JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Determination of 15 Isoflavone Isomers in Soy Foods and Supplements by High-Performance Liquid Chromatography

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ABSTRACT: Soy isoflavone is the generic name for the isoflavones found in soy. We determined the concentrations of 15 soy isoflavone species, including 3 succinyl glucosides, in 22 soy foods and isoflavone supplements by high-performance liquid chromatography (HPLC). The total isoflavone contents in 14 soy foods and 8 supplements ranged from 45 to 735 μg/g and from 1,304 to 90,224 μg/g, respectively. Higher amounts of succinyl glucosides were detected in natto, a typical fermented soy product in Japan; these ranged from 30 to 80 μg/g and comprised 4.1−10.9% of the total isoflavone content. In soy powder, 59 μg/g of succinyl glucosides were detected, equivalent to 4.6% of the total isoflavone content. These data suggest that the total isoflavone contents may be underestimated in the previous studies that have not included succinyl glucosides, especially for *Bacillus subtilis*-fermented soy food products.

KEYWORDS: *Isoflavones, succinyl glucosides, soy foods, dietary supplements, high-performance liquid chromatography*

INTRODUCTION

Soy isoflavone is a generic name for the polyphenolic compounds in soy that have phytoestrogenic effects because of their structural similarities to 17β-estradiol, which enable binding to the estrogen receptor.[1,2] Epidemiological studies suggest that the low incidence of osteoporosis and heart disease caused by estrogen deficiency in Asian women is attributable to their higher consumption of soy foods compared to American and European women.[3] A number of studies have elucidated health benefits of soy isoflavones in humans and animals, such as amelioration of lipid metabolism,[4] prevention of bone loss,[5] and antioxidant actions.[6] The binding affinities to estrogen receptors differ with the types of isoflavones.[7,8] Thus, quantitative determination of each isoflavone molecular species is required to elucidate the biological effects of isoflavones in detail.

Isoflavones are contained in soybeans, soy-based foods, and multiple soy food products worldwide. Daizein, glycitein, and genistein are the most abundant soy isoflavones, and each group has corresponding β-glucosides, acetyl-β-glucosides, malonyl-β-glucosides, and succinyl-β-glucosides. Succinyl-β-glucosides are isoflavone derivatives found in soybeans fermented by *Bacillus subtilis* (natto) and are reported to have a biological activity.[9,10] 6″-*O*-Succinylated isoflavone glucoside administration prevented bone loss in ovariectomized rats that were fed a calcium-deficient diet.[10] However, several purification processes are required from soybeans fermented with *B. subtilis*, to yield the following succinylated types of isoflavones: 6″-*O*-succinyl-daidzin, 6″-*O*-succinyl-glycitin, and 6″-*O*-succinyl-genistin, for quantitative determinations. To the best of our knowledge, these compounds were not readily commercially available. Previous studies have determined total isoflavone content by summing the contents of the 12 isoflavone isomers or the 3 aglycons (daidzein, glycitein, and genistein) with hydrolysis and have calculated the contents as aglycone equivalent in soy foods.[11−14] In the case of the total isoflavone determined by summing the content of the 12 isoflavone isomers without hydrolysis, it might be underestimated if soy foods and supplements contained the succinyl-β-glucoside forms.

Park et al. reported a method to identify the succinyl glucoside forms 6″-*O*-succinyl-genistin and 6″-*O*-succinyl-daidzin based on the coeluted retention time from a previous report of high-performance liquid chromatography (HPLC) analysis.[9] Although, in this case, it is certainly possible to analyze the succinyl glucoside form, without standardization, there is a risk of misidentifying a peak.

Thus, in the present study, 15 isoflavone isomers, including 3 succinyl glucoside forms provided by Nagara Science Co., Ltd., were quantitatively analyzed by HPLC in 14 soy foods and 8 isoflavone supplements obtained from several countries. Furthermore, we determined the succinyl glucoside content and its proportion in the total isoflavone content in soy foods and supplements.

MATERIALS AND METHODS

The method described below is based on a guideline for use of Foods for Specified Health Use containing soy isoflavones from the Ministry of Health, Labour and Welfare, Japan.[15]

Chemicals. The isoflavone glucosides [daidzin (purity of >99%), glycitin (purity of >99%), and genistin (purity of >99%)] and their corresponding aglycones (daidzein, glycitein, and genistein), malonyl glucosides (6″-*O*-malonyl-daidzin, 6″-*O*-malonyl-glycitine, and 6″-*O*-malonyl-genistin), and acetyl glucosides (6″-*O*-acetyl-daidzin, 6″-*O*-acetyl-glycitin, and 6″-*O*-acetyl-genistin) (all of them had a purity level of >90%) were purchased from Nagara Science Co., Ltd. (Gifu, Japan). The 3 succinyl glucoside isoflavone compounds (6″-*O*-succinyl-daidzin, 6″-*O*-succinyl-glycitin, and 6″-*O*-succinyl-genistin) (purity

Received: August 16, 2011
Revised: March 9, 2012
Accepted: March 21, 2012
Published: March 21, 2012

Table 1. Isoflavone Contents of Soy Foods and Supplements (μg of Aglycone Equivalent/g)[a]

		glucoside			malonyl-glucoside			acetyl-glucoside			succinyl-glucoside			aglycone				
country	product	D	Gl	G	MD	MGl	MG	AD	AGl	AG	SD	SGl	SG	De	Gle	Ge	total (μg/g)	succinyl glucoside form (%)
Japan	soy foods																	
	natto A	190	14	177	7	88	94	0	0	0	15	0	21	17	0	40	664	5.4
	natto B	181	29	212	1	6	4	8	0	19	24	13	26	18	3	41	587	10.9
	natto C	220	38	288	27	8	12	9	0	26	33	0	47	8	0	19	735	10.9
	natto D	218	31	304	0	63	0	0	0	10	15	0	15	22	3	51	731	4.1
	miso	154	9	199	0	0	0	6	1	15	2	0	0	80	12	66	546	0.4
	tempeh	20	8	83	58	8	114	7	10	17	1	0	0	137	11	174	649	0.2
	soymilk	49	2	59	23	1	33	2	1	3	0	0	0	4	0	3	178	0.0
	supplements																	
	soy powder A	79	21	98	194	37	0	465	0	35	0	59	0	128	14	174	1304	4.6
	soy powder B	83	24	162	319	56	639	10	4	43	0	0	0	113	13	144	1611	0.0
	supplement A	83	0	74	0	0	0	0	0	0	0	0	0	40784	0	49282	90224	0.0
Thailand	soy foods																	
	bean curd skin	4	0	20	8	0	35	0	3	1	0	0	0	108	0	245	425	0.0
	tofu	83	17	75	1	1	2	2	0	3	0	0	1	0	0	1	186	0.6
	pressed tofu	24	0	98	0	0	0	0	7	5	0	0	0	168	0	357	659	0.0
China	soy foods																	
	powdered soymilk	23	8	51	80	7	158	0	19	6	0	0	0	87	5	118	562	0.0
	food fiber biveradge	8	0	4	5	1	1	0	0	0	0	0	0	8	3	16	45	0.0
	cookie A	52	11	59	0	2	0	36	8	2	0	0	0	3	57	3	232	0.0
	cookie B	53	12	66	0	0	0	37	5	46	0	0	0	3	3	4	229	0.0
U.S.A.	supplements																	
	supplement A	8023	1796	6875	179	134	41	261	0	267	98	0	67	1770	127	598	20237	0.8
	supplement B	26017	5965	24702	485	170	830	286	0	423	0	0	327	0	0	271	59476	0.5
	supplement C	10035	5891	1895	119	112	0	482	380	150	94	0	144	172	489	70	20032	1.2
	supplement D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9986	9986	0.0
	supplement E	17805	3978	22654	134	77	78	715	141	922	41	74	0	440	216	267	47540	0.2

[a]All samples were measured in triplicate. D, daidzin; Gl, glycitin; G, genistin; AD, 6″-*O*-acetyl-daidzin; AGl, 6″-*O*-acetyl-glycitin; AG, 6″-*O*-acetyl-genistin; MD, 6″-*O*-malonyl-daidzin; MGl, 6″-*O*-malonyl-glycitine; MG, 6″-*O*-malonyl-genistin; SD, 6″-*O*-succinyl-daidzin; SGl, 6″-*O*-succinyl-glycitin; SG, 6″-*O*-succinyl-genistin; Da, daidzein; Gle, glycitein; and Ge, genistein.

dx.doi.org/10.1021/jf205154x | J. Agric. Food Chem. 2012, 60, 4012−4016

level of >90%), were standardized by Nagara Science Co., Ltd. Acetonitrile (HPLC grade), acetic acid, and ethanol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Food Samples and Extraction. Soy foods and isoflavone supplements were purchased at random from local markets in Japan, Thailand, China, and the U.S.A. Solid foods were crushed or pasted in a mortar before samples were weighed. If the isoflavone contents were listed on the labels, food samples were weighed to contain 1−10 mg of the total isoflavones, and if not, 5−8 g of food samples was weighed. The samples were added to 25 mL of 70% ethanol in water. After stirring for 30 min at room temperature, the sample was centrifuged (1,942*g* at 4 °C for 15 min) (Himac CF7D2, Hitachi, Tokyo, Japan) and the supernatant was collected. Then, 25 mL of 70% ethanol in water was added to the residue. The residue was stirred and centrifuged twice in the same manner. The collected supernatants were combined and made up to a volume of 100 mL with 70% ethanol in water. Rather than being stirred, tofu from Thailand was processed by continuous shaking at 2*g* at room temperature for 30 min using the shaker (SR-2DS, Taitec, Saitama, Japan). Soymilk was extracted by mixing with 70 mL of 70% ethanol in water for 2 min. The range of the isoflavone concentration was adjusted to 1−10 mg/100 mL. All sample extracts were prepared in triplicate from the same lots of the soy foods and supplements (Table 1). The extracts were centrifuged (1,942*g* at 4 °C for 15 min) and filtered through 0.45 μm cellulose acetate filters prior to HPLC analysis. To evaluate accuracy, we had estimated recoveries of external standards, including 12 isoflavone standards daizein, glycitin, and genistin, and corresponding agrycones, malonyl glucosides, and acetyl glucosides in different food matrix, powder or liquid. The recovery of the 12 isoflavone isomers was 97.2−108.0% in powder matrix and 98.5−99.7% in liquid matrix (data not shown). The extraction and analysis methods were the same as above.

HPLC Conditions. A Shimadzu HPLC system was used with a SCL-10A system controller, a DGU-14A degasser, 2 LC 10AD liquid chromatography pumps, a SIL-10A auto injector, a CTO-10A column oven, and a SPD-10A UV detector set at a wavelength of 254 nm. The column used was 250 × 4.6 mm, S-5 μm, YMC pack ODS AM-303 C_{18} reverse-phase column (YMC Co., Ltd.), with a guard cartridge column (23.0 × 4.0 mm, S-5 μm, YMC Co., Ltd.) and eluted with a linear gradient from solvent A [15:85 (v/v) acetonitrile/water, containing 0.1% acetic acid] to solvent B [35:65 (v/v) acetonitrile/water, containing 0.1% acetic acid] for 50 min. The column was then re-equilibrated to the initial conditions for 20 min. To identify 15 isoflavone isomers in soy foods, all of the 15 isoflavone standards were injected with every analysis, prior to food sample injections. All samples were analyzed using 10 μL injections under ambient temperature. The flow rate was 1.0 mL/min. The column temperature was maintained at 35 °C.

Determination of the Isoflavone Content. Each isoflavone standard was dissolved in 70% ethanol in water to the concentration of 200 mg/mL and stored at −30 °C in the dark under a nitrogen atmosphere. The qualitative standard solution was prepared to contain all 15 molecular species of isoflavone at the concentration of approximately 10 mg/mL in 70% ethanol in water. The quantitative standard solution was prepared to contain 3 isoflavone glucoside species (daidzin, glycitin, and genistin) at the concentration of 200 mg/mL in 70% ethanol in water, because the availability of high-quality standards (>99% pure) was limited to these 3 glucosides. The exact concentrations of the stock solutions for these 3 isoflavones were calculated by using molar extinction coefficients (daidzin, 26 830 M^{-1} cm^{-1}, 249 nm; glycitin, 26 713 M^{-1} cm^{-1}, 259 nm; and genistin, 41 700 M^{-1} cm^{-1}, 254 nm[16]). Each peak in the sample was identified by the retention time of the qualitative standard solution. The individual isoflavones were quantified by comparing the peak area of each isoflavone in the sample to that of the corresponding isoflavone glucoside in the quantitative standard solution. For example, the peak areas of daidzein, daidzin, 6″-*O*-malonyl-daidzin, 6″-*O*-acetyl-daidzin, and 6″-*O*-succinyl-daidzin in the sample were compared to that of daidzin in the quantitative standard solution.

Assuming that molar extinction coefficients of the isoflavones possessing the same aglycone moiety are identical, the isoflavone concentration (mg of aglycone equivalent/mL) in the sample extract could be calculated using the following formula:

$$\text{isoflavone concentration (mg of aglycone equiv/L)} = \frac{A_{\text{sample}}}{A_{\text{std}}} MC_{\text{std}} M_{\text{agl}}$$

where, A_{sample} is the peak area of each isoflavone detected in the sample extract, A_{std} is the peak area of the corresponding glucoside in the standard solution, MC_{std} is the molar concentration of the corresponding glucoside in the standard solution (mmol/L), and M_{agl} is the molecular weight of the corresponding aglycone.

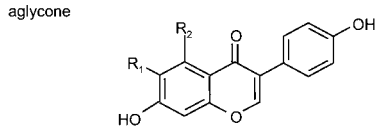
Then, the content of total isoflavone in the original food samples was calculated using the following formula:

$$\text{isoflavone aglycone content (}\mu\text{g/g)} = \text{isoflavone concentration (mg of aglycone equiv/L)} \frac{100}{1000} \frac{1}{\text{sample wt (g)}} \text{dilution factor} \times 1000$$

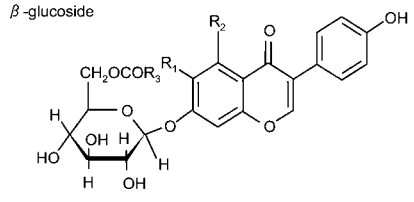
The isoflavone contents in the foods and supplements were shown as aglycone equivalents (μg of aglycone equivalent/mL or g). The total isoflavone content was calculated as the sum of the contents of 15 individual isoflavone molecular species.

RESULTS AND DISCUSSION

Of the 22 varieties of soy foods and dietary supplements, natto as well as several other soy foods and supplements were determined by HPLC analysis to contain the succinyl glucoside form of isoflavone. Isoflavone analysis had previously been performed to evaluate the total isoflavone content as aglycones by summing the amounts of 12 isoflavone spices or 3 aglycones (daidzein, glycitein, and genistein) with hydrolysis, and this quantization was used to estimate the daily intake of total isoflavones (Figure 1).[11−14] However, it is possible to evaluate the total isoflavone content, including the succinyl glucoside form with hydrolysis, but difficult to estimate the composition of individual isoflavone conjugates.[11] The bioavailability of soy



Compound	R_1	R_2
Daidzein	H	H
Glycitein	OCH_3	H
Genistein	H	OH



Compound	R_1	R_2	R_3
Daidzin	H	H	H
Glycitin	OCH_3	H	H
Genistin	H	OH	H
Malonyl-β-glucoside			CH_2COOH
Acetyl-β-glucoside			CH_3
Succinyl-β-glucoside			CH_2CH_2COOH

Figure 1. Chemical structures of the isoflavones.

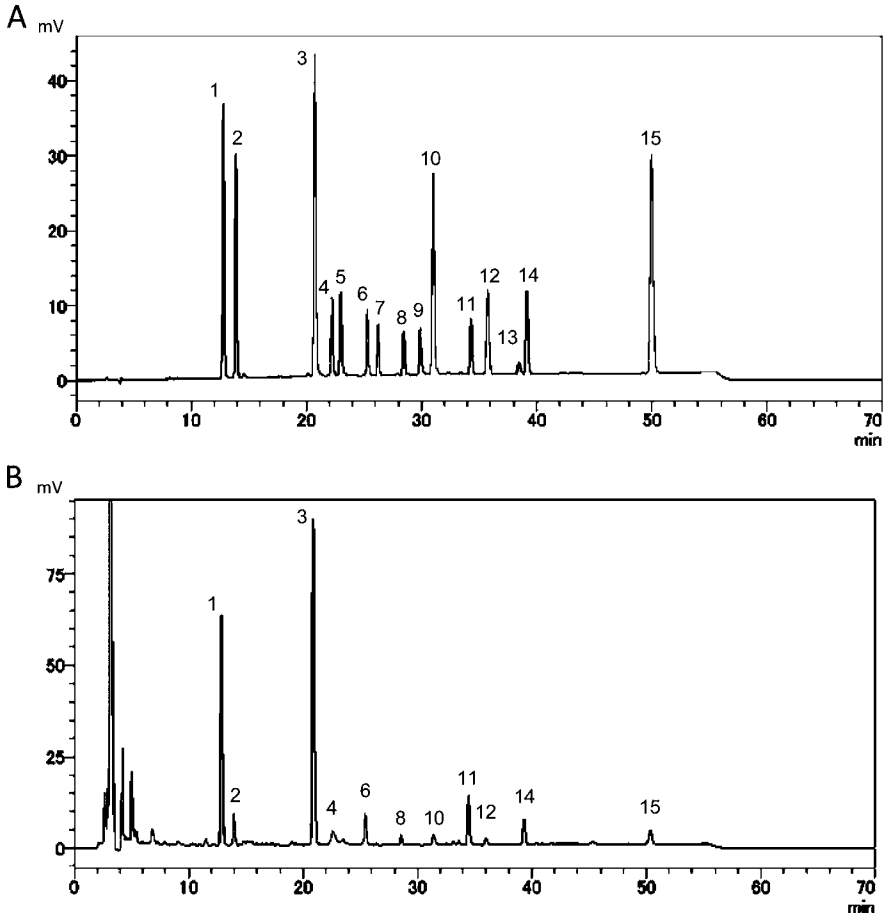


Figure 2. HPLC chromatograms of a sample containing 15 isoflavones, as recorded at 254 nm. Column: YMC pack ODS AM-303 C_{18} reverse phase (250 × 4.6 mm, S-5 μm). Elution (1.0 mL/min) performed using a linear gradient of acetonitrile in water, containing 0.1% acetic acid, from 15 to 35% for 50 min. (A) 15 isoflavone standards prepared in 70% ethanol in water. (B) Ethanolic (70%) extract of natto. Numbers of the peaks are as follows: 1, daidzin; 2, glycitin; 3, genistin; 4, 6″-*O*-malonyl-daidzin; 5, 6″-*O*-malonyl-glycitin; 6, 6″-*O*-succinyl-daidzin; 7, 6″-*O*-succinyl-glycitin; 8, 6″-*O*-acetyl-daidzin; 9, 6″-*O*-acetyl-glycitin; 10, 6″-*O*-malonyl-genistin; 11, 6″ -*O*-succinyl-genistin; 12, daidzein; 13, glycitein; 14, 6″-*O*-acetyl-genistin; and 15, genistein.

isoflavones differs based on the isoflavone isomer,[17−19] and 6″-*O*-succinyl daidzin and 6″-*O*-succinyl genistin administration can prevent bone loss in ovariectomized rats.[10] There were a limited number of the studies that have reported the bioavailabilites of 6″-*O*-succinylated isoflavone glucosides, and the difference in bioavailabilities between the succinyl glucoside form and other isoflavone glucoside forms is not clear. Thus, to understand the health benefits of 15 individual isoflavone isomers in more detail, it is important to determine the total isoflavone content, including the 3 succinyl glucoside forms in soy products.

The analytical conditions of HPLC analysis were appropriate for the 15 independent isoflavone isomer standards and food extraction samples. Every analysis was performed under completely separate conditions without acid hydrolysis of glucosides (panels A and B of Figure 2). In soy foods, the peak areas corresponding to the retention times of 3 succinyl glucoside forms were detected in natto (peaks 6 and 11 in Figure 2B), which is fermented with *B. subtilis*. The succinyl glucoside forms were not detected in miso or tempeh, which are soy foods fermented by another *Bacillus* species. Differences in the type of fungus may result in these differences. The amount of the 2 succinyl glucoside forms was 30−80 μg of aglycone equivalent/g in natto; this was evaluated to equal 4.1−10.9% of the total isoflavone content. Higher proportions of 6″-*O*-succinyl-daidzin and 6″-*O*-succinyl-genistin were detected in the succinyl glucoside form, and 6″-*O*-succinyl-glycitin was hardly detected in the fermented foods. These data for the succinyl glucoside form and its proportions were almost identical to the data from a previous report.[10]

As for supplements, 39−327 μg of aglycone equivalent/g in the succinyl glucoside was detected in 5 of the 8 kinds of supplements. Some supplements contained these compounds at a higher concentration than natto, and the ratio ranged from 0.1 to 4.6% of the total isoflavone content. The highest ratio of the succinyl glucoside form was observed at 4.6%, and its content was 59 μg of aglycone equivalent/g in soy powder A. However, because the volume of the supplement is not so large, the total intake of succinyl glucoside forms from the supplement might be lower than that from natto.

It should be noted that there was no indication of whether or not this product had been produced by fermentation. The isoflavone contents were shown on the labels of all 8 supplements, but in 5 supplements, it was unclear whether they were aglycone equivalents. Therefore, it is difficult to compare the labeled values with the analyzed values in this experiment. If the labeled values were shown to be those of the aglycone forms, the ratio of the isoflavone content to the

dx.doi.org/10.1021/jf205154x | *J. Agric. Food Chem.* 2012, 60, 4012−4016

labeling value ranged from 39 to 134% in the supplements (data not shown).

Previous studies calculated the total isoflavone content by summing the contents of the 12 or 15 individual isoflavone isomers; we followed the same method. However, the binding affinities of each isomer to estrogen receptors differ with their chemical structures,[7,8] suggesting that each isomer might confer different health effects. Accordingly, in future studies, it may be preferable to calculate the total isoflavone content by calculating the weighted sum of the contents of the individual isoflavone isomers. Further studies are needed to explore the bioavailability of succinyl glucoside isomers, which have not yet been completely elucidated.

This study used 70% ethanol in water as the solvent for extraction, but the efficiencies of solvent extraction for individual isoflavones from various soy food samples may differ depending upon the food matrix.[20] The evaluation of isoflavone contents of foods and supplements needs to be further refined from both biological and methodological points of view.

We have identified individual isoflavone succinyl glucosides in various soy foods and supplements by using the standardized reagent purified from soybean. The data presented here suggested that the total isoflavone content calculated using 12 isoflavone standards without hydrolysis in the previous studies was underestimated by a maximum value of about 10%. However, it may not affect the total isoflavone content in most soy foods, because the succinyl glucoside form was not detected in any of the soy foods studied, except natto. On the other hand, in the supplements, the succinyl glucoside form was detected to constitute 4.6% of the total isoflavone content. It is considered that the analysis of the 15 isoflavone isomers, including the succinyl glucoside form, is necessary for constitutive isoflavone analysis.

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Funding

This work was supported by a Grant-in-Aid H20-Shokuhin-Ippan-003 from the Ministry of Health, Labour and Welfare of Japan.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the staff of Nagara Science Co., Ltd. (Gifu, Japan) for the standardization of the three succinyl glucoside isoflavone compounds.

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