed through the origin. In the standard substance spike recovery tests for the processed soybean samples, the recovery rate was 85.7–103.2%. The detection times of the hydroxylated isoflavones contained in tofu, soymilk and natto were 8.8 min for 8-OHD, 10.8 min for 8-OHG and 13.6 min for 8-OHGL (Fig. 2).

Table 1 shows the measured values of the concentrations of 8-OHD, 8-OHG and 8-OHGL in

Table 1 Hydroxylated isoflavone content of tofu, soymilk and natto.

	Hydroxylaed isoflavone (μg／g)			
Food	8-OHD	8-OHG	8-OHGL	Total
Silken tofu	9.4±0.9	12.6±0.6	2.1±0.3	24.1±1.8
Firm tofu	11.4±1.3	8.0±1.1	24.6±2.1	44.0±4.5
Unadjusted soymilk	3.8±0.6	4.5±0.7	8.0±0.8	16.3±2.1
Functional soymilk	5.3±0.8	2.5±0.3	4.3±0.6	12.1±1.7
Nattto－1	69.0±5.9	Not detected	Not detected	69.0±5.9
Nattto－2	60.2±6.3	Not detected	Not detected	60.2±6.3

Explanation: Hydroxylaed isoflavone (μg/g)
The hydroxylated isoflavone content of silken tofu and firm tofu was 24.1±1.8 μg/g and 44.0±4.5 μg/g, respectively. The hydroxylated isoflavone content of unadjusted soymilk and functional soymilk was 16.3±2.1 μg/g and 12.1±1.7 μg/g, respectively. The 8-OHD content of Natto-1 and Natto-2 was 69.0±5.9 μg/g and 60.2±6.3 μg/g, respectively.Values are mean±SD.

each processed soybean food based on the calibration curves. The hydroxylated isoflavone content of silken tofu and firm tofu was 24.1 µg/g and 44.0 µg/g, respectively. The hydroxylated isoflavone content of unadjusted soymilk and functional soymilk was 16.3 µg/g and 12.1 µg/g, respectively. The hydroxylated isoflavone content of natto-1 and natto-2 was 69.0 µg/g and 60.2 µg/g, respectively. However, 8-OHG and 8-OHGL were not detected in natto, which only contained 8-OHD. When comparing silken tofu and firm tofu, the hydroxylated isoflavone content was higher in firm tofu. When comparing unadjusted soymilk and functional soymilk, 8-OHD was more abundant in functional soymilk, whereas 8-OHG and 8-OHGL were more abundant in unadjusted soymilk. Only 8-OHD was measured in natto-1 and natto-2, as 8-OHG and 8-OHGL could not be detected. However, natto contained significantly more 8-OHD than the other processed soybean foods.

4. Discussion

The total amount of hydroxylated isoflavones in tofu was higher in firm tofu than in silken tofu. This difference may be due to the production methods used for each type. Silken tofu is produced by heating and grinding the soybeans, and then adding a coagulant to promote solidification. To make firm tofu, the soybeans are similarly heated, ground and solidified by the addition of a coagulant, but the tofu is then crumbled, pressed to squeeze out the moisture, and solidified again. Firm tofu contains less moisture than silken tofu and is produced with the soybean solids in a concentrated state, which is presumably why the hydroxylated isoflavone content per unit volume of raw material was higher than that found in silken tofu. When comparing the amount of each hydroxylated isoflavone in unadjusted and function soymilk, 8-OHD content was greater in functional soymilk, whereas 8-OHG and 8-OHGL were more abundant in unadjusted soymilk. The total content of hydroxylated isoflavones was higher in unadjusted soymilk. This may be because unadjusted soymilk, as defined by Japanese Agricultural Standards, has a solid soybean content of ≥8% and soybean protein equivalent of ≥3.8%, and thus contains more soybean protein than functional soymilk. The hydroxylated isoflavone with the highest biological activity is 8-OHG[11]. When comparing unadjusted soymilk and functional soymilk, 8-OHG was more abundant in functional soymilk, suggesting that consumption of functional soymilk is slightly more effective for 8-OHG intake. Only two types of soymilk were analyzed in this study, so in order to better understand the disease-preventing effects of these compounds it will be necessary to analyze other soymilk foods, as well as to consider regional differences in soybean quality.

Natto contained considerably more of 8-OHD than the other soybean foods. Unlike tofu and soymilk products, natto must undergo fermentation during the production process, and the high levels of 8-OHD are considered to result from this fermentation. Further, tofu and soymilk require grinding after heating as part of the production process, whereas natto production does not include grinding.
Isoflavone glycosides are most abundant in the germ of the soybean, and these isoflavones are presumably not generated in hydroxylated form in natto.

Isoflavone glycosides are absorbed by the body as aglycones after cleavage of the sugar by β-glycosidase by gut bacteria. A recent study reported that 8-OHD and 8-OHG are produced from isoflavone glycosides in large amounts by formation with koji mold[12]. Previous studies using foods such as miso have shown that naturally occurring microorganisms such as *Pseudomonas sp.* and *Arthrobacter sp.* enhance the production of hydroxylated isoflavones[13]. In miso, in particular, it is thought that aglycones are produced by the action of β-glucosidase, and that 8-OHD and 8-OHG are produced from the deglycosylated isoflavones by the action of hydroxylase at the sporulation stage[14].The results of this study suggest that the fermentation period in the natto production process causes a marked increase in the amount of the hydroxylated isoflavone 8-OHD compared to tofu and soymilk. Previous research on the isoflavone content of foods containing soybean consists of many studies that describe glycoside and aglycone contents; however, relatively few studies focusing on hydroxylated isoflavone content. Natto is a familiar and readily available food in Japan, and is high in hydroxylated isoflavones that have attracted attention in recent years due to their biological regulation functions. The results of this study suggest that natto is a highly effective fermented food for the intake of hydroxylated isoflavones. Natto is also superior to other processed soybean foods in that it can provide increased levels of hydroxylated isoflavones after a short period of fermentation.

Future research is needed to investigate whether hydroxylated isoflavone levels can be increased at an earlier stage using different bacterial strains in natto, and by adding microorganisms such as koji mold and lactic acid bacteria to processed soybean foods.

Conflicts of interest

The authors declare no conflict of interest.

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