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Antioxidant Properties of Soybean Isoflavone Extract and Tofu in Vitro and in Vivo

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Isoflavones in soybean were extracted in the crude form using 80% food-grade ethanol at 80 °C for 6 h and followed by concentration and dehydration. The soy extract contained isoflavones primarily in the forms of glucosides. In vitro antioxidant activities of the soy extract containing 20−500 ppm isoflavones were conducted using a Rancimat method. The results showed that soy isoflavone extract had strong in vitro antioxidant activity. There was a dose-dependent response for the in vitro antioxidant activity at the lower concentrations but not at the higher concentrations. In vivo antioxidant property was determined by measuring the antioxidant enzymes, superoxide dismutase, and catalase in various organs of rats that were fed with diets containing partially oxidized oil and various levels of isoflavones for up to 24 weeks. Neither short-term (8 weeks) feeding nor low isoflavone content (50 ppm) induced changes in superoxide dismutase or catalase activities in rats. Only diets containing high isoflavone contents (150 and 250 ppm) showed obvious elevated enzymatic levels in various organs. In addition, a laboratory-prepared tofu containing approximately 50 ppm isoflavones had better effects than the soy extract with the 250 ppm isoflavone group, which indicated that molecules other than isoflavones may have a synergistic effect on in vivo antioxidant enzyme inductions of tofu.

KEYWORDS: Isoflavones; antioxidant activity; soy extract; tofu; SOD; catalase

INTRODUCTION

Epidemiological studies have shown that the consumption of soybean products is inversely correlated to human cancer, osteoporosis, and cardiovascular diseases (*1*−*6*). Isoflavones are believed to contribute to the biological effects of soybeans. Various animal studies indicate that isoflavones have antioxidant, anticarcinogenic, and osteoporosis-preventing characteristics (*7*, *8*).

Depending upon the method of antioxidant assays, various forms of isoflavones have been shown to possess antioxidant activities. Purified isoflavones can inhibit lipid peroxidation in vitro by acting as free radical scavengers or as metal-chelating agents (*9*). Among the three isoflavone aglycones, genistein is the most potent antioxidant in a carotene bleaching assay (*10*). The phenolic acids such as hydroxylated cinnamic acid derivatives in the aqueous extract of soybean also have antioxidant activity (*10*). The combined isoflavone and phenolic acids account for nearly all the in vitro antioxidant activity of soybean, soy flour, and concentrate (*10*). However, a separate study (*11*) shows, among all aglycones, that only genistein has in vitro antioxidant activity as measured by a Rancimat method in a lard system. Phospholipids can synergistically promote antioxidant activity of genistein (*11*). Naim et al. (*12*) shows that aglycones have greater antioxidant activities than their glucosides. Therefore, glycosylation of isoflavones may depress the antioxidant activities of aglycones. However, malonyl isoflavones also have been shown (*13*) to exhibit strong antioxidant activities using a chicken olein storage test; but at the end of the storage, all malonyl forms are in the forms of glucosides. Recently, Yen and Lai (*14*) showed that supplementations with either purified isoflavones (genistein and daidzein) or 80% methanol extract (containing isoflavones) from several soy foods, including tofu, inhibited reactive nitrogen species-induced oxidation both in vitro and in vivo. The inhibition was positively correlated with the total isoflavone contents in the extract.

Genistein exhibits antioxidant properties by preventing hemolysis of the red blood cells by dialuric acid or H_2O_2, by protecting against microsomal lipid peroxidation induced by an Fe^{2+}−ADP complex and NADPH (*15*−*17*), by suppressing the NADH oxidase and respiratory chain in the rat liver mitochondria (*18*). Genistein also suppresses H_2O_2 production by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-stimulated human polymorphonuclear leukocytes and superoxide anion ($O_2^{\bullet}$) formation by xanthine/xanthine oxidase through the c-fos pathway (*19*). Genistein might directly target mitogen-activated protein and

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leads to the deregulation of cellular response (*20*). Genistein inhibits DNA topoisomers II (*21*) and ribosomal S6 kinase (*22*), which may lead to protein-linked DNA strand breaks. Genistein also can inhibit tumor cell growth and differentiation induction of several malignant cell lines (*23*−*25*). Other isoflavones or isoflavone metabolites, such as equol and O-DMA, also show potent antioxidant effect (*26*).

The in vivo feeding of a 250 ppm genistein (pure compound) diet to SENCAR mice for 30 days significantly increased the activities of catalase in small intestine, liver, and kidney; the activities of superoxide dismutase and glutathione peroxidase in skin; and the activity of glutathione reductase in skin and small intestine (*27*). The same study also shows that 50 ppm genistein feeding elevated catalase activity in the small intestine and glutathione-*S*-transferase activities in skin, small intestine, liver, kidney, and lung. The greatest enhancement by feeding genistein is in the skin and small intestine. The authors suggested that the increased activities of antioxidant enzymes in various organs might be a mechanism of genistein chemopreventive action on cancers. However, a conflicting report (*28*) on the effect of feeding Okara Koji, a fermented Okara that had a higher total antioxidant activity than unfermented Okara, on glutathione peroxidase activity was reported by Matsuo (*28*), who found that feeding Okara Koji had the same effect as adding vitamin E to unfermented Okara to decrease the activity of glutathione peroxidase activity in rat plasma or liver after feeding for 36 days. Therefore, further research is needed to clarify the effect of antioxidant on antioxidant enzyme activities in various tissues. If soy extract, which contains a mixture of various forms of isoflavones, could achieve the antioxidant effect in vivo, soy extract may be used as an economical material for chemopreventive purpose since pure isoflavones are costly.

Long-term feeding up to 24 weeks with various concentrations of soy isoflavone extract using oxidized oil to evaluate the in vivo effects has not been reported. The objective of this study was to compare the in vitro antioxidant activity of soy extract with vitamin E and to study the antioxidant enzymes induction ability of the soy extract containing various isoflavone levels and of tofu using a diet containing partially oxidized oil. The aim of these dose−response determinations was to estimate how much isoflavones and how long would provide the best effect. Hopefully, our study would contribute to the application of isoflavones in the food industry. Frying is frequently used in the food industry and in the food service industry. Oxidized fatty acids in the frying oil may increase significantly after a period of time of extensive use. Frying oil was partially oxidized and used in this in vivo feeding study. Our intention was trying to accentuate the needs for antioxidants in vivo. The information obtained may have implications to the food industry and to the population who consume fried foods frequently.

MATERIALS AND METHODS

Soybean. Soybean (the Kato variety) was obtained from Sinner Brothers and Bresnahan Co. (Casselton, ND). Soybean was ground using a plate mill (Straub model 4E grinding mill, Philadelphia, PA) to pass through a 30-mesh screen.

Chemicals. Chemicals used in this study were of the reagent grades. Diethylenetriamine-pentacetic acid (DTPA), Tris, pyrogallol, potassium phosphate, and hydrogen peroxide were purchased from Aldrich Chemical (Milwaukee, WI). Vitamin E is from Nature Made Co. (Mission Hill, CA).

Preparation of Soy Extract Containing Isoflavones. Batches of 50 kg of soybean were ground using a plate mill (Straub model 4E grinding mill, Philadelphia, PA) to pass a 30-mesh size screen. Soybean flour was mixed with 80% food grade ethanol (1:10 wt/v) in a 200 L enclosed jacketed kettle (Lee Industries In., Philipsburg, PA) for 6 h. A water circulation system (VWR Scientific, Marshalltown, IA) was applied to control the extraction temperature at 80 °C. The extract solution was collected and concentrated by a climbing-film evaporator (Standard Industries, Fargo, ND) for two passes, and then the concentrated extract containing isoflavones in ethanol was concentrated in a vacuum shelf drier (Buffalo Technologies Co., Buffalo, NY) for 16 h under room temperature. The resulting syrup was freeze-dried (Virtis Co., Gardiner, NY). The dried solid was then ground to pass through a 30-mesh screen.

Tofu Processing. Tofu was produced from the Kato soybean using a pilot-scale method developed in our laboratory using calcium sulfate as a coagulant (*29*). Tofu was then freeze-dried and ground to fine powder to pass a 60-mesh screen.

Analysis of Isoflavones and Proximate Chemical Analysis. The soy extract and tofu samples were sent to Dr. Patricia Murphy's laboratory at the Iowa State University for isoflavone analysis using an established method developed in their laboratory (*30*). AOAC methods (*31*) were used for other chemical compositions, including moisture (Vacuum Oven Method 925.09), protein (Method 955.04 using 6.25 as conversion factor), lipids (Method 945.39), and ashes (Method 924.05). Carbohydrates were determined by difference.

In Vitro Experiment. Soy extract containing 25, 50, 100, 150, 250, 300, and 500 ppm isoflavones (in the forms of glucosides and aglycones, excluding the acetyl and malonyl glucosides) was tested for antioxidant activity in 5 g of sunflower oil (from Cargill, Fargo, ND) by the Rancimat method (*13*), and vitamin E was selected as a reference. Autoxidation of sunflower oil was induced by a stream of air (17.5 L/h) at 98 °C in a model 679 Rancimat (Metrohm, Switzerland) according to the method described in the instrument manual. The experiments were conducted in duplicate. The ratio of the induction time of the oil containing isoflavones or vitamin E with that of the oil without isoflavones or vitamin E was used as an indicator for the in vitro antioxidant activity.

Diet Preparation. The pretest diets were formulated as AIN 93G (*32*), and all components were obtained from Dyets Inc. (Bethlehem, PA). The experimental diets also were formulated according to the formulation of AIN 93G, except that soybean oil (Cargill Co. Fargo, ND) without any added antioxidants was used. The Vitamin Mix without vitamin E was used for the diet. Soybean oil was held at 160 °C in a fryer for 5∼7 d until the oil reached the peroxide value of 50 mequiv as determined by the AOCS Method Cd 8-53 (*33*). The method determines mequiv of peroxide/1000 g of sample that oxidizes potassium iodide under the conditions of the test. The partially oxidized oil was then stored in a freezer at −20 °C until the diet preparation.

The freeze-dried crude extract containing isoflavone was added to the control diet so that the diet contains 50, 150, and 250 ppm isoflavones (glucosides and aglycones). If counting the acetyl and malonyl glucosides, the isoflavone 50, 150, and 250 ppm diets contained 67, 200, and 334 ppm of the total isoflavones, respectively. A tofu-based diet containing 50 ppm isoflavones (corresponding to 60 ppm total isoflavones) was also formulated to evaluate the effects of nonalcohol extractable compounds by comparing the results with the crude soy extract group. A positive control containing vitamin E (25 ppm) was included for comparison. A negative control was formulated without any soy extract and vitamin E. All prepared diets were kept in a −20 °C freezer until feeding. The peroxide value was determined every 2 months. Results showed that there were no significant changes during the 6-month storage in the freezer.

Feeding Experiment. A total of 126 male, 3-week-old Sprague−Dawley rats were purchased from Harlan Co. (Indianapolis, IN) and housed individually in stainless steel cages. Upon delivery to the animal research facility at North Dakota State University, rats were acclimatized for 2 weeks on the pretest diet without vitamin E as described by Anderson et al. (*34*). Rats were randomized, and 21 rats were assigned to each of the six diets (negative control, positive control, tofu base, and three isoflavone diets: 50, 150, and 250 ppm). Seven rats in each group were fed for 8, 16, and 24 weeks, respectively. The feed consumption by each rat was recorded. At the end of each time period, seven rats in each group were sacrificed by using ether.

Table 1. Chemical Composition of Freeze-Dried Tofu and Soy Extract (g/100 g of sample)[a]

composition	tofu	extract
moisture	2.58 ± 0.28	4.63 ± 0.45
ash	7.39 ± 0.01	15.70 ± 2.10
protein	47.89 ± 0.32	6.71 ± 0.06
fat	23.17 ± 0.16	45.19 ± 2.12
CHO	18.96 ± 0.17	27.77 ± 1.02

[a] Data are means ± standard deviations of triplicate determinations.

Table 2. Isoflavone Composition in Freeze-Dried Tofu and Crude Isoflavone Extract[a]

	tofu (mg/100 g)	extract (mg/100 g)
daidzin	25.7 ± 1.9	471.6 ± 13.3
glycitin	4.1 ± 0.2	55.3 ± 3.9
genistin	35.1± 3.1	626.0 ± 16.7
6-*O*-malonyldaidzin	4.2 ± 0.1	130.8 ± 8.9
6-*O*-malonylglycitin	0.5 ± 0.5	17.5 ± 2.2
6-*O*-malonylgenistin	1.3 ± 0.1	31.5 ± 6.2
6-*O*-acetyldaidzin	0.8 ± 0.1	10.8 ± 2.2
6-*O*-acetylglycitin	0.5 ± 0.4	51.3 ± 4.8
6-*O*-acetylgenistin	6.4 ± 0.4	166.4 ± 13.1
daidzein	1.7 ± 0.1	7.9 ± 1.1
glycitein	0.8 ± 0.1	48.8 ± 3.2
genistein	2.5 ± 0.2	7.5 ± 0.8

[a] All samples were quantified in triplicate. Data are means ± standard deviations.

Sample Collection and Preparation. Tissue/organ samples were collected as previously described by Maestro and McDonald (*35*). In brief, the dorsal hair of rats was shaved immediately after sacrifice. Dorsal skin, small intestine, liver, kidneys, and lungs were removed and immediately placed in ice-cold 50 mM potassium phosphate buffer (pH 7.4). Tissues were cleaned by rinsing in buffer carefully and homogenized at 20000 rpm in 50 mM potassium phosphate buffer (pH 7.4) for 15−25 s in a tissue homogenizer (model T25-S1, IKA-Labortechnik, Staufen, West Germany). Tissue sample specimens ranging in size from 25 to 100 mg were further homogenized for 45 s (in 1-s bursts to minimize heat production) in 1.0 mL of ice-cold buffer. The homogenates were centrifuged (Beckman High-Speed Refrigerated Centrifuge, model J2-HS) for 10 min at 10000*g* at 4 °C. The supernatants were used for immediate enzyme activity assays or stored at −70 °C until analysis. All enzymes were assayed within 3 weeks.

Assay for SOD and Catalase Activity. Superoxide dismutase (SOD) activities were assayed as described by Maestro and McDonald (*35*). Catalase (CAT) activities were assayed as described by Aebi (*36*).

Statistical Analysis. Statistical analysis was conducted by using the SAS package (SAS Institute, Inc., Cary, NC). ANOVA was performed on all isoflavone data collected in this factorial, cross-over design study. ANOVA was also applied for the analysis of body weight and diet consumption. The correlation of Rancimat value to the isoflavone concentration was determined by Pearson's correlation coefficient. Effects of different diets feeding were analyzed by Duncan multiple range difference test at $\alpha = 0.05$.

RESULTS

Proximate Chemical Composition and Isoflavones in Tofu and Soy Extract. Table 1 shows that proximate chemical compositions of tofu and soy extract. The tofu composition was generally consistent with the literature report (*29*). Soy extract was high in crude lipids (presumably phospholipids or pigments), carbohydrates (presumably soluble sugars), and ashes. **Table 2** shows the isoflavone compositions in tofu and soy extract. Tofu contains approximately 70 mg/100 g of total isoflavones, predominantly in the form of glucosides. The concentration of the tofu made from the Kato variety was slightly higher than that reported in the literature (*37*, *38*). Soy extract contained much higher isoflavone content (1217 mg/100 g) than tofu. It is well-known that most of the isoflavones in raw soybean are in the form of malonyl glucosides. Heat treatments converted some malonyl glucosides to acetylglucosides and glucosides (*38*, *39*).

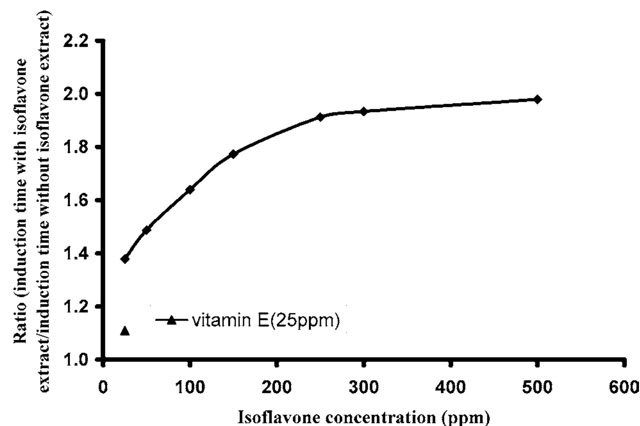

Figure 1. In vitro antioxidant activity of crude soybean isoflavone extracts. Activities were quantified by the Rancimat method. Vitamin E at 25 ppm concentration was used as a positive control.

In Vitro Study. Soy isoflavones were believed to have antioxidant activity in vitro. The extract of *Garcinia kola* was reported to inhibit in vitro lipid peroxidation of rat liver homogenate in a dose-dependent manner, and the active components were tentatively identified as isoflavones (*40*). In our study, crude soy isoflavone extract showed strong in vitro antioxidant activities. There were significant differences in the induction time ratios between the oil with isoflavone extract and the oil without isoflavones ($p < 0.05$). At the lower isoflavone concentrations in the range between 25 and 150 ppm, a linear dose-dependent response was noted ($R^2 = 0.978$). When the isoflavone content was relatively high, however, its antioxidant power reached a plateau (**Figure 1**).

The antioxidant property of isoflavones contributed to their power of inhibition in a non-zero-order manner. Once the inhibitions were saturated, there were no dose-dependent effects. When added at the same concentration of 25 ppm, soy isoflavone extract showed stronger in vitro antioxidant activities than vitamin E (**Figure 1**).

In Vivo Study. There were no significant differences in the diet consumed (ranged from 2800 to 3000 g for 24 weeks) and the body weight of rats among different feeding groups (ranged from 499 to 522 g by the end of 24 weeks) during the course of the feeding experiment.

Changes of SOD Activities in Various Organs. Table 3 illustrated that the SOD in various organs/tissues induced by the diets containing vitamin E, soy extract with different isoflavone levels, and tofu and compared to that in the negative control. The 50 ppm isoflavone feeding did not contribute to any significant increases of SOD activity during the entire 24 weeks of feeding experiment. The 150 ppm isoflavone groups did not have any effects except in the small intestine after 24 weeks.

Considering all diet groups, in 8 weeks of feeding, only a few significant ($p < 0.05$) effects were produced in various organs. The isoflavone group produced a significant effect in the liver only at the 250 ppm level. The positive control—the vitamin E group—produced a significant effect only in the skin. However, the tofu group produced significant SOD increases both in the liver and skin.

Table 3. Effect of Diets Containing Various Levels of Isoflavones on SOD Activity[a,b]

diet	8 weeks	16 weeks	24 weeks
	Small Intestine		
negative	$15.04^{a} \pm 4.36$	$26.24^{b} \pm 7.34$	$24.11^{c} \pm 6.54$
positive	$17.72^{a} \pm 5.79$	$32.64^{ab} \pm 8.84$	$31.13^{ab} \pm 8.17$
50 ppm	$16.60^{a} \pm 3.21$	$28.47^{b} \pm 9.73$	$27.26^{bc} \pm 7.41$
150 ppm	$15.95^{a} \pm 7.91$	$31.11^{ab} \pm 12.31$	$31.25^{ab} \pm 8.13$
250 ppm	$17.52^{a} \pm 4.75$	$33.40^{ab} \pm 6.17$	$35.73^{a} \pm 9.08$
tofu	$17.97^{a} \pm 4.37$	$36.48^{a} \pm 8.33$	$34.67^{a} \pm 8.65$
	Kidney		
negative	$55.87^{ab} \pm 11.56$	$93.20^{a} \pm 13.92$	$82.26^{c} \pm 17.87$
positive	$64.60^{a} \pm 13.93$	$107.52^{a} \pm 21.12$	$104.30^{ab} \pm 18.35$
50 ppm	$54.15^{b} \pm 11.67$	$97.86^{a} \pm 25.00$	$88.47^{c} \pm 18.55$
150 ppm	$59.36^{ab} \pm 10.79$	$101.77^{a} \pm 11.66$	$94.26^{bc} \pm 21.84$
250 ppm	$58.00^{ab} \pm 9.90$	$98.67^{a} \pm 19.53$	$108.76^{ab} \pm 18.01$
tofu	$62.08^{ab} \pm 9.41$	$102.90^{a} \pm 24.05$	$110.44^{a} \pm 18.28$
	Liver		
negative	$91.41^{c} \pm 20.20$	$99.99^{c} \pm 24.79$	$106.67^{b} \pm 22.09$
positive	$103.22^{bc} \pm 17.73$	$121.83^{ab} \pm 24.88$	$119.95^{ab} \pm 13.27$
50 ppm	$95.56^{bc} \pm 17.73$	$106.04^{bc} \pm 30.96$	$118.44^{ab} \pm 22.66$
150 ppm	$104.54^{bc} \pm 18.43$	$112.96^{abc} \pm 22.24$	$122.91^{ab} \pm 28.48$
250 ppm	$108.53^{b} \pm 20.58$	$117.23^{abc} \pm 18.56$	$119.61^{ab} \pm 18.29$
tofu	$122.51^{a} \pm 16.20$	$130.90^{a} \pm 24.09$	$128.18^{a} \pm 23.40$
	Lung		
negative	$18.23^{a} \pm 8.39$	$30.02^{d} \pm 7.79$	$28.59^{b} \pm 8.75$
positive	$19.97^{a} \pm 5.82$	$39.91^{abc} \pm 15.26$	$37.64^{a} \pm 10.38$
50 ppm	$17.73^{a} \pm 5.27$	$34.17^{bcd} \pm 9.91$	$33.71^{ab} \pm 9.09$
150 ppm	$19.03^{a} \pm 7.08$	$31.50^{cd} \pm 9.26$	$33.84^{ab} \pm 10.56$
250 ppm	$20.92^{a} \pm 9.85$	$40.38^{ab} \pm 9.50$	$37.70^{a} \pm 8.37$
tofu	$20.93^{a} \pm 7.64$	$48.02^{a} \pm 10.47$	$39.60^{a} \pm 9.34$
	Skin		
negative	$4.45^{c} \pm 1.11$	$4.30^{b} \pm 2.25$	$4.45^{c} \pm 1.31$
positive	$6.20^{b} \pm 1.57$	$7.88^{a} \pm 2.17$	$6.90^{a} \pm 2.07$
50 ppm	$4.54^{c} \pm 1.08$	$4.96^{b} \pm 1.58$	$5.06^{c} \pm 1.17$
150 ppm	$4.96^{c} \pm 1.75$	$4.93^{b} \pm 1.27$	$5.53^{bc} \pm 1.65$
250 ppm	$5.29^{bc} \pm 1.25$	$5.79^{b} \pm 2.31$	$6.79^{ab} \pm 1.87$
tofu	$7.80^{a} \pm 1.60$	$7.66^{a} \pm 1.71$	$7.20^{a} \pm 1.86$

[a] Values are means±SD of duplicate assays in seven animals. Unit was μg of SOD/g. [b] Values in a column under each organ with different superscripts are different at $\alpha \leq 0.05$.

In the 16 weeks feeding, the 250 ppm isoflavone group significantly enhanced SOD activity in the lungs. The vitamin E group produced a significant increase in SOD in lungs, liver, and skin after 16 weeks of feeding. The tofu group produced a significant effect in the small intestine, liver, lungs, and skin.

In the 24 weeks feeding, both the vitamin E group and the 250 ppm isoflavone group produced increases in SOD in the small intestine, kidneys, lungs, and skin. However, the tofu group produced significant increases in all five organs tested. Considering all dietary groups and feeding duration combinations, tofu produced the most frequent effects (11 combinations), whereas the vitamin E group produced effects in 8 combinations, and the 250 ppm isoflavone diet produced 6 combinations. Therefore, it is reasonable to conclude that tofu is more potent than 250 ppm isoflavones or 25 ppm vitamin E in the diet to enhance SOD production. We did not observe any adverse effects to decrease SOD activities in all isoflavone diets when compared to the negative control.

When the SOD activities within each group were compared with respect to the durations of feeding (8 weeks vs 16 weeks vs 24 weeks), it was striking to observe that SOD activities increased sharply from the 8th week to the 16th week of feeding in the small intestine, kidneys, and lungs. **Figure 2** illustrates the changes of SOD in the small intestine in all diet groups. From the 16th week to the 24th week, the SOD decreased slightly in most diet groups except the 250 ppm isoflavone group showed a slow increase in this time period.

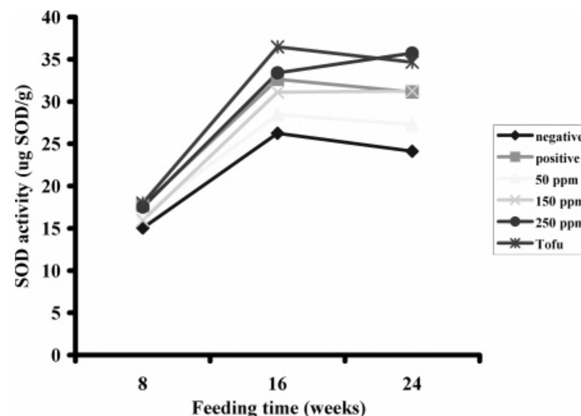

Figure 2. SOD activity changes vs feeding time. Each group contained seven independent samples, and each sample was assayed in duplicate.

Changes of Catalase Activities during Feeding. Table 3 showed that the activities of catalase were not significantly different among all five organs or tissues after feeding for 8 weeks. However, after 16 weeks, a significant increase in catalase activity was observed in the small intestine and liver of rats fed 250 ppm isoflavones. The 150 ppm isoflavone diet demonstrated significant activity increases in the small intestine, liver, and skin, whereas the 50 ppm isoflavone diet had little effect on catalase activities except in the liver. The tofu and vitamin E positive control group also produced significant increases in intestine and liver after 16 weeks of feeding.

After 24 weeks of feeding, significant differences were observed in the small intestine, liver, and kidneys of rats fed 250 ppm isoflavones; in the small intestine and liver of rats fed 150 ppm isoflavones; and in liver of rats fed 50 ppm isoflavones. The vitamin E group had significant increases in catalase activity in the small intestine, liver, and kidney. However, the tofu group had only a significant increase in the small intestine.

DISCUSSION

Both in vitro and in vivo studies suggested that oxidized fatty acids may contribute to cardiovascular system disease as well as cancers (*41*−*45*). Therefore, we chose heated soybean oil with peroxide value of 50 mequiv to accentuate the possible needs of more antioxidants to overcome the oxidative stress. Our intention was to examine if isoflavones, as natural antioxidants, could exhibit in vivo antioxidant activity and what concentrations of the isoflavones would be effective within the period of 6 months. Accumulating evidence suggests that reactive oxygen species and their subsequent modification of macromolecules (such as DNA, lipid, and protein) play an important role in cytotoxicity, genotoxicity, and carcinogenic processes (*46*, *47*). Antioxidant enzymes are capable of eliminating reactive oxygen species and products of lipid peroxidation, thereby protecting cells and tissues from oxidative damage. Antioxidant enzymes include SOD, which dismutates the superoxide radicals to molecular oxygen and H_2O_2, and catalase, which decomposes H_2O_2 to molecular oxygen and water. There is a variety of evidence indicating that antioxidant enzyme activities are lower in tumor tissues (*46*) and that certain carcinogenic agents cause oxidative damage to biological macromolecules (*47*, *48*). Genistein had been found to suppress H_2O_2 production by TPA-stimulated human polymorphonuclear leukocytes and HL-60 cells in a dose-dependent manner over the concentration range of 1−150 μM concentrations (*20*).

Short-time duration feeding, such as 8 weeks, did not induce many significant differences in enzyme activities. Neither did lower isoflavone concentrations (most of the 50 and 150 ppm groups) in the diet produce a significant effect over the 24 weeks of feeding. Webb et al. (*49*) reported that diets supplemented with soybean (3.13−25% of soybean in the diet) were more potent in chemoprevention of cancers in the rats as compared to the control at 3 months; however, only two high-soybean levels, 12.5% and 25%, were significantly different at 1 month. Therefore, isoflavones may exhibit observable antioxidant properties at a relatively high concentration level or after a long time period.

Our results indicated that SOD activity increased by the tofu and the isoflavone diets the most significantly in the small intestine and lungs, whereas catalase activity increased significantly in the small intestine and liver. Our finding that few significant differences were observed in the skin is inconsistent with others (*27*). Feeding rats with a genistein-containing diet significantly increased the activities of antioxidant enzymes (15−28% increase), suggesting that genistein was able to enhance the defense mechanism against oxidative damage to macromolecules in vivo (*27*).

Cai and Wei (*27*) reported the enhancement of antioxidant enzymes in the skin and small intestine by purified dietary genistein was more evident than in organs such as liver, kidney, and lungs. Their results showed that the activities of SOD increased significantly in skin, whereas catalase activities increased significantly in the small intestine. Their study demonstrated that dietary genistein also increased activities of antioxidant enzymes in various organs, and they also suggested that the antioxidant properties and the elevation of antioxidant enzyme activities might, at least in part, be responsible for genistein's anticarcinogenic mechanism. However, Breinholt et al. (*50*) reported that genistein significantly inhibited catalase and SOD activity, which was explained by the down-regulation with increasing antioxidant potential. In our studies, we observed the increasing trends of the antioxidant enzyme activities in many groups over time, and this was probably due to the application of oxidized soybean oil with peroxide value of 50 mequiv in the diet. Since oxidized oil could accentuate the need for antioxidant enzymes, this led to the increases in SOD and catalase activities over time.

Since soy extract containing isoflavones possessed in vitro antioxidant activities, the enzyme activities should decrease if those isoflavones acted directly as antioxidants against lipid oxidation in vivo. However, our results indicated that there were increases of those antioxidant enzymes. This is probably due to the up-regulation of the enzyme activities. Although oxidized oil was used in our research, the components, such as oxidized fatty acid and polymerized fatty acid fraction, and the secondary products of fatty acids might induce the antioxidant enzyme activities. However, the increase in enzyme activities should not be due to the oxidized oil used in the diet since oxidized oil was also used in the control diet. Isoflavones might have their effects on the pathways by inducing the gene expression or modifying the enzyme activity rather than acting directly as free radical scavengers (*27*, *51*, *52*). In addition to the animal study conducted by Cai and Wei (*27*), Choi et al. (*51*) observed significant increases in SOD and catalase in genistein-treated (50−100 μM) RAW 264.7 marcrophages. Furthermore, Rohrdanz et al. (*52*) attributed the increase in catalase activity in daidzein-treated (300 μM) rat hepatoma H4IIE cells to an increase in the increase in catalase mRNA. However, the increase in catalase did not protect against oxidative stress

Table 4. Effect of Diets Containing Various Levels of Isoflavones on Catalase Activity[a,b]

diet	8 weeks	16 weeks	24 weeks
	Small Intestine		
negative	2.29[a] ± 0.37	6.40[c] ± 1.03	4.74[c] ± 1.04
positive	2.52[a] ± 0.54	7.26[ab] ± 1.42	5.4[ab] ± 0.99
50 ppm	2.00[a] ± 0.37	6.80[bc] ± 1.10	4.93[bc] ± 0.86
150 ppm	2.31[a] ± 0.39	7.20[ab] ± 1.23	5.84[a] ± 0.81
250 ppm	2.49[a] ± 0.53	7.34[a] ± 1.27	5.89[a] ± 1.15
tofu	2.53[a] ± 0.52	7.97[a] ± 1.40	5.96[a] ± 1.31
	Kidney		
negative	131.81[a] ± 25.13	105.59[a] ± 17.01	50.91[b] ± 11.52
positive	141.67[a] ± 28.62	112.33[a] ± 12.54	60.70[a] ± 10.65
50 ppm	135.87[a] ± 23.50	106.42[a] ± 18.32	59.32[a] ± 11.73
150 ppm	141.45[a] ± 26.22	103.84[a] ± 13.86	58.27[ab] ± 12.33
250 ppm	146.90[a] ± 21.07	108.29[a] ± 16.86	62.18[a] ± 14.73
tofu	143.38[a] ± 24.78	104.45[a] ± 16.24	56.28[ab] ± 7.25
	Liver		
negative	148.11[a] ± 35.56	244.35[c] ± 31.90	223.21[c] ± 45.76
positive	146.16[a] ± 21.42	285.22[b] ± 36.83	265.76[ab] ± 37.57
50 ppm	157.03[a] ± 25.30	282.05[b] ± 44.71	263.56[ab] ± 38.40
150 ppm	161.17[a] ± 25.82	310.41[a] ± 39.11	268.82[a] ± 45.07
250 ppm	159.59[a] ± 27.41	285.07[b] ± 20.88	260.23[ab] ± 42.19
tofu	153.70[a] ± 25.49	268.44[b] ± 44.65	238.94[bc] ± 46.60
	Lung		
negative	23.12[a] ± 4.82	17.58[a] ± 2.93	16.90[a] ± 2.66
positive	23.69[a] ± 3.97	17.49[a] ± 2.53	18.26[a] ± 3.35
50 ppm	22.80[a] ± 3.56	18.83[a] ± 4.62	16.83[a] ± 2.50
150 ppm	23.38[a] ± 3.77	18.47[a] ± 3.03	17.52[a] ± 2.49
250 ppm	23.29[a] ± 3.61	19.43[a] ± 2.72	16.78[a] ± 3.13
tofu	21.90[a] ± 3.77	19.65[a] ± 3.69	17.35[a] ± 3.02
	Skin		
negative	3.64[a] ± 0.52	3.73[b] ± 0.53	3.76[a] ± 0.62
positive	3.43[a] ± 0.77	3.80[b] ± 0.60	3.65[a] ± 0.56
50 ppm	3.68[a] ± 0.57	3.66[b] ± 0.63	4.06[a] ± 0.64
150 ppm	3.57[a] ± 0.52	4.52[a] ± 0.66	3.76[a] ± 0.82
250 ppm	3.33[a] ± 0.51	3.80[b] ± 0.62	3.96[a] ± 0.71
tofu	3.46[a] ± 0.67	4.04[b] ± 0.66	4.10[a] ± 0.72

[a] Values were means ± SE of triplicate assays in seven animals. Unit was micro moles H_2O_2 consumed/min/mg tissue. [b] Values in a column under different organs with different superscripts were different at $\alpha \leq 0.05$.

resulting from H_2O_2 exposure. Daidzein itself at 200−500 μM actually exerted a mild oxidative stress. On the contrary, the treatment with genistein (50−100 μM) effectively suppressed the oxidative stress in RAW 264.7 marcrophage. Therefore, the antioxidant effect at the cellular levels may be affected by the type of isoflavones and concentrations. More research in this area is needed.

The tofu group contained only 50 ppm isoflavones. However, tofu showed the most pronounced effect in the enhancement of SOD activity in terms of having the highest number of the tissue/organ−time duration combinations during 24 months of feeding (**Table 3**). The overall antioxidant enzymes induction of tofu was apparently more potent than the isoflavones 250 ppm group. Tofu also enhanced catalase activity (**Table 4**) in the small intestine and in the liver at 16 weeks ($p < 0.05$). Our findings that tofu, which contained approximately 50 ppm isoflavones, had greater antioxidant enzyme-inducing effect than the 250 ppm isoflavone (in the form of soy extract) group might be attributed to the other tofu components, including proteins and other aqueous ethanol-unextractable residues such as tocopherols and phospholipids, which had been shown to increase antioxidant activities in vitro. These unextractable constituents may have a synergistic effect on in vivo antioxidant activities. Further research on isolation, identification, and characterization of the effects of the isolated and recombined components is needed to understand this phenomenon. Tofu is a popular traditional

Oriental food. The antioxidant property of tofu implied that consuming tofu may bring health benefits to fight against aging or some chronic diseases.

Comparison the in Vitro and in Vivo Studies. In the in vitro study, we found out that isoflavone extract had strong antioxidant activity, even under low concentrations. Their power was stronger than vitamin E once applied under the same low concentration (25 ppm). However, their in vivo antioxidant activities at the levels of 50−150 ppm were not so strong as they were estimated in the in vitro study. There was no dose−response relationship between isoflavone intake and the antioxidant enzyme activities in vivo. At the low concentration level of 50 ppm isoflavones, most organs/tissues showed no significant differences in the antioxidant enzyme activities when compared with the negative control group. The differences only became significant in several organs/tissues once the dietary isoflavone contents were increased to 250 ppm.

In the SOD studies, it seems that 250 ppm isoflavone group had similar in vivo effect as the 25 ppm Vitamin E positive control group. The bioavailability of isoflavones might contribute to the differences between their in vitro and in vivo effects. Only a fraction of the ingested isoflavones is absorbed. Setchell et al. (*53*) reported that isoflavone glycosides are not absorbed directly unless they are hydrolyzed by the intestine enzymes or microbial enzymes. Izumi et al. (*54*) reported that aglycones were absorbed faster and in greater amounts than glucosides in humans. Andlauer et al. (*55*), however, reported that some aglycones and glucosides from tofu are absorbed directly in the small intestine of rats, whereas the malonyl forms are not.

In addition to the differences in absorption, some in vivo metabolites of flavonoids were better antioxidants than the parent compounds (*54*). Isoflavone metabolic products were more potent inhibitors of lipoprotein oxidation in their in vitro study, and this made the explanation for the isoflavones antioxidant activities more complex (*56*). The complexity of the isoflavone affects enzymatic processes, and additionally, the effect on a given enzyme system may be related to the structures of the isoflavones, the specific tissue, and systemic bioavailability. The levels of isoflavones that were applied in the previous study exceeded the human daily intake by several 100-fold (*50*). Isoflavones in the diet in conjunction with other phytochemicals may contribute to the overall protective activity of foods against cancer, cardiovascular disease, and other illness. Further research using purified isoflavones is essential to verify the long-term antioxidant effects of isoflavones at various concentrations since the crude soy isoflavone extract may contain other phenolic compounds, saponins, peptides, and phytate that may possess or enhance antioxidant activities.

Conclusion. Soy isoflavone extract showed a strong in vitro antioxidant activity using the Rancimat induction time method. At lower isoflavone concentrations (<250 ppm), there was a dose-dependent relationship. However, once the isoflavone content was relatively high, its antioxidant power reached a plateau.

Low concentrations of isoflavones and short-term rat feeding (8 weeks) did not induce significant differences in the SOD or catalase activities in a great majority of tissues/organs studied. High isoflavone concentraitons (250 ppm) showed enhanced enzymatic levels in several tissues/organs at 16 and 24 weeks. The need for high isoflavone concentrations might be partly due to the use of oxidized oil in the diets.

The reason tofu with approximately 50 ppm isoflavone had a greater enzyme induction effect than the 250 ppm isoflavone group may be due to the synergistic effects with other biochemical components, including proteins and other unextractable residues. Tofu is an inexpensive food as compared to isoflavone concentrate pills. Eating tofu may be better to achieve potential health benefits than eating crude isoflavone extract concentrate. However, the crude isoflavone extract may be incorporated in non-soy foods to achieve similar health benefits of soy foods.

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