Antimutagenic Activity of Isoflavones from Soybean Seeds (*Glycine max* Merrill)

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Two isoflavones, daidzein (**1**) and genistein (**2**), were isolated from soybean hypocotyls. Daidzein and genistein showed a suppressive effect on *umu* gene expression of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen 3-amino-1,4-dimethyl-5*H*-pyrido[4,3*b*]indole (Trp-P-1), which requires liver metabolizing enzymes. Compound **1** suppressed 73% of the SOS-inducing activity at concentrations <0.74 μmol/mL, and the ID_{50} value was 0.37 μmol/mL. Compound **2** suppressed 95% of the SOS-inducing activity at concentrations <0.74 μmol/mL, and the ID_{50} value was 0.17 μmol/mL. Compounds **1** and **2** were also assayed with the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide) and activated Trp-P-1. In addition to the antimutagenic activities of daidzein and genistein against Trp-P-1, frylfuramide and activated Trp-P-1 were assayed by an Ames test using *S. typhimurium* TA100.

Keywords: *Soybean; isoflavonoid; daidzein, genistein; SOS response; antimutagenic activity; umu test; Ames test*

INTRODUCTION

Asian diets, such as those consumed in China and Japan, are typically lower in total and saturated fat and higher in dietary fiber; these are characteristics that may contribute to the relatively low rates of breast, prostate, and colon cancer in these countries (Saio, 1984). Several investigators (Setchell et al., 1984; Barnes et al., 1990; Adlercreutz, 1990) have suggested that soy consumption may contribute to the lower rates of these cancers in Asian countries. In 1990, a National Cancer Institute (Bethesda, MD) workshop identified several anticarcinogens in soybean and recommended that the relationship between soy intake and cancer risk be investigated (Messina et al., 1991). Particularly noteworthy was that soybean is a rich and relatively unique source of isoflavones. Soybeans are known to contain the five isoflavone glycosides genistin, daidzin, glycitein 7-*O*-β-D-glucoside (glycitin), 6″-*O*-acetylgenistin, and 6″-*O*-acetyldaidzin and their corresponding aglycons, daidzein, glycitein, and genistein (Walz, 1931; Naim et al., 1973; Ohta et al., 1979, 1980). It has been reported that these isoflavone compounds possess antihemolytic, antioxidative, antifungal, estrogenic, and antitumorial activities and also exhibit undesirable bitter and astringent tastes (Naim et al., 1976; Farmakalidis et al., 1985; Akiyama et al., 1987; Huang et al., 1981; Iijima et al., 1987).

In the evaluation of the carcinogenicity or mutagenicity of environmental chemicals, it is quite important to determine factors present in the environment that may affect these activities. With the development of techniques for detecting possible environmental carcinogens and mutagens (Ames et al., 1975), it has been shown that ordinary diets contain many kinds of mutagens and antimutagens. Kada et al. (1981) have studied the antimutagenic activity of foodstuffs using microbial mutation assay systems. Ishii et al. (1984) reported on the screening of the bioantimutagenic capacities of plant extracts with criteria of suppressing UV-induced mutations in *Escherichia coli* B/r WP2 (*trp*) and the mutator activity in *Bacillus subtilis* NIG 1125 (*his met*). Protoanemonin was identified as the factor responsible for the antimutagenicity of ranunculus and anemone plants against UV- and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced *E. coli* B/r WP2 *trp* (Minakata et al., 1983) reversions.

The *umu* test system was developed to evaluate the genotoxic activities of a wide variety of environmental carcinogens and mutagens, using the expression of one of the SOS genes to detect DNA-damaging agents (Oda et al., 1985; Nakamura et al., 1987). We have searched for new naturally occurring antimutagenic compounds in plants that have a history of safe use as Chinese crude drugs (Miyazawa et al., 1995a−c, 1996, 1997). In this paper, we report the antimutagenic activity of isoflavones contained in soybean as determined with the Ames (Ames et al., 1975) and *umu* tests.

MATERIALS AND METHODS

General Procedure. Electron-impact mass spectra (EI-MS) were obtained on a Hewlet Packard 5972A mass spectrometer. IR spectra were determined with a Perkin-Elmer 1760-x infrared Fourier transform spectrometer. Nuclear magnetic resonance (NMR) spectra (δ, J in hertz) were recorded on a JEOL GSX 270 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H NMR spectra measured in DMSO-d_6. This solvent was used for ^{13}C NMR spectra.

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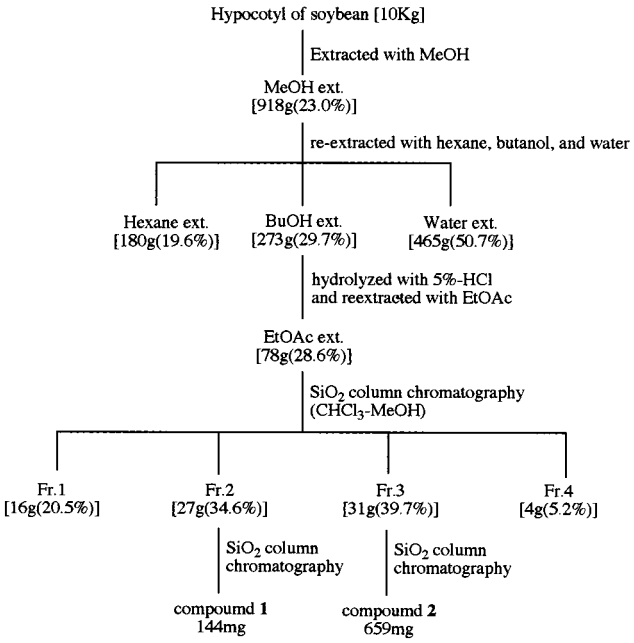


Figure 1. Isolation scheme for the isoflavones from soybean seed hypocotyls.

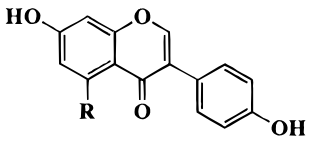
Materials. Soybean hypocotyls were a gift from Fuji Oil Co., Ltd. (Hannan R&D Center, 1 Sumiyoshi-cho Izumisanoshi, Osaka, Japan). Furylfuramide and Trp-P-1 were purchased from Wako Pure Chemicals Co. S9 metabolizing enzyme was purchased from Oriental Yeast Co.

***Umu* Test.** The *umu* test is based upon the abilities of carcinogens and mutagens to induce expression of an *umu* gene in *Salmonella typhimurium* TA1535/pSK1002 (Oda et al., 1985), in which a plasmid (pSK1002) carrying a fused gene (*umuC′-lac*Z) had been introduced. The SOS-inducing potency is estimated by the measurement of the level of *umu* operon expression in terms of cellular β-galactosidase activity. The SOS response appears after DNA damage or interference with DNA replication (Littele et al., 1982; Walker, 1984).

Briefly, an overnight culture of the tester bacterial strain in Luria broth (1% Bactotryptone, 0.5% NaCl, and 0.2% glucose; supplemented with 20 mg/L ampicillin) was incubated at 37 °C until the bacterial density at 600 nm reached 0.25−0.30. The culture was divided into 2.3 mL portions in test tubes. The test compound (50 μL, dissolved in DMSO), 0.1 M phosphate buffer (300 μL, pH 7.4), and furylfuramide (50 μL, 1 μg/mL in DMSO) were added to each tube. In the case of Trp-P-1 (50 μL, 40 μg/mL in DMSO), 300 μL of S9-metabolizing enzyme instead of 0.1 M phosphate buffer mixture was added. After 2 h of incubation at 37 °C with shaking, the culture was centrifuged to collect cells, which were resuspended in 25 mL of PBS; the cell density was read at 600 nm with one portion (1.0 mL) of the suspension. Using the other portion (0.25 mL), the level of β-galactosidase activity in the cell was assayed according to the method of Miller (1972).

Ames Test. The mutation test was carried out by the preincubation method (Yahagi et al., 1977), which is a modification of Ames method (Ames et al., 1975). The test compound (50 μL), Trp-P-1 (50 μL, 40 μg/mL in DMSO), and 500 μL of S9-metabolizing enzyme mixture instead of 0.1 M phosphate buffer were added to each tube. In the case of furylfuramide, 0.1 M phosphate buffer (500 μL) and furylfuramide (50 μL, 0.2 μg/mL in DMSO) were added. The culture of tester bacterial strain (*S. typhimurium* TA100) was divided into 100 μL portions into the test tube. The mixture was preincubationed at 37 °C for 20 min, mixed with 2.0 mL of top agar at 45 °C, and poured onto a minimal-glucose agar plate. After incubation for 2 days at 37 °C, the colonies on the plate were counted.

Isolation of Isoflavones (Figure 1). The soybean hypocotyls (10 kg) were refluxed with methanol for 12 h to give a methanol extract (918 g). This extract was suspended in water (2 L) and partitioned between hexane (1 L), butanol (1 L), and water successively. Each soluble fraction was concentrated under reduced pressure to give hexane (83 g), butanol (153 g), and water (682 g) fractions. The butanol fraction was hydrolyzed with hydrochloric acid. The reaction mixture was extracted with ethyl acetate (1 L). The soluble fraction was concentrated under reduced pressure to give an ethyl acetate fraction (78 g). To prepare isoflavones, the ethyl acetate fraction was repeatedly fractionated by SiO_2 column chromatography. Finally, compounds **1** (144 mg) and **2** (659 mg) were isolated.



1 : R=H
2 : R=OH

*Compound **1***. Compound **1** was isolated as colorless needles: mp 300 °C; MS, *m/z* 254 $[M]^+$, 137, 118; IR γ_{max}^{KBr} 3160, 1595, 1520, 1460 cm^{-1}. The ^1H NMR and the ^{13}C NMR corresponded with those of daidzein (Kinjo et al., 1987). Compound **1** was identified as daidzein [4*H*-benzopyran-4-one-7-hydroxy-3-(4-hydroxyphenyl)] from these spectral data and physical properties.

*Compound **2***. Compound **2** was isolated as a colorless plate: mp 297−298 °C; MS, *m/z* 270 $[M]^+$, 153; IR γ_{max} 3390, 1615, 1515, 1460 cm^{-1}. The ^1H NMR and the ^{13}C NMR corresponded with those of genistein (Kinjo et al., 1987). Compound **2** was identified as genistein [4*H*-benzopyran-4-one-5,7-dihydroxy-3-(4-hydroxyphenyl)] from these spectral data and physical properties.

RESULTS

Fractionation of the Extract from Soybean Hypocotyls and Isolation of Compounds 1 and 2. The methanol extract of soybean hypocotyls was partitioned between hexane, butanol, and water successively. The butanol fraction was hydrolyzed with hydrochloric acid, and the reaction mixture was extracted with ethyl acetate. To prepare isoflavones, the ethyl acetate fraction was repeatedly fractionated by SiO_2 column chromatography. Finally, compounds **1** (144 mg) and **2** (659 mg) were isolated.

Structure Determination of Compounds 1 and 2. The identities of compound **1** as daidzein and compound **2** as genistein were established by comparison of spectral data and physical constants with literature data.

Suppression of the SOS-Inducing Activity of Compounds 1 and 2. The suppressive effects of **1** and **2** were determined in the *umu* test. As shown in Figure 2, **1** and **2** exhibited inhibition of the SOS induction of Trp-P-1, which requires metabolic activation. Compounds **1** and **2** suppressed 73 and 95% of the SOS-inducing activity at concentrations <0.74 μmol/mL, and the ID_{50} values were 0.37 and 0.17 μmol/mL, respectively. Compounds **1** and **2** also showed suppression of the SOS-inducing activity of activated Trp-P-1 and furylfuramide. Compounds **1** and **2** suppressed 69 and 94% of the SOS-inducing activity of activated Trp-P-1 at concentrations <0.74 μmol/mL, and the ID_{50} values were 0.43 and 0.16 μmol/mL, respectively. Compounds **1** and **2** suppressed 69 and 72% of the SOS-inducing activity of furylfuramide at concentrations <0.74 μmol/

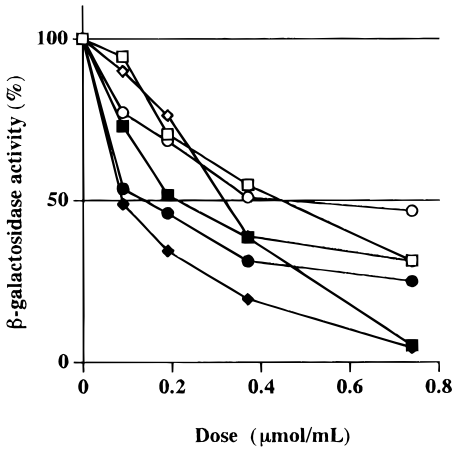


Figure 2. Suppressive effect of **1** and **2** on the SOS-inducing activity of Trp-P-1 in *S. typhimurium* TA1535/pSk1002: (□) **1** of Trp-P-1; (■) **2** of Trp-P-1; (○) **1** of activated Trp-P-1; (●) **2** of activated Trp-P-1; (◇) **1** of furylfuramide; (◆) **2** of furylfuramide. Furylfuramide (1 μg/mL in DMSO) was added at 50 μL. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL.

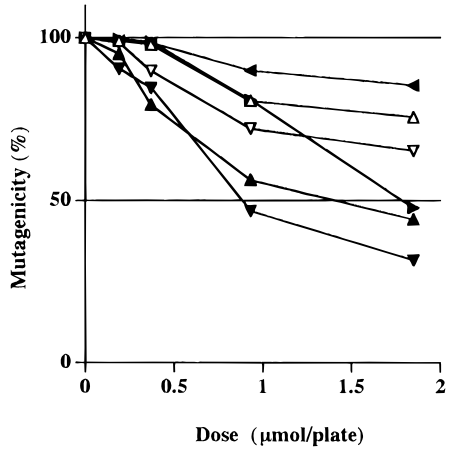


Figure 3. Effect of **1** and **2**, on the mutagenicity of Trp-P-1, activated Trp-P-1, and furylfuramide in *S. typhimurium* TA100: (△) **1** of Trp-P-1; (▲) **2** of Trp-P-1; (▽) **1** of activated Trp-P-1; (▼) **2** of activated Trp-P-1; (▲, pointing left) **1** of furylfuramide; (▲, pointing right) **2** of furylfuramide. Furylfuramide (1 μg/mL in DMSO) was added at 50 μL. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL.

mL, and the ID_{50} values were 0.32 and 0.30 μmol/mL, respectively.

Antimutagenic Activity of Compounds 1 and 2. The antimutagenic activities of these compounds against Trp-P-1, activated Trp-P-1, and furylfuramide were also demonstrated by the Ames test using *S. typhimurium* TA100. As shown in Figure 3, compounds **1** and **2** suppressed 25 and 56% of the mutagenicity of Trp-P-1 at <1.85 μmol/plate, respectively. Compounds **1** and **2** also suppressed 35 and 69% of the mutagenicity of activated Trp-P-1 at <1.85 μmol/plate, respectively. Compounds **1** and **2** also suppressed 42 and 44% of the mutagenicity of furylfuramide at <1.85 μmol/plate, respectively. The ID_{50} values of **2** against Trp-P-1, activated Trp-P-1, and furylfuramide were 1.38, 0.91, and 1.73 μmol/plate, respectively.

DISCUSSION

Compounds **1** and **2** were clearly identified as antimutagenic compounds. These compounds had a suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against furylfuramide and Trp-P-1, which requires liver metabolizing enzymes (Table 1). Compounds **1** and **2** were examined for their ability to suppress the metabolic activation of Trp-P-1. As shown in Figure 2, compound **2** exhibited a greater suppressive effect on the SOS-inducing activity of every mutagen than did compound **1**. The difference in structure between **1** and **2** is a hydroxyl group at the C-5 position. This result of the *umu* test indicates that a hydroxyl group at the C-5 position is important for the suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against every mutagen. In the Ames test using *S. typhimurium* TA100, compounds **1** and **2** suppressed the mutagenicity of furylfuramide, Trp-P-1, and activated Trp-P-1 (Figure 3). Compound **2** exhibited more suppression of the mutagenicity of all mutagens than did **1**. It was also suggested that **2** had a more suppressive potency than did **1**. As shown by the result of the *umu* test, clearly the presence of a hydroxyl group at the C-5 position is important for antimutagenic activity against chemical mutagens. In addition, from the results of suppressive effect against activated Trp-P-1 in the *umu* and Ames tests, it may be expected that the suppressive effect of Trp-P-1 did not rely on inhibition of metabolic activation by the S9-mix.

Previously, isoflavones were isolated from plant extracts and investigated for their antimutagenicity (Wall et al., 1988a,b; Manikumar et al., 1989). Soybeans are known to contain the five isoflavone glycosides genistin, daidzin (Waltz, 1931), glycitein 7-*O*-β-D-glucoside (glycitin) (Naim et al., 1973), 6″-*O*-acetylgenistin (Ohta et al., 1980), and 6″-*O*-acetyldaidzin (Ohta et al., 1979) and their corresponding aglycons, daidzein (Shibata et al., 1959), glycitein, and genistein (Hudson et al., 1969). It has been reported that these isoflavone compounds possessed antihemolytic (Naim et al., 1976), antioxidative (Naim et al., 1976), antifungal (Naim et al., 1976), estrogenic (Farmakalidis et al., 1985), and antitumorial (Akiyama et al., 1987) activities and also exhibited undesirable bitter and astringent tastes (Huang et al., 1981; Iijima et al., 1987).

Table 1. Suppressive Effect of Daidzein and Genistein on Furylfuramide,[a] Activated Trp-P-1, and Trp-P-1[b] Using *S. typhimurium* TA1535/PSK1002

					dose response[c]			
compd	furylfuramide	activated Trp-P-1	Trp-P-1	control	0.74 μmol/mL	0.37 μmol/mL	0.19 μmol/mL	0.09 μmol/mL
1	445			162	250	272	378	430
2	365			123	134	170	206	241
1		463		149	296	309	392	432
2		463		149	228	247	294	318
1			404	152	267	327	349	365
2			460	113	131	247	292	366

[a] Furylfuramide (1 μmg/mL in DMSO) was added at 50 μL. [b] Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. [c] β-Galactocidase activity (units).

In this paper, we have shown that daidzein and genistein were antimutagenic compounds against chemical mutagens.

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