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Radiation-Induced Enhancement of Antioxidant Contents of Soybean (*Glycine max* Merrill)

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Soybean samples were treated with γ-radiation doses between 0.5 and 5 kGy for achieving insect disinfestation and microbial decontamination. Nutritional quality of soybeans with respect to antioxidant isoflavone content was tested in radiation-treated and untreated samples. Changes in major isoflavones such as genistein, diadzein, glycetein, and their glycosides were monitored by high-performance liquid chromatography. Interestingly, a decrease in content of glycosidic conjugates and an increase in aglycons were noted with increasing radiation dose. Antioxidant potential measured as percent 1,1-diphenyl-2-picrylhydrazyl scavenging activity showed an increasing trend with dose, indicating that radiation processing as a method of food preservation has a positive nutritional implication.

KEYWORDS: Antioxidant activity; γ-irradiation; HPLC; isoflavones; soybean (*Glycine max* Merrill)

INTRODUCTION

Soybean and its processed products have been acclaimed as health foods due to their high content of protein and essential amino acids, omega-3 fatty acids, fat-soluble vitamins, polysaccharides, and insoluble fibers (*1*). Besides these constituents, soybeans also contain isoflavones that are of wide interest due to their beneficial effects on humans, such as prevention of cancer, cardiovascular diseases, osteoporosis, and menopausal symptoms (*2*). The ability to prevent cancer and cardiovascular diseases has been strongly linked to their antioxidant activity (*3*, *4*). The main isoflavones of soybeans are genistein, diadzein, and glycitein, each of which mainly exists as its aglycon (genistein, diadzein, and glycitein), β-glucoside (genistin, diadzin, and glycitin), and malonylglucoside (6″-*O*-malonylgenistin, 6″-*O*-malonyldiadzin, and 6″-*O*-malonylglycitin). They are also present to a lesser extent as their acetylated glucosides (6″-*O*-acetylgenistin, 6″-*O*-acetyldiadzin, and 6″-*O*-acetylglycitin). These bioactive substances are present in high concentrations in soybean (*5*). High-performance liquid chromatography (HPLC) has been widely used for the direct analysis of free and conjugated forms of isoflavones, facilitating their rapid quantification (*5*−*8*).

Radiation processing by γ-radiation up to a dose of 1 kGy has been recommended for quarantine treatment of legumes including soybeans, whereas exposure to higher doses (up to 5 kGy) resulted in improvement in quality such as reduction in cooking time and improvement in texture without production of off-flavor (*9*). Degradation of isoflavones has been shown to occur in soy and soy products during food processing and storage, resulting in changes in their bioactivities (*10*). Thermal degradation during heating, baking, frying, etc., with lowering in isoflavone conjugates and increase in free isoflavone has been reported (*11*−*14*). Free isoflavones were shown recently to be more rapidly and extensively absorbed than the glycosides in humans (*15*). However, the effect of radiation on the isoflavone content and the impact of these changes on the antioxidant properties of soybeans have not been investigated so far.

EXPERIMENTAL PROCEDURES

Commercial soybean samples, variety JS 335 (1000 g), were obtained from three different local markets of Mumbai, India. The average protein, fat, and ash contents as provided by Soybean Processors Association of India (Indore, Madhya Pradesh, India) for this variety were 38.97, 19.51, and 1.2%, respectively. Each of the three samples was divided into two lots. One lot (250 g) was used as non-irradiated control. The other lot was further subdivided into three equal lots (250 g) and then exposed to γ-radiation at 25 °C to doses of 0.5, 1, and 5 kGy using a cobalt-60 package irradiator (AECL, Ottawa, Canada; 14 Gy/min). Samples were analyzed within 1 week of storage. For analysis of isoflavones 10 g of each of the above samples was analyzed in triplicate. Solvents used were obtained from E. Merck (Mumbai, India) and were of analytical grade. Genistein, Trolox, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Isolation of Total Isoflavones. Isoflavones were extracted from soybeans essentially according to the procedure of Eldridge and Kwolek (*16*). Coarsely ground soy flour (10 g) was defatted by refluxing with hexane (100 g) in a water bath for 2 h at 80 °C. The mixture was then filtered under suction through a Büchner funnel, and the residual defatted flour was then subsequently refluxed with 150 mL of 80% aqueous methanol for 4 h as above. The extract thus obtained was concentrated under vacuum to a small volume (40 mL) and then successively extracted with diethyl ether followed by *n*-butanol. The diethyl ether and butanol fractions were evaporated to dryness, and the residue in each case was dissolved in methanol to obtain a 10% solution.

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Thin-Layer Chromatography (TLC). Analytical TLC of the above diethyl ether and butanol fractions was carried out on ammonium sulfate (5%) impregnated silica gel G (E. Merck, Darmstadt, Germany) plates [10 cm (width) × 20 cm (height) × 1.1 mm (thick) glass plate, 0.25 mm thickness of adsorbent]. The laboratory-prepared plates were developed using toluene/ethyl formate/formic acid (5:4:1) as the developing solvent system. Separated spots were visualized under UV light (254 nm) after the plate had been either exposed to ammonia vapor or heated for 15 min at 180 °C. Comparison of their R_f values with those of standard genistein as well as purified isoflavones obtained from the experimental samples, as described below, identified the spots of interest. Because the diethyl ether fraction was devoid of isoflavones as noted on TLC, this fraction was not studied further.

Column Chromatography. The butanol extract (100 mg) containing isoflavones of interest was loaded on the top of a silica gel 60 column (17.25 cm length × 2.5 cm internal diameter) and eluted with chloroform followed by increasing proportions of methanol. Fractions (5 mL each) were collected in separate test tubes, concentrated to a small volume, and then monitored on TLC using the same solvent system as above. Those fractions containing identical compounds were pooled and evaporated to dryness to obtain partially purified isoflavones. Whereas the genistein, diadzein, and glycitein eluted in the chloroform/methanol (90:10), the glycosides were detected in the 85:15 fractions. The isoflavones thus obtained were further purified by preparative (0.5 mm thickness) TLC using the above solvent system. The identity of these compounds was confirmed by mass spectral analysis. These isolated compounds were used as standards in the present study. A part of the individual glycosides were separately subjected to acid hydrolysis (1 N HCl, 1 h, 80 °C), and the hydrolysate was then extracted with *n*-butanol in each case. The butanol extract was washed free of acid with distilled water in a separating funnel, concentrated to a small volume, and then subjected to analytical TLC as described above. The remaining aqueous hydrolysate was freeze-dried and the residue subjected to acetylation using pyridine/acetic anhydride (1:1) overnight at room temperature. Solvent was removed under vacuum and the residue dissolved in chloroform. This solution was analyzed by gas chromatography−mass spectrometry (GC-MS) to identify the sugar residue.

Gas Chromatography−Mass Spectrometry. The acetylated fractions containing sugar residues as obtained above were subjected to GC-MS analysis using a Shimadzu QP-5050A series GC-MS instrument provided with a direct inlet facility (DI-50). The instrument was equipped with a GC-17A gas chromatograph and provided with a DB-1 (dimethyl polysiloxane, J&W Scientific) capillary column (length, 30 m; i.d., 0.25 mm; and film thickness, 0.25 μm). The operating conditions were as follows: column temperature, programmed from 60 to 200 °C at the rate of 4 °C/min, held at initial temperature and at 200 °C for 5 min, and further to 280 °C at the rate of 10 °C/min, held at final temperature for 20 min; injector and interface temperatures, 210 and 230 °C, respectively; carrier gas, helium (flow rate, 0.9 mL/min); ionization voltage, 70 eV; electron multiplier voltage, 1 kV. Acetylated fractions in each case gave a single peak accounting for 98% of the chromatogram. This peak was identified as glucose by comparing its mass fragmentation pattern with that of standard spectra available in the spectral library (Flavor and Fragrance and Wiley/NIST libraries) of the instrument.

The purified isoflavones obtained from the column were directly introduced into the mass spectrometer and bombarded with electrons accelerated at 70 eV. Samples were heated from 60 to 350 °C at the rate of 40 °C over a period of 20 min.

HPLC. HPLC analysis was carried out on a Pharmacia LKB system equipped with a C-18 reversed phase stainless steel column (250 mm × 4.6 mm). Samples were analyzed according to the method proposed by Seo and Morr (*17*). Samples (10 μL each of the butanol fraction, 1% solution) were injected onto the column and then eluted with water/acetic acid (95:5) as solvent A and methanol as solvent B. A gradient elution from 100% A for the first 5 min, followed by a linear increase to 100% B in A over a period of 55 min at a flow rate of 1.0 mL/min, was used. Peaks were identified by comparing their retention times with available authentic genistein as well as with the isolated isoflavones obtained above injected under identical conditions. Hold-up time (t_0) was obtained by determining the retention time of uracil injected under similar conditions as above. Retention factor (K) was then calculated.

Quantitative Estimation of Isoflavones. Genistein (1 mg) was dissolved in 1 mL of methanol to obtain a stock solution of the standard. Aliquots of this solution ranging in concentration from 1 to 10 μg were injected onto the HPLC column under the same conditions as above. A graph of concentration versus peak area was then drawn to obtain a standard curve (correlation coefficent of 0.996). The graph was found to be linear in range of 1−8 μg. Content of the individual isoflavones present in each of the butanol extracts was estimated from the standard curve and expressed as milligrams of isoflavone per 100 g of soybean.

Antioxidant Activity. Antioxidant activity of butanol extracts was assayed according to the method of Li et al. (*18*) using DPPH. An aliquot of sample solution (0.5 mL) was treated with 2.5 mL of DPPH (4.5 mg in 100 mL of methanol). The solution was thoroughly mixed, and the decrease in OD was recorded on a spectrophotometer over a period of 10 min at 517 nm. Distilled water (0.5 mL) was used as blank. Trolox (2 mmol/mL) was used as standard. Antioxidant activity was measured as percent DPPH scavenging activity, which is defined as

$$\%\ \text{DPPH scavenging activity} = \frac{\text{absorbance of blank} - \text{absorbance of extract}}{\text{absorbance of blank}} \times 100$$

Data Analysis. Data collected are an average of three independent determinations, each carried out in triplicate. Thus, a total of nine estimations were carried out for each sample, and standard deviations were calculated. Statistical analysis was done using a paired *t* test and ANOVA (Microcal Origin 4.1 software), and the results expressed as significant/nonsignificant at $p \leq 0.01$. Correlation between increase in dose with increase in aglycon content and decrease in glycoside content as well as between dose and antioxidant activity was also determined.

RESULTS AND DISCUSSION

Radiation processing of foods by ionizing radiation such as γ- and X-rays and electron beams has in recent years assumed considerable importance as a technology to reduce postharvest food losses by increasing shelf life and to eliminate food-poisoning microorganisms. The overall goal of this study was to determine the stability of isoflavones, a major bioactive constituent of soybean, and to examine the effect of these changes on the soybean's antioxidant activity after radiation processing.

The 80% aqueous methanol extract as well as its subsequent butanol fraction gave absorption maxima at 262 nm, suggesting the presence of isoflavones in these isolates. The butanol fraction when subjected to TLC resolved into six major spots at R_f values of 0.40, 0.35, 0.32. 0.3, 0.27, and 0.22. The spots at R_f 0.40 and 0.32 were identified as genistein and diadzein by comparing their R_f values with those of authentic standards as well as by their brown and brilliant blue fluorescence when visualized under a UV lamp (254 nm) after exposure to ammonia vapor (*19*). Components at R_f 0.3, 0.27, and 0.22 after acid hydrolysis migrated to 0.40, 0.35, and 0.32, respectively, on TLC, suggesting their glycosidic nature. The identities of each of the purified spots were confirmed as genistein (R_f 0.40, m/z 270 [M^+]), glycitein (R_f 0.35, m/z 284 [M^+]), diadzein (R_f 0.32, m/z 254 [M^+]), genistin (R_f 0.3, m/z 432 [M^+]), glycitin (R_f 0.27, m/z 446 [M^+]), and diadzin (R_f 0.22, m/z 416 [M^+]) by their mass spectral data.

Table 1 gives the distribution of the above isoflavones as estimated by HPLC in non-irradiated control as well as samples exposed to various doses of radiation. The total isoflavone content in the control sample was found to be 152.05 mg/100 g of soybean. Wide variations in contents of isoflavones (100−300 mg/100 g) have been reported in the literature depending

Table 1. Total and Individual Isoflavone Contents (Milligrams per 100 g) in Non-irradiated and γ-Irradiated Samples As Estimated by HPLC

	glucoside (retention time in min) (retention factor)				aglycon (retention time in min) (retention factor)				
sample	diadzin (23.53) (8.4)	glycitin (24.95) (8.98)	genistin (26.85) (9.74)	total glucoside	diadzein (29.25) (10.7)	glycitein (30.24) (11.1)	genistein (31.92) (11.77)	total aglycon	total isoflavones
non-irradiated	39.11 ± 2.73	31.15 ± 1.44	75.45 ± 4.58	145.71 ± 6.37	1.8 ± 0.24	1.26 ± 0.13	3.28 ± 0.06	6.34 ± 0.25	152.05 ± 6.3
γ-irradiated									
0.5 kGy	30.57 ± 0.87	26.14 ± 1.68	57.16 ± 2.37	113.87 ± 1.83	2.62 ± 0.13	1.0 ± 0.05	3.43 ± 0.08	7.05 ± 0.09	120.92 ± 1.87
1 kGy	28.27 ± 0.92	24.04 ± 0.76	53.13 ± 2.47	105.44 ± 2.41	2.45 ± 0.07	0.88 ± 0.002	4.01 ± 0.15	7.34 ± 0.27	112.78 ± 2.61
2 kGy	22.59 ± 1.73	17.06 ± 0.2	42.25 ± 1.22	81.9 ± 1.23	2.27 ± 0.28	0.87 ± 0.001	4.63 ± 0.25	7.77 ± 0.19	89.67 ± 1.35
5 kGy	20.19 ± 0.58	16.43 ± 0.16	40.96 ± 0.77	77.58 ± 0.99	1.91 ± 0.17	0.85 ± 0.005	5.29 ± 0.28	8.05 ± 0.24	85.63 ± 1.15

[a] All means (± SD, $n = 9$) are significantly different at $p \leq 0.01$ (within columns).

upon changes in crop year, location, and variety as well as climactic, environmental, and genetic factors (*20*). The content of individual isoflavones is also comparable with the reported literature values (*20*).

Glucosides account for 95% of the total isoflavones. Besides the above isoflavone glucosides identified in this study, soybeans have been reported to have a high content of malonylated glucosides of genistein, glycitein, and diadzein (*20*). These constituents were, however, not detected here. Heating during extraction is known to result in rapid transformation of malonylated derivatives to the corresponding glucosides (*21*), which could possibly explain their absence in the present study. As the study deals with a comparative profile of isoflavones and a similar extraction technique was followed, it is assumed that the changes due to thermal treatment are similar in the other isoflavone constituents in the different soybean samples presently studied.

A significant ($r = -0.9678$, $p < 0.01$) decrease in total isoflavone content as well as glucosides with increasing dose could be clearly noted (**Table 1**). These decreases were more prominent at doses >1 kGy. The aglycon content, on the other hand, showed an increasing trend ($r = 0.98443$, $p < 0.01$). The results suggest a radiation-induced breakdown of glycosides resulting in release of free isoflavones. Isoflavone content and composition are known to vary in different soy foods depending upon manufacturing practices. Thermal treatments such as extrusion, baking, and frying were shown to alter the profile of isoflavone conjugates and increase free isoflavones without affecting the total isoflavone content (*13*, *14*). Ungar et. al. (*22*), in their recent work on the thermal degradation of genistein and diadzein, have demonstrated higher thermal stability of genistein compared to diadzein. This was attributed to the stabilization of the 5-hydroxy group of genistein attached to ring A of the molecule by the 4-oxo moiety in ring C of the isoflavone.

A similar trend in radiation stability of genistein and diadzein was also observed in the present study (**Table 1**). Whereas the content of genistein increased with radiation dose, that of diadzein showed an initial increase at a dose of 0.5 kGy and then decreased at higher doses. Degradation of diadzein beyond 0.5 kGy could thus be assumed. Glycitein appears to be the least stable among the three aglycons as its content decreased at all of the doses studied.

Genistein and diadzein are known to inhibit low-density lipoprotein (LDL) oxidation and iron-mediated free radical reaction by scavenging peroxy radicals (*23*). Previous studies (*24*) have demonstrated a higher antioxidant activity for genistein compared to diadzein. Antioxidant activities of their degradation products, however, showed a reverse trend, with diadzein products being more active than that formed from genistein (*25*).

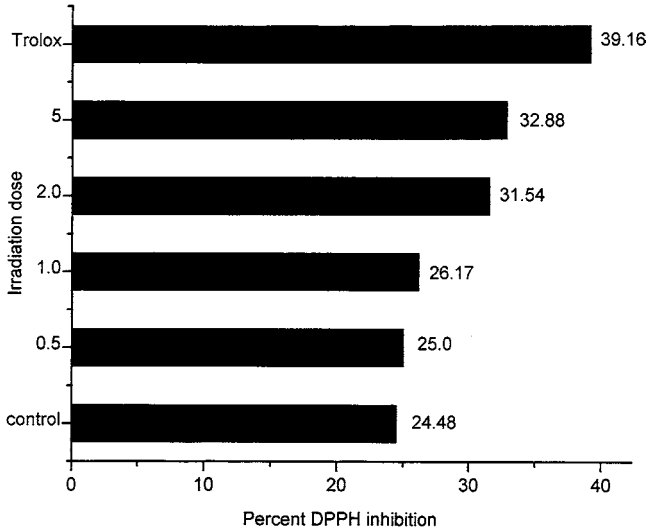

Figure 1. Percent DPPH inhibition of non-irradiated control and γ-irradiated samples at various doses compared to Trolox as standard.

Butanol extract of control soybean as well as that of samples irradiated at 0.5, 1.0, 2.0, and 5.0 kGy exhibited DPPH scavenging activities of 24.48, 25.0, 26.17, 31.54, and 32.88%, respectively (**Figure 1**). Increase in DPPH radical scavenging activity co-varied positively with increase in aglycon ($r = 0.94436$, $p < 0.01$). Higher antioxidant activity of irradiated samples could thus be attributed to the increased levels of genistein and to a lesser extent on the antioxidant activities of diadzein degradation products. Beyond 1 kGy the antioxidant activity increased substantially (**Figure 1**) with the 5 kGy treated sample showing activities approaching that of standard Trolox (39.16%). This increase could be correlated to the higher levels of free isoflavones in samples treated with radiation doses >1 kGy.

Free flavonoids have been shown to have a greater antioxidant effect than glycosides. Miyake et al. (*26*) have demonstrated higher antioxidant activities for aglycons than for their glycosides in lemon fruit. In a recent study on the antioxidant effects of isorhamnetin and its diglucosides in rats, Yokozawa et al. (*27*) have shown that although isorhamnetin had a potent antioxidant effect in vitro and in vivo, the glucoside had no DPPH radical scavenging activity. They hypothesized that the glucoside was metabolized by intestinal bacteria to isorhamnetin, resulting in reduced levels of serum glucose and glycosylated protein, thereby protecting serum and tissue mitochondria against lipid peroxidation.

There is a misconception among consumers that radiation-processed foods have a lower content of bioactive nutrients brought about by their radiolytic degradation, resulting in lower

nutritive value. This study strongly suggests that radiation treatment causes an increased availability of free isoflavones, resulting in greater bioavailability of these antioxidant phenolic compounds. Thus, besides reducing microbial load and preventing insect infestation, radiation processing of soybean also increases nutritional quality of this legume by enhancing the levels of antioxidants.

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