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Soy isoflavones: Database development, estrogenic activity of glycitein and hypocholesterolemic effect of daidzein

by

Tongtong Song

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Major Professor: Patricia A. Murphy

Iowa State University

Ames, Iowa

1998

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ABSTRACT

Soy isoflavones are proposed to have certain health protective activities, including prevention of cancer, lowering blood cholesterol levels and reducing postmenopausal bone loss. To evaluate the health-enhancing properties of isoflavones, a stable and inexpensive source of these compounds is necessary. Chemical synthesis is a practical way to obtain pure isoflavones. The synthesis of daidzein was modified to a simple, fast and high yield method by cyclizing 2,4,4'-trihydroxydeoxybenzoin (THB) with boron trifluoride etherate and methanesulphonyl chloride. The yield was >86%, and the purity of synthesized daidzein was >98%. The intermediate of daidzein synthesis, THB, with a similar structure to the isoflavones, proved to be an excellent internal standard in soy isoflavone analysis.

The estrogenic activity of glycitein was investigated by using *in vivo* mice uterine enlargement assay and *in vitro* estrogen receptor (ER) binding assay. Glycitein demonstrated an estrogenic response in mice uterine enlargement assay 3 times greater than genistein, but 30,000 times less than diethylstillbestrol (DES). Glycitein bound to mice uterine cytosol estrogen receptor proteins with an affinity similar to daidzein but 20 times lower than genistein. This is the first report demonstrates that glycitein has estrogenic activity comparable to daidzein and genistein. Glycitein, although only accounts for 5 to 10% of the total isoflavones in soybeans, can not be neglected in evaluating isoflavones' health enhancing properties and in development of isoflavone database in foods.

The dietary effects of soy protein, with or without isoflavones, daidzein and soygerm on plasma cholesterol levels were investigated in golden Syrian hamsters. Daidzein, soygerm or soygerm extract, when added to casein diet, lowered plasma total cholesterol by 15% to 28% ($P<0.01$) and LDL-cholesterol by 15 to 50% ($P<0.01$) in both male and female hamsters compared with the casein control group. Soy protein and isoflavone-depleted soy protein, when compared with the casein control, significantly lowered total cholesterol ($P<0.01$) and LDL-cholesterol ($P<0.01$) levels for both genders. These data suggest that the isoflavone daidzein, soygerm and soy protein with or without isoflavones, have the ability to lower plasma cholesterol levels in hamsters.

GENERAL INTRODUCTION

A. Introduction

Soybeans contain significant amounts of isoflavones at 1-3 mg/g. Three types of isoflavones are found in soybeans: daidzein, genistein, and glycitein. These isoflavones predominantly occur as glucoside forms in soybeans. The isoflavones have been associated with different health-enhancing properties such as prevention of breast and prostate cancers; lowering blood cholesterol levels, thus, decreasing the risk of cardiovascular diseases; prevention of bone resorption and reduction of postmenopausal symptoms such as hot flash.

The need to perform studies on isoflavone's possible health-enhancing properties requires an inexpensive, stable source of pure isoflavones. However, commercial isoflavones are very expensive and from limited sources. Isolation of isoflavones from soybeans is time consuming and provides very low yields. Chemical synthesis is a practical way to produce large quantity of pure isoflavones. To develop a simple, fast and high yield protocol to synthesize isoflavones is one important objective of my research.

In order to fully and safely utilize the health-enhancing properties of soy isoflavones, a database of isoflavone content of soy products is needed. Development of a database of isoflavones in foods requires accurate and precise evaluation of different food systems. Usually a good internal standard is required to perform recovery studies and quality control. Selection of an appropriate internal standard and performance of quality control during the database development is another objective of my research.

Numerous studies have been performed on the isoflavones, daidzein and genistein. They have weak estrogenic activity, which may be associated with their health-enhancing properties. In addition, genistein is a specific inhibitor of protein tyrosine kinase, a critical enzyme in cellular signal transduction and cellular proliferation. Genistein inhibits the activity of DNA topoisomerase II, an enzyme that is very important in DNA replication and cell proliferation. The antioxidant activity of daidzein and genistein may contribute to their observed anticancer activities by relieving oxidative stress. However, no biological activity of glycitein has been reported to date. Because glycitein has similar structure to the other isoflavones, we hypothesize that it would have weak estrogenic activity comparable to daidzein and genistein. Therefore, one goal is to investigate the estrogenic activity of glycitein.

The cholesterol-lowering effects of soy protein have been reported for more than 80 years. Numerous studies have been carried out to confirm this observation and to elucidate the mechanisms of this hypocholesterolemic effect. Recent studies have been focused on the roles that soy isoflavones might play in this effect. Although several animal-feeding studies using soy isoflavone extract or isoflavone-free soy proteins suggested that soy isoflavones may be one of the major factors responsible for this hypocholesterolemic effect, no direct evidence has been provided. The final objective of my research is to use pure isoflavones to investigate the possible hypocholesterolemic effect of isoflavones.

B. Dissertation Organization

This dissertation consists of a literature review and three papers. The first paper, " Isoflavone analysis: new internal standard and quality control" has been accepted for publication in the American Journal of Clinical Nutrition. The second paper, "Estrogenic activity of glycitein, a soy isoflavone", has been submitted to the Journal of Agriculture and Food Chemistry and examines the estrogenic activity of glycitein using an *in vivo* mice uterine enlargement assay and an *in vitro* estrogen receptor binding assay. The third paper, entitled " Soy protein, with or without isoflavones, daidzein and soygerm lower plasma cholesterol levels in hamsters", will be submitted to the Journal of Nutrition. This paper investigates the cholesterol lowering effects of soy isoflavone daidzein, soygerm as well as isoflavone-depleted soy protein. The papers follow the format of the journals to which they have been or will be submitted. A general conclusion chapter follows the three papers.

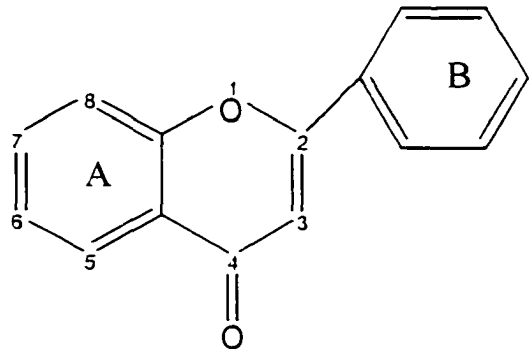
LITERATURE REVIEW

A. Plant Sources and Analytical Chemistry of Isoflavones

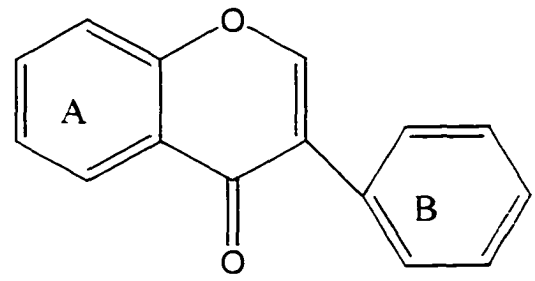
Flavonoids are phenolic compounds occurring widely in plants and almost all plant tissues are able to synthesize flavonoids (Ho, 1992; Kühnau, 1976). There are a wide variety of types with at least 2,000 different naturally occurring flavonoids. The basic structure of these compounds consists of two aromatic rings (A-ring and B-ring) linked by a three carbon aliphatic chain which is condensed to form a pyran ring or a furan ring (C-ring) (Figure 1).

In many plants, flavonoids are present in high concentrations, suggesting that they may have very important roles in the life of plants. These functions may include activities as phytoalexins by inhibiting the action of invading microorganisms (Barz and Welle, 1992) and as specific molecular signals to other microorganisms that take part in symbiotic relationships with the plants (Phillips, 1992). *Bradyrhizobium sp.*, which forms root nodules on leguminous plants, are attracted to the plant when specific flavonoids and isoflavonoids are secreted from the plant roots. The root nodules containing the bacterium convert atmospheric nitrogen to ammonia that can be used directly by the plants. The functions of isoflavones in soybeans are as inducing compounds of *nod* genes in *Bradyrhizobium japonicum* (Kosslak *et al.*, 1990) and as precursors for inducible pterocarpan phytoalexin, glyceollin I – III, in soybeans (Ebel and Grisebach, 1988).

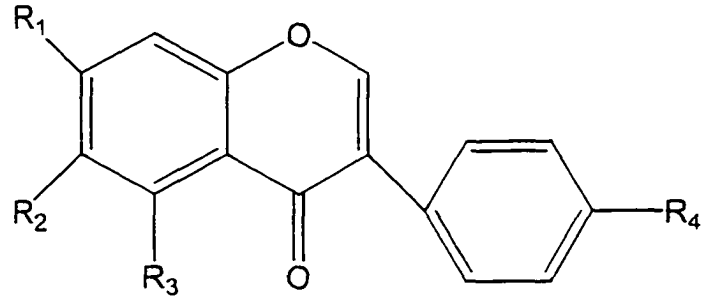
A. Flavone



B. Isoflavone



C. Common isoflavone aglycones

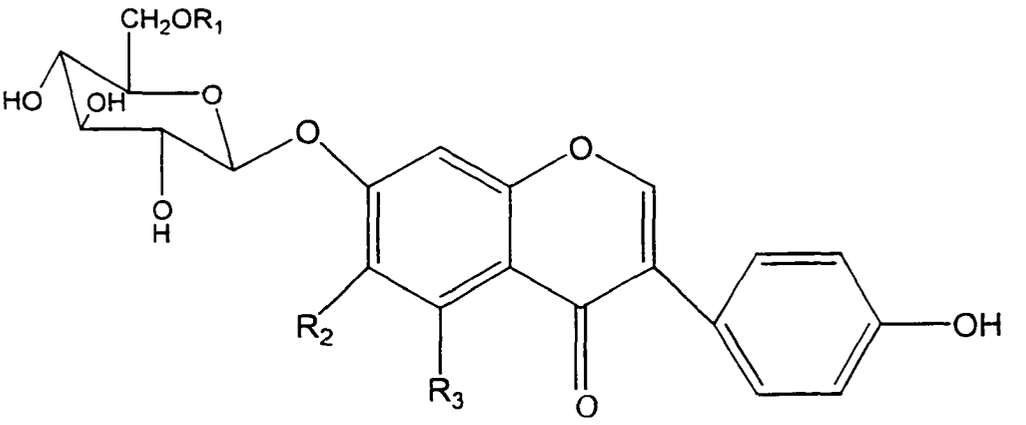


Compounds	**R1**	**R2**	**R3**	**R4**
Daidzein	OH	H	H	OH
Genistein	OH	H	OH	OH
Glycitein	OH	OCH3	H	OH
Biochanin A	OH	H	OH	OCH3
Formononetin	OH	H	H	OCH3

Figure 1. Chemical structures of flavone, isoflavone and isoflavone aglycones.

Isoflavonoids differ from other flavonoid compounds in that they have a rearranged 15-carbon skeleton derived from 3-phenylchroman (Figure 1). The isoflavonoid family includes isoflavones, isoflavanones, isoflavans, pterocarpans, coumestans and rotenoids. They differ from each other in the oxidation level of the skeletal structures. Isoflavones have B ring joined at position 3 on the C-ring and not at position 2 as in flavones (Figure 1).

The common isoflavone aglycones include daidzein, genistein, glycitein, formononetin and biochanin A (Figure 1). Unlike other flavonoid compounds, isoflavonoids have a very limited distribution in plants. Isoflavones are found primarily, and almost exclusively, in legumes. C. *arietinum* or chickpeas, mainly accumulate formononetin and biochanin A. The soybeans have the highest content, ranging from 1 to 3 mg/g (Wang and Murphy, 1994), of the isoflavones daidzein, genistein, glycitein and their glucosides of seeds consumed by humans. The aglycones, daidzein and genistein were first isolated and identified from soybean meal by Walter (1941). The third isoflavone in soybeans, glycitein, was first isolated by Naim *et al.* (1973). However, in soybeans, these isoflavones are mainly present in the plant in conjugated from, principally with glucose residue linked through one or more of the phenolic groups. Kudou *et al.* (1991) isolated and characterized nine β-glucoside forms of isoflavones from soybean hypocotyl. They are daidzin, glycitin, genistin, 6"-O-acetyl daidzin, 6"-O-acetyl glycitin, 6"-O-acetyl genistin, 6"-O-malonyl daidzin, 6"-O-malonyl glycitin and 6"-O-malonyl genistin (Figure 2). Both acetylation and malonylation of isoflavones occurs at position 6 of the glucose moiety in soybeans. The malonylated glucosides were very heat labile. At 80 °C, the majority of the glucosides were converted to daidzin, genistin and glycitin (Kudou *et al.* 1991). Kudou *et al.* (1991) reported that most of the soy isoflavones are concentrated in the hypocotyl and are four times higher concentrated



Compounds	**R1**	**R2**	**R3**
Daidzin	H	H	H
6"-O-Malonyl daidzin	$COCH_2COOH$	H	H
6"-O-Acetyl daidzin	$COCH_3$	H	H
Genistin	H	H	OH
6"-O-Malonyl genistin	$COCH_2COOH$	H	OH
6"-O-Acetyl genistin	$COCH_3$	H	OH
Glycitin	H	OCH_3	H
6"-O-Malonyl glycitin	$COCH_2COOH$	OCH_3	H
6"-O-Acetyl glycitin	$COCH_3$	OCH_3	H

Figure 2. Chemical Structures of soy isoflavone glucosidic forms.

than in cotyledons. Glycitein and its glucoside derivatives are almost exclusively found in the hypocotyl. The conjugated forms of isoflavones can be transformed to aglycones or free isoflavones through enzymatic (β-glucosidase) or acidic (2 N HCl at 98 °C for 2 hours) hydrolysis (Franke *et al.*, 1994).

Separation of isoflavones can be accomplished by using the following techniques: paper chromatography (PC), thin-layer chromatography (TLC), column liquid chromatography, reverse-phase high-performance liquid chromatography (HPLC) and gas chromatography (GC). The detection and identification of isoflavones can be achieved by using UV absorbency detection, mass spectrometry, coulometric array detection (CAD), IR and NMR analysis.

Ultraviolet (UV) spectroscopy is very effective for the identification of isoflavones (Marbry *et al.*, 1970). Since there is little or no conjugation between A- and B-rings of isoflavones, they typically exhibit an intense Band II absorption in the range of 245 – 270 nm with only a shoulder or low intensity Band I peak above 300 nm (Marbry *et al.*, 1970) (Figure 3). Band II maximal absorption wavelength is relatively unaffected by increased hydroxylation of the B-ring. For example, 5,7-dihydroxyisoflavone has maximal Band II absorption at 259 nm and 5,7,4'-trihydroxyisoflavone (genistein) has maximal Band II absorption at 261 nm. However, Band II can be shifted bathochromically by increased oxygenation in the A-ring. For example, daidzein has maximal Band II absorption at 249 nm, while genistein, with an additional 5-hydroxy group, has a maximum at 261 nm, and 6-hydroxygenistein has a maximum at 270 nm.

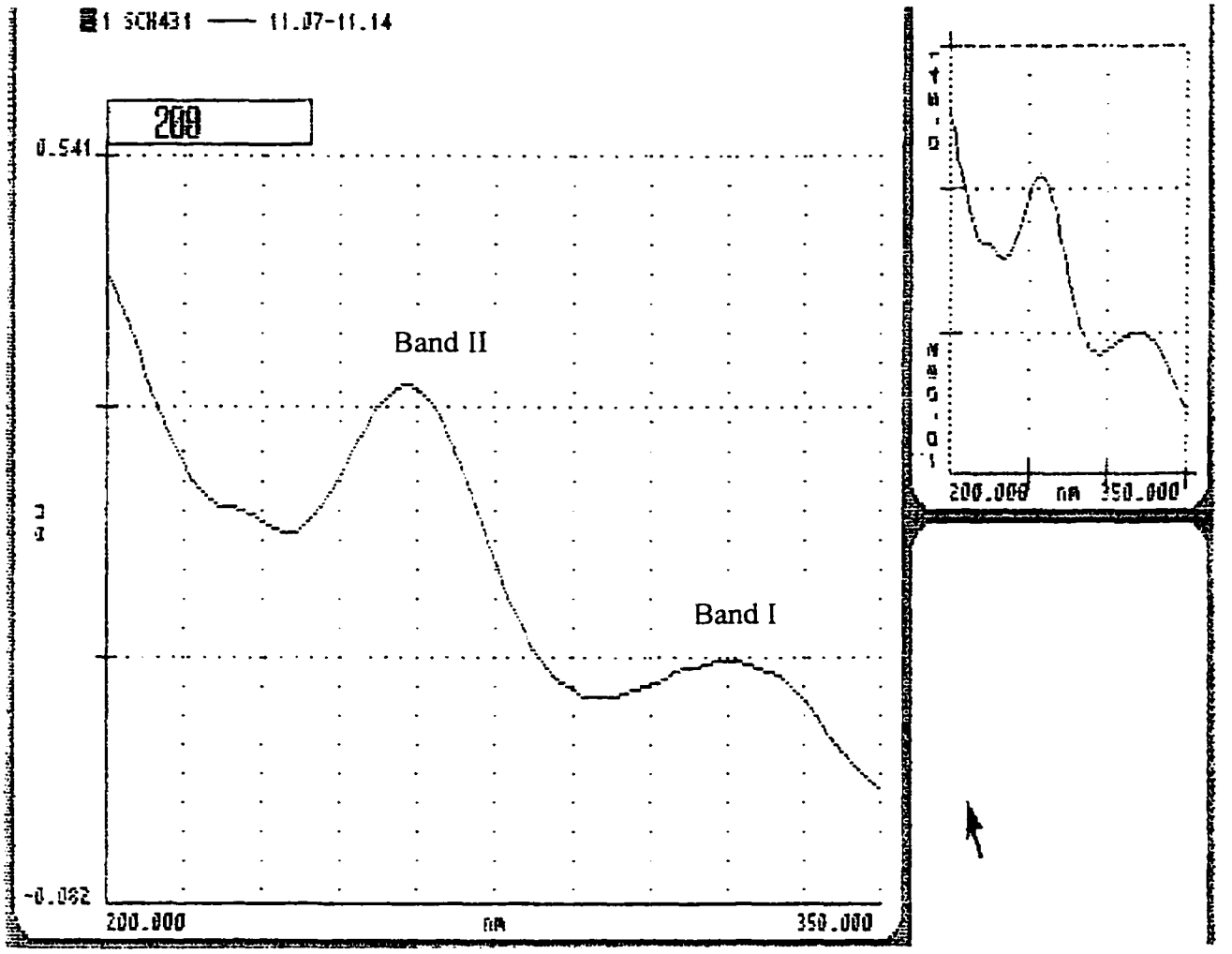


Figure 3. UV spectrum of glycitein from 200 to 350 nm. Band II maximal absorption at 262 nm and band I maximal absorption at 323 nm.

Certain reagents, such as sodium methoxide (NaOMe), aluminum chloride ($AlCl_3$), HCl, sodium acetate (NaOAc) and boric acid, can be used to perform specific oxidation and cause UV spectral shifts for identification purposes with isoflavones. The spectra of isoflavones containing A-ring hydroxyl groups usually show bathochromic shifts on both Band I and Band II in the presence of NaOMe. In addition, the peaks in the UV spectrum of a 3'4'-dihydroxyisoflavone show reduced intensity within a few minutes with added NaOMe. NaOAc specifically ionizes the 7-hydroxyl groups in isoflavones and causes Band II to show bathochromic shift of 6 – 20 nm, and can be used to detect the presence of 7-hydroxyl group. The detection of A-ring ortho-dihydroxyl groups can be done by the effects of $AlCl_3$ and $AlCl_3$ / HCl on the spectrum. When compounds contain ortho-dihydroxyl groups at position 6,7 or 7,8, the $AlCl_3$ spectrum exhibits bathochromic shifts with respect to the $AlCl_3$ /HCl spectrum. Band II of 5-hydroxyisoflavones undergoes a consistent 10-14 nm bathochromic shift compared to the spectrum in methanol in the presence of $AlCl_3$ /HCl (Mabry *et al.*, 1970).

The extraction of isoflavones usually involves organic solvents. Methanol, ethanol, acetone and acetonitrile are the most widely used solvents. Because isoflavones are neither very hydrophilic nor very lipophilic, aqueous solutions of these organic solvents were used. Jones *et al.* (1989), Coward *et al.* (1993) and Eldridge *et al.* (1982) used 80% methanol. Naim *et al.* (1973) and Seo *et al.* (1984) used 80% ethanol while Farmakalidis and Murphy (1985) used acetone-0.1 N HCl. Wang and Murphy (1994) used 70% aqueous acetonitrile + 0.1 N HCl to extract isoflavones. In some procedures, pH was adjusted to achieve a better extraction efficiency (Murphy, 1981). Farmakalidis and Murphy (1985) compared the extraction of isoflavones from defatted soyflakes using acetone - 0.1N HCl and using 80%

methanol. The extract of 80% methanol had much lower levels of acetyl-daidzin and acetyl-genistin compared to acetone-HCl extract, while other major isoflavones including daidzin and genistin in the two extracts were at the same level. There was selective extraction of the isoflavones with 80% methanol.

The most popular technique for isoflavone analysis is octadecylsilanes (C_{18}) reverse-phase HPLC with UV detection. However, other methods, including TLC (Pratt and Birac, 1979), PC (Knuckles *et al.*, 1976), GC (Lu *et al.*, 1995), GC-MS (Dwyer *et al.*, 1994), HPLC-MS (Barnes *et al.*, 1998) and HPLC-CAD (Gamache and Acworth, 1998) are employed in the analysis of isoflavones. UV detection works well for most soybean food samples, which have a relatively high content of isoflavones in the mg/g range. However, because of its detection limits at about 1.8 μM in the injection solution, UV detector is not a very good tool for the analysis of biological samples such as urine and plasma, which containing levels of 0.1 to 10 μM in isoflavones. Mass-spectrometry has been used successfully with HPLC and GC to detect 0.1 to 10 μM levels of isoflavones (Barnes *et al.*, 1998; Dwyer *et al.*, 1994). Electrochemical detector has been successfully used to detect low levels of isoflavone with the detection limit of as low as 0.005 μM (Gamache and Acworth, 1998).

There have been reports on the isoflavone content in different soybeans and soybean foods. Murphy (1982) used an HPLC method to analyzed the content of daidzein, daidzin, genistein and genistin in several processed soybean products, including toasted soy flakes, tofu, soy sprouts, soy isolate and soy sauce. The total isoflavone content ranged from zero in soy sauce to 1 mg/g in toasted soy flakes. Franke *et al.* (1994) used HPLC to quantify daidzein and genistein levels in more than 40 foods, mostly legumes. They used acid

hydrolysis to produce the free forms and only measured the total aglycones of daidzein and genistein. They used flavone as an internal standard to adjust for losses during the extraction and analysis. The recovery of the internal standard achieved was between 94% to 104%. Daidzein and genistein were detected in soybean seeds, soy flours and tofu at levels from 18 μg/g in soybean hulls to 2.2 mg/g in certain variety of dry soybeans. Wang and Murphy (1994) used HPLC method to analyze the isoflavone content in 29 commercial soybean foods, categorized into soy ingredients, traditional and second-generation soy foods. They analyzed all 12 isoflavone isomers, including glycitein and its glucosidic forms. External standards daidzein and genistein were used to spike the sample to estimate recoveries. Recoveries of 70% to 100% were achieved in different food samples. In addition to individual isoflavone levels, they reported normalized total isoflavone content. All the glucosidic form concentrations of isoflavones were converted back to the concentrations of free forms by normalization of molecular weight differences. Simply adding concentrations of each individual forms will overstate the isoflavone content, since the sugar portion in glucosidic forms are counted in. Soy ingredients, except soy concentrate which has been alcohol washed during the processing, including soybeans, soy flour, soy granule, soy isolate and textured vegetable protein (TVP), had total normalized isoflavone content from 466 μg/g to 1640 μg/g. Traditional soy foods include tofu, soymilk, tempeh and miso, all had significant amount of isoflavones, ranging from 100 to 1600 μg/g. However, the second generation of soy products, such as soy hot dog, soy berger, soy bacon, had very low levels of isoflavone (<100 μg/g). The isoflavone composition of American and Japanese soybeans

in Iowa was studied by Wang and Murphy (1994). Total isoflavones ranged from 1176 μg/g to 3309 μg/g. Crop year seems to have great impact on total isoflavone content.

Coward *et al* (1993) used 80% aqueous methanol (10 mL/g) to extract isoflavones from food samples and used HPLC-UV detection to analyzed the isoflavone content in soybean foods from American and Asian diets. The Asian soy foods tested, including soymilk, tofu, soy flour, soy powder and soy nuts, which were not diluted by the addition of non-soybean components, had total isoflavone content in the range of 1.3 to 3.8 mg/g. Fermented soy foods, including tempeh, miso and soybean paste, had lower total content of total isoflavones, ranging from 0.6 to 1.4 mg/g. The commercial soy products used in American foods, including soy flour and soy protein concentrate, had total isoflavone content from 1 to 2.7 mg/g. However, soy sauce had negligible amount of isoflavones.

The isoflavone content in soy-based infant formulas was investigated by Setchell *et al* (1997). Five major brands of soy-based infant formulas, including Nursoy powdered formula (Wyeth Laboratories, Philadelphia, PA), Isomil powdered formula (Ross Products Division Abbott Laboratories, Columbus, OH), Isomil "Ready to Feed" liquid formula, Alsoy liquid formula concentrate (Carnation Nutritional Products Division, Nestle Food Company, Glendale, CA) and Prosobee liquid formula concentrate (Mead Johnson, Evansville, IN), were analyzed by HPLC method. All of the soy formulas contained mainly glycoside forms of genistein and daidzein, ranging from 32 to 47 μg/mL of reconstituted formula. Murphy *et al* (1997) analyzed the isoflavone content in 6 major brands of soy-based infant formulas. In addition to the five soy-based formulas analyzed by Setchell et al (1997), Gerber's soy formula was analyzed. Total isoflavones in these formulas ranged from 214 to 285 μg/g of dry formula and 25 to 30 μg/mL reconstituted formula.

Mazur *et al.* (1998) used GC-MS to analyze the isoflavone content in 48 cultivars of 16 food legume species. The highest total concentration of isoflavones was found in kudzu root (over 200,000 μg/g), followed by soybeans (from 373 μg/g to 1410 μg/g) and chickpea (12 to 36 μg/g).

Recently, Reinli and Block (1996) summarized 21 studies on isoflavone analysis and tried to create a phytoestrogen database in foods. Of the 21 studies reviewed on isoflavone analysis, 17 studies used HPLC to measure isoflavones, others used either gas chromatography or thin-layer chromatography. Most of the studies only analyzed daidzein and genistein families. Wang and Murphy (1994) reported glycitein content in different food products, in addition to daidzein and genistein. The normalized total isoflavone content, including daidzein and genistein, in different soy foods varied from 1-2 μg/g to 2-3 mg/g. Because different researches used different extraction and analysis procedure, and only a few of them used internal standards to account for losses during the extraction and analysis process, the database set up by Reinli and Block showed inconsistency in the literature.

To fully utilize the health promoting benefits of soy isoflavones, a complete database of isoflavone content in foods is needed. To build a good database of isoflavone content in foods, a systemic, standardized, and well-controlled analysis need to be carried out. The food samples should cover the mostly consumed soy products, the analysis method should be well recognized and widely tested, in addition, both internal and external standards are needed for a good quality control.

B. Sources of Isoflavone Standards:

Isolation, purification and chemical synthesis

To isolate milligrams of isoflavones from soybeans, column chromatography is a good choice. Defatted soybeans or soy flours are the common starting materials. Eighty percent aqueous ethanol (Setchell *et al.*, 1987) or acetone-0.1 N HCl (Farmakalidis and Murphy, 1985) was used to extract isoflavones. The crude isoflavone fractions can be separated to individual pure isoflavones by fractionating through different column materials including silica gel, Sephadex LH-20 and polyamide (Kudou *et al.*, 1991; Agarwal *et al.*, 1984). Usually these column materials have to be used in sequential order to achieve the isolation of pure isoflavones. The semi-preparative HPLC is a faster, simpler separation method, but requires extensive sample treatment before chromatography and has relatively lower capacity due to solubility limits of isoflavones (Farmakalidis and Murphy, 1984).

Since the discovery of isoflavones, several methods have been introduced to chemically synthesize them. In many cases, deoxybenzoins and chalcones can serve as the starting materials for natural isoflavones. The synthesis of deoxybenzoin can be achieved by the Friedel-Crafts reaction of the phenylacetyl chloride and phenol, or the boron trifluoride catalyzed reaction of the phenol and the phenylacetic acid. Chalcones are readily obtained by the condensation of acetophenones and the aromatic aldehydes. The cyclization of deoxybenzoin can be achieved by a wide variety of C_1 reagents such as ethoxallyl chloride (Iyer *et al.*, 1989), ethyl formate (Shaw *et al.*, 1990) and dimethyl formamide (DMF) (Parmar *et al.*, 1987; Jain *et al.*, 1986). Sometimes the protection of other functional groups, except

for the hydroxyl required to condense the C_2 ring may be necessary. The protective groups are then removed by acid hydrolysis.

The chemical synthesis of isoflavones can be time-consuming and with low yields. However, Chang *et al.* (1994) reported the microwave-mediated synthesis of the isoflavones, daidzein, genistein, formononetin and biochanin A. By using microwave energy, they reduced synthesis time. However, in their synthesis procedure for daidzein and formononetin, they had to use TLC purification to obtain pure isoflavones from the reaction mixture, which lowered the yield and increased the total operation time.

Wähälä *et al.* (1995) successfully synthesized more than 20 different isoflavones, include daidzein and genistein, by using the "one-pot" procedure for deoxybenzoin and isoflavones. A phenol and an arylacetic acid were dissolved into freshly distilled boron trifluoride etherate under argon. The mixture was stirred and heated at 65-70 °C. Then the mixture was cooled to room temperature and dry dimethylformamide (DMF) was added. The mixture was heated to around 50°C and methanesulphonyl chloride was added slowly. After reaction at 60–70 °C, the reaction mixture was cooled down. Cold water was added to the mixture, the crude isoflavone was then recrystallized from aqueous methanol. The yield of daidzein and genistein was 98% & 53%, respectively. They demonstrated that deuterium (D)–labeled daidzein and genistein could be produced by using OH/OD exchange of phenolic groups in isoflavones.

Although numerous methods have been reported on the chemical synthesis of daidzein and genistein, no method on glycitein synthesis was reported until early 1995. Nógrádi and Szöllösy (1995) have reported the synthesis of glycitein and its glucoside form, glycitin. Glycitein was prepared by oxidative rearrangement of the fully protected chalcone

by $TI(NO_3)_3$ in methanol, followed by deprotection and ring closure. The glucosylation of glycitein with acetobromoglucose and subsequent saponification yields glycitin.

C. How Do Plants Make Isoflavones?

The biosynthesis of isoflavones has been studies extensively in both chickpea and soybeans; thus, the essential enzymes of the general phenyl-propanoid pathway and chalcone synthase, chalcone isomerase as well as isoflavone synthase are well known (Braz *et al.*, 1990; Ebel *et al.*, 1986). It is clear that A-ring arises from acetate and the B-ring-C_3 unit may originate from various C_9 precursors such as phenylalanine and caffeic acid (Birch, 1957). Chalcone synthase, the key enzyme of isoflavone biosynthesis, catalyzes the formation of a chalcone with a phloroglucinol substitution pattern in ring A. The resulting 6'-hydroxychalcone is isomerized to naringenin, the intermediate for genistein and biochanin A formation. A central step in isoflavone biosynthesis is the molecular rearrangement catalyzed by isoflavone synthase in which a (2S)-flavanone is converted to an isoflavone such as daidzein or genistein. 4'-O-Methylation, catalyzed by isoflavone methyltransferase, of daidzein yielding formononetin, of genistein yielding biochanin A (Wengenmayer *et al.*, 1974). These aglycons undergo further enzymatic glucosylation and malonylation to form the predominant glucoside forms of isoflavones in soybeans (Figure 4).

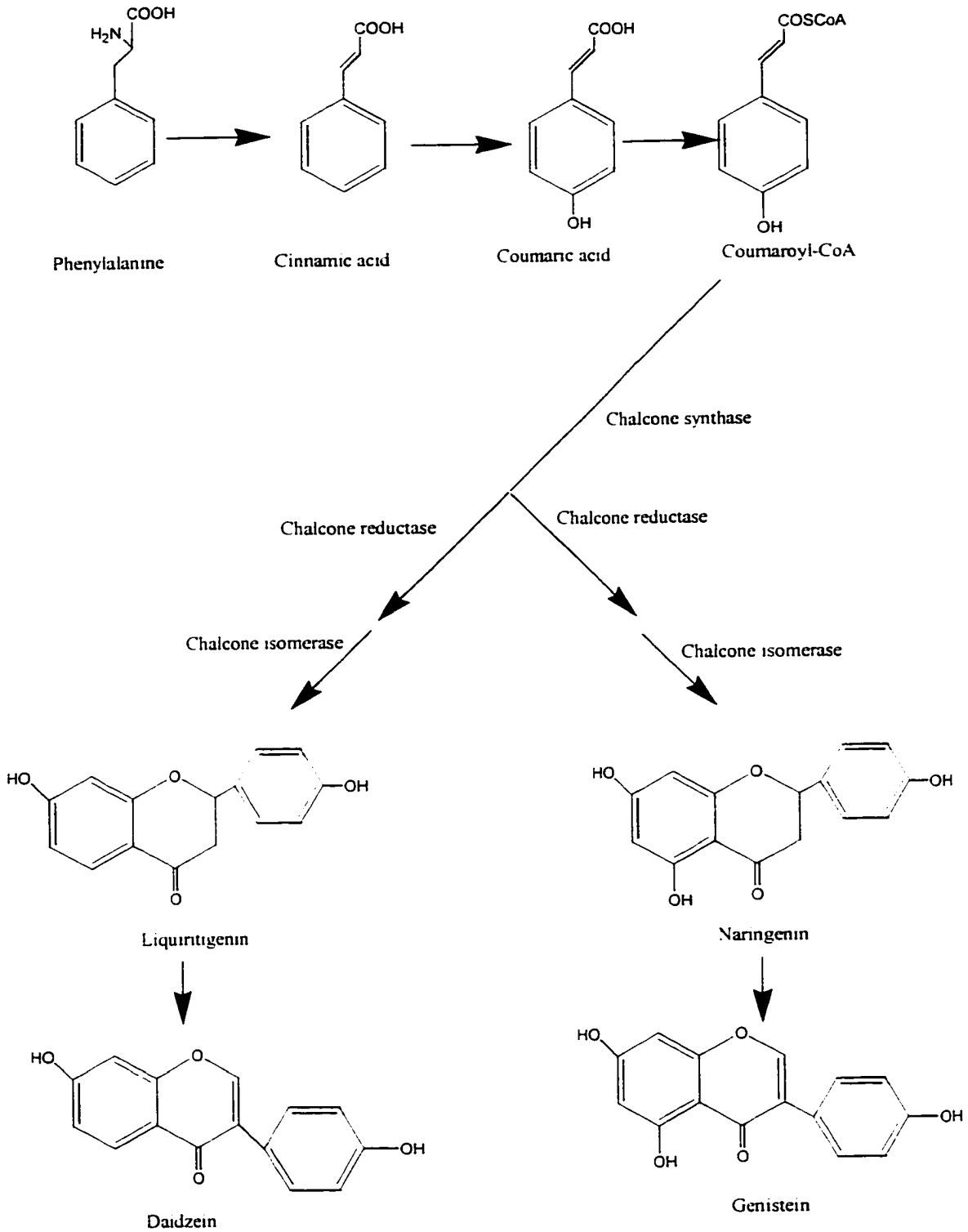


Figure 4. Biosynthesis pathway to isoflavone daidzein and genistein in soybeans.

D. Are Isoflavones Good Antioxidants?

Soybeans, defatted soybean flours, soy protein concentrates and soy protein isolates have appreciable antioxidant activity in lipid-aqueous systems (Pratt and Birac, 1979). The antioxidant activity of fermented soybean products such as tempeh and natto was investigated by exposing the lipids in these samples to air and measuring the extent of lipid peroxidation and significant antioxidant activity was reported (Esaki *et al.* 1994). The antioxidant activity of these soy products was thought to be due to tocopherols, phospholipids, amino acids and peptides in soybeans, as well as isoflavones. The antioxidant activity of soybean isoflavones was demonstrated in *in vitro* systems by measuring the extent of inhibition of lipoxygenase (Naim *et al.*, 1976) and by the Rancimat method with lard at 100°C (Dziedzic and Hudson, 1983). The degree of the antioxidant capacity of isoflavones was thought to be positively correlated to the number of hydroxyl groups in the structure (Naim *et al.*, 1976). Isoflavones, genistein, genistin, daidzein and daidzin at 10^{-3} M inhibited linoleate oxidation 70%, 30%, 26% and 15%, respectively (Pratt and Birac, 1979). Genistein seems to have the greatest antioxidant activity among these isoflavones.

Fleury *et al.* (1992) used Rancimat, storage test, heat and UV light-induced oxidation to test the antioxidant activity of malonyl isoflavones. No antioxidant activity was observed in the Rancimat test performed at 100°C and the heat-induced oxidation of β-carotene/linoleic acid system. They suggested that isoflavones might not act as radical scavengers. However, malonyl isoflavones at 500 ppm have been shown to be good antioxidants in the storage test at 37°C and in UV light-induced oxidation of β-carotene/linoleic acid system (Fleury *et al.* 1992).

There is considerable evidence to suggest that reactive oxygen species may induce a series of oxidative reactions which could cause modification of some macromolecules and lead to mutagenesis and carcinogenesis (Frankel, 1992). The antioxidant activity of isoflavones may contribute to their anticarcinogenic effects. Record *et al.* (1995) tested the antioxidant activity of genistein. Significant protection was observed on incubation of liposomes containing genistein in the presence of the peroxyl radical generating agent. Genistein gave 50% protection against UV- and peroxyl-induced lipid peroxidation in liposomes at about 25 nmole genistein/mg of phosphatidylcholine (PC). Other peroxidative systems, involving hydrogen peroxide, such as metmyoglobin peroxidase activity and Fe/ascorbate/hydrogen peroxide oxidation of liposomes, were inhibited by genistein. But genistein was ineffective in preventing conjugated diene formation in linoleic acid micelles. Wei et al. (1995) compared the effects of different isoflavones on the hydrogen peroxide production by tumor promoter activated HL-60 cells. A dose-dependent effect was observed for the inhibition by these isoflavones. Among the tested compounds at concentrations of 10, 50, 100 and 200 μM, genistein (IC_{50} = 25 μM) and daidzein (IC_{50} = 150 μM) were the most potent inhibitors of hydrogen peroxide formation. In contrast, apigenin and biochanin A showed little inhibitory effect. They proposed that the hydroxyl group at 4' position is crucial for isoflavone antioxidant activity.

Cai and Wei (1996) investigated the *in vivo* effects of genistein on antioxidant enzyme activities in SENCAR mice. Dietary administration of genistein at 50 or 250 ppm in diets for 30 days significantly increased the activities of antioxidant enzymes such as catalase, superoxide dismutase and glutathine peroxidase in liver, kidney, lung and intestine of the SENCAR mice compared to groups receiving no genistein.

Although antioxidant activities have been reported for genistein and daidzein, the isoflavone concentrations used in most of the systems are in the range of 50 μM to 1m M, which are well above the physiological concentrations, which are 0.1 to 10 μM. The antioxidant activities achieved by isoflavones, in most of the cases, were lower than some of the positive antioxidant controls, such as α-tocopherol. In addition, the highest physiological isoflavone concentration in human is probably around 10 μM. So it is unlikely that the antioxidant activity of isoflavones explains some of their health enhancing properties.

E. Estrogenic or Anti-estrogenic?

The estrogens are a family of hormones synthesized in a variety of tissues that are required for the development of female secondary sex characteristics. 17-β-Estradiol is the primary estrogen of ovarian origin. In some species, estrone, synthesized in numerous tissues, is more abundant. Estrogens are formed by the aromatization of androgens in a complex process that involves three hydroxylation steps, each of which requires O_2 and NADPH. The aromatase enzyme complex is the key enzyme in estrogen synthesis, and estradiol is formed from testosterone, whereas estrone results from the aromatization of androstenedione. These estrogens are produced in the ovary, adipose and muscle tissues, and liver. Women cease having regular menstrual cycles at about age 46 to 53, coincident with loss of all follicles and ovarian estrogen production. Estrone, a weak estrogen, is produced in postmenopausal women as the principal estrogen, whereas in premenopausal women, estradiol is the major estrogen. The loss of estrogen production in postmenopausal women is thought to be the main reason for accelerated bone resorption causing osteoporosis, which

can be a major health problem in older individuals. In addition, the loss of estrogen increases total cholesterol, LDL cholesterol and triglycerides, and decreases HDL cholesterol (Kannel *et al.*, 1976; Gaspard *et al.*, 1995). Over the years, life expectancy of women has increased dramatically, while the average age of menopause has remained relatively stable. As a result, women today will spend more than one third of their lives beyond menopause (Burnett, 1987) with a natural estrogen deficit and with greater risk of health problems such as osteoporosis and heart diseases. To treat these problems, certain natural or synthetic estrogenic compounds have been used as estrogen replacements.

A number of chemicals in the environment possess estrogenic activity in a variety of biological systems. These compounds are capable of perturbing the normal hormonal melieu in human and animals. They may exhibit their endocrine and reproductive effects through: 1) mimicking the effects of endogenous hormones such as estrogens; 2) antagonizing the effects of normal endogenous hormones; 3) altering the pattern of synthesis and metabolism of natural hormones; and 4) modifying hormone receptor levels. These compounds include plant products such as isoflavonoids and fungal products such as zcaralenone; pesticides such as DDT and dieldrin; plasticizers such as polyclorinated biphenyls (PCB); synthetic estrogenic agents administered to livestock such as diethylstilbestrol (DES), and a variety of other chemicals such as bisphenol A (Soto et al., 1995). It is clear that pharmacological doses of estrogenic compounds, such as DES, can cause toxicity. For example, women exposed to DES *in utero* developed reproductive tract abnormalities and endometrial cancers (Herbst *et al.*, 1971). Chronic exposure to these environment estrogens may have adverse or beneficial effects on health.

With the growing awareness and concern regarding the estrogens in the environment, dependable biological assays for estrogenic activity are needed. One important issue is that various investigators use different endpoints to define estrogens. Some researchers require the growth of rat or mouse uterus to define estrogen bioactivity. Others rely on the ability to bind to estrogen receptors, while still others use the estrogen-dependent cell lines to test estrogenic activity. Recently, estrogen receptor-dependent transcriptional expression (Soto *et al.*, 1995) was used to evaluate estrogenicity. Between 1920 and 1996, numerous studies have been carried out to test the estrogenic activity of environmental compounds. These methods can be grouped into four categories: (1) reproductive tract response; (2) non-reproductive tract target tissue response; (3) estrogen receptor binding assay; and (4) estrogen receptor-dependent transcriptional expression (Reel *et al.*, 1996). A possible test array for potential estrogenic/anti-estrogenic compounds is proposed by Reel *et al.* (1996). They propose the following decision tree. The first step is estrogen receptor binding assay (*in vitro*). A negative response means the compound is unlikely to be a direct acting estrogen and/or anti-estrogen. If the compound gives a positive response in the receptor-binding assay, the next step is the estrogen receptor-dependent transcriptional expression assay (*in vitro*). A negative response means the compound is a possible antagonist, a positive response suggests potential estrogen. The third step involves uterotrophic activity (*in vivo*) and vaginal cornification (*in vivo*). A negative response from either of these test means the compound is unlikely to be estrogenic *in vivo*, while a positive response means the compound is estrogenic.

Phytoestrogens include a wide variety of plant chemicals with weak estrogenic activity. Phytoestrogens have been identified in over 300 different plants (Bierman, 1992).

These phytoestrogens vary widely in both estrogenic potency, physiological effects and may have both estrogen agonistic and antagonistic properties (Dwyer et al., 1994). The estrogen-agonist and estrogen-antagonist effects depend, in part, on the ambient estrogen concentrations at the site of action (Anderson, 1996). Table 1 summarizes some of the best-characterized phytoestrogens (Anderson, 1996).

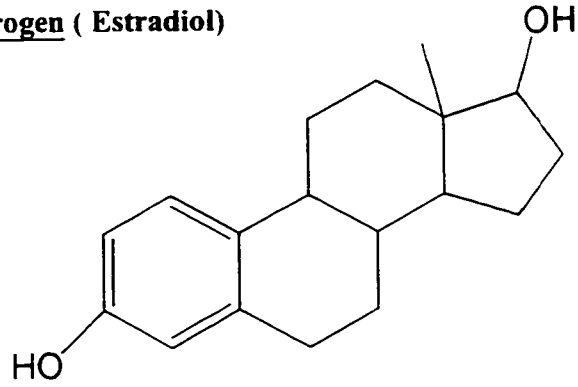
Isoflavones have similar structures as the natural estrogen, estradiol, and the synthetic estrogen, DES. (Figure 5). Genistein and daidzein are roughly 10^5 times less effective than DES and estradiol in the mice uterine enlargement assay (Bickoff *et al.*, 1962). Farmakalidis and Murphy (1985) used inbred B6D2F1 mice to test the estrogenic responses of daidzin, genistin and genistein. The estrogenic response to 1.5 mg of genistin was equivalent to 1 mg of genistein or a 1:1 molar relationship in estrogenic activity between genistin and genistein. Daidzin had a relative potency of 40% of that of genistin.

Animal species differ in their response to phytoestrogens. In sheep, daidzein, formononetin and their metabolite, equol, shows potent estrogenic activity in uterine enlargement assay. In contrast, genistein and biochanin A are much less potent (Braden *et al.*, 1967). However, in mice and rats uterine growth assay, daidzein and formononetin were found to be much less potent than genistein (Bickoff *et al.*, 1962). The response to phytoestrogen may differ within the same species, the Swiss CD-1 mouse did not respond, and ICR mouse showed a slight response, to daidzein and genistein, while inbred B6D2F1 mouse showed significant responses to these phytoestrogens (Farmakalidis and Murphy, 1984).

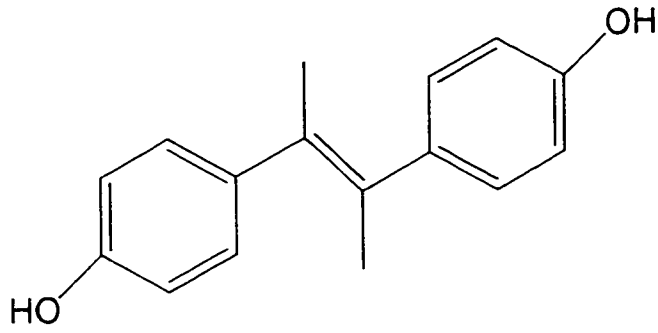
Table 1. Major phytoestrogen classes, representative compounds and sources.

Class	Example	Source	Comments
Isoflavone	Genistein Daidzein Formononetin Biochanin A	Legumes, mainly soybeans, chickpeas and cloves.	Metabolized in body by human and other animals (rats, mice and sheep) to a series products including equol, dihydrodaidzein, dihydrogenistein and O-desmethylangolensin
Lignans	Matairesinol Lariciresinol Isolarciresinol Secoisolaricerison	Seeds and Grains, such as sesame seed and flaxseed.	Metabolized in humans to enterolactone, enterodiol and 7-hydroxymatairesinol.
Coumestans	Coumestrol	Legumes, includes alfalfa, clovers and soybean sprout	

Natural estrogen (Estradiol)



Synthetic estrogen (Diethylstilbestrol, DES)



Soy isoflavones	R1	R2
Daidzein	H	H
Genistein	H	OH
Glycitein	OCH3	H

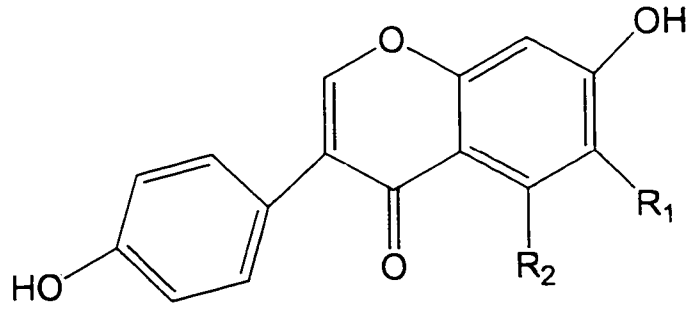


Figure 5. Structures of natural estrogen, synthetic estrogen and isoflavones.

Verdeal *et al.* (1980) compared the estrogen receptor (ER) binding affinity of different isoflavones using rat uterine cytosol preparation. Genistein had about 100 times lower binding affinity to ER than estradiol, but 15 times higher than daidzein and 20 times higher than biochanin A. The estrogen receptor binding assay was performed in rabbit (Shemesh, 1979), sheep (Shutt and Cox, 1972) and calf (Lee *et al.*, 1977) uterine cytosol and human cancer cell line MCF-7 (Martin *et al.*, 1978). The binding affinity of genistein was between 50 to 160 times lower compared with that of estradiol. Daidzein was about 10 times lower than genistein in binding affinity.

The effect of genistein on the estrogen receptor (ER) positive human breast cancer cell line MCF-7 has been investigated by Thomas *et al.* (1996). Genistein stimulated estrogen-responsive pS2 mRNA expression at concentration as low as 10 nM. Thus, it may act through an ER-mediated transcriptional event. When both genistein and estradiol were added together, the expression of pS2 mRNA was 20% lower than when estradiol was added alone. MCF-7 cells, treated with genistein, showed a decrease in the response to estrogen as well as a decrease in ER mRNA levels. In another study, genistein injected subcutaneously at a dose of 1.6 mg was shown to inhibit DES-stimulated vaginal and uterine growth by almost 40% in rats (Messina *et al*, 1994). When 300 to 900 μg of genistein are injected into mice with estradiol, the uptake of estradiol by the uterus and vagina was significantly reduced by 71% and 90%, respectively (Folman *et al*, 1969). These results suggested that genistein interfered with the effects of estradiol and DES, and thus, has antiestrogeic activity.

Estrogen can act through the classical ER, now called ERα. ERα is a 65-66 kD protein possessing the following domains: an N-terminal portion (regions A/B) which

contains transactivation domains possessing constitutive and hormone-dependent transcription-activating functions. Region C is a DNA-binding domain with two "zinc-finger" motifs in the middle. D is the hinge region and E is the area of high-affinity (hormone) ligand-binding domain in the C-terminal region. Ligand binding induces a conformational change in ERα and subsequent DNA binding to specific estrogen response elements (EREs) present in target genes. ERα then activates transcription initiation through protein : protein interactions with accessory factors. The estrogen receptor knockout (ERKO) animals are good models to study the *in vivo* effects of estrogens through ER (Korach *et al.*, 1994). For example, classic estrogen responses in the uterus such as water imbibition, hyperemia and gene induction are absent in the ERKO mice (Couse *et al.*, 1995). Synthetic estrogen tamoxifen can also induce these responses in normal mice but is ineffective in ERKO mice.

Experiments in ERKO mice have suggested that some estrogenic effects were not mediated solely by ERα. For example, progesterone receptor mRNA is induced in the preoptic area of ERKO mice by estrogen (Merchenthaler *et al.*, 1996). This led to the recent cloning of a new ER, called ERβ, by Kuiper *et al.* (1996). ERβ is a member of the steroid hormone superfamily like ERα. ERβ binds to estrogen with high affinity; it activates transcription in response to estrogen; and it is expressed in estrogen target tissues such as pituitary and uterus. ERβ binds estradiol with a dissociation constant of 0.4 nM versus the 0.1 nM of ERα under the same assay conditions (Kuiper *et al.*, 1997). In general, other estrogenic or antiestrogenic compounds, such as tamoxifen, do not discriminate between ERα and ERβ. There is overlapping pattern of expression of ERβ relative to ERα. ERβ is

highly expressed in prostate and ovary with low expression in brain, uterus and testis (Messelman *et al.*, 1996). The discovery of ERβ raises important questions on the mechanisms of the actions of estrogen. The tissue distribution of ERα and ERβ, and the differences in certain ligand binding affinity between these two ERs may contribute to the selective action of ER agonists and antagonists in different tissues (Kuiper *et al.*, 1997).

The phytoestrogens will compete with the natural estrogens for estrogen receptors but may be limited to the binding stages and fail to transmit the hormonal message to RNA. The ability of some phytoestrogens to send messages to nuclei once they are bound to the uterine receptors has been studied by Tang and Adams (1980). They observed that equol can bind to estrogen receptors as does estradiol, but the rate of synthesis of DNA and protein by rat uterus was 80% higher in the rats receiving estradiol (5 μg subcutaneously) than that by rats receiving equol (5 mg subcutaneously). These data showed that, although phytoestrogens can bind to estrogen receptors in different tissues, their cellular responses were different from when natural estrogens were used.

Daily intake of 60 grams of soy protein containing 45 mg of soy isoflavones for one month significantly increased follicular phase length and delayed menstruation in six premenopausal women with regular menstrual cycles. This effect was similar to that caused by the antiestrogen tamoxifen (Cassidy *et al*, 1994). Lu *at al.* (1996) fed soymilk to healthy females 22 to 29-year-old for one month on a metabolic unit. The daily intake of daidzein and genistein was 100 mg each. Serum 17 β-estradiol levels on menstrual cycle days 5-7, 12-14, and 20-22 decreased by 31%, 81% and 49% respectively, during soymilk feeding. Decreases persisted for two or three menstrual cycles after withdrawal for soymilk feeding. Menstrual cycles were increased from 28 days to 32 days, remained at 32 days at one cycle

after termination of soy feeding and returned to pre-soy diets length five to six cycles later. They concluded that consumption of soy foods containing isoflavones might reduce circulating ovarian steroids and adrenal androgens and increase menstrual cycle lengths.

Isoflavones can stimulate sex hormone binding globulin (SHBG) synthesis in the liver, in this way, they may reduce the biological effects of sex hormones (Adlercreutz *et al.*, 1987). SHBG binds estrogens to provide a circulating reservoir of estrogen. An increase in SHBG decreases the relative amount of free estrogens, thus decreases the metabolic clearance rate of estrogen and reduces estrogen uptake and action in peripheral tissues, thereby lower their biological activity. Adlercreutz *et al.* (1987) showed the positive correlation between the excretion of total phytoestrogen metabolites with plasma SHBG levels and negative correlation between total phytoestrogen metabolites with percentage free estradiol in plasma. *In vitro* studies using HepG2 liver cancer cells demonstrated that genistein and daidzein stimulate SHBG synthesis (Mousave and Adlercreutz 1993).

The activity of aromatase, the rate-limiting enzyme in estrogen synthesis, is inhibited by isoflavonoids and lignans (Campbell and Kurzer 1993; Wang *et al.*, 1994). Lignans are a group of diphenolic compounds with molecular weight similar to those of steroid estrogens. This inhibitory effect on aromatase may be another mechanism for isoflavones' antiestrogenic effects. Enterloactone, the most abundant lignan with mammalian activity, is a moderate inhibitor of placental aromatase and competes with the natural substrate androstenedione for binding to the enzyme (Adlercreutz *et al.*, 1993).

Numerous evidences support both estrogenic and antiestrogenic activity of isoflavones. Whether isoflavones are estrogenic or antiestrogenic depends upon the

concentrations of exogenous isoflavones vs. the levels of endogenous estrogens, as well as the sensitivity of target tissues and cells to these compounds.

F. Isoflavone Metabolism and Bioavailability

The predominant isoflavones in normal soy products are the glucoside forms of daidzein, ganistein and glycitein (Wang and Murphy, 1994). Once ingested by humans, the glucoside forms may undergo chemical hydrolysis by gastric acid or enzymatic hydrolysis by intestinal microflora (Xu *et al.*, 1995) to yield aglycones. These aglycones can be absorbed from the intestinal lumen and undergo phase I and II biotransformation reactions, or they can be metabolized and broken down by the gut microflora to different metabolites. Equol, O-desmethylangolensin (O-dma) and dihydrodaidzein have been identified as the major metabolites of daidzein in sheep as well as in humans (Kelly *et al.*, 1993). Genistein can be metabolized to dihydrogenistein, or p-ethylphenol in sheep (Shutt, 1976) (Figure 6). However, no metabolites have been identified for glycitein to date. The glucosides of isoflavones are very poorly absorbed in the small intestine compared with their aglycones because they have relatively higher molecular weight and hydrophilicity (Brown, 1988).

Bioavailability refers to the proportion of certain substance absorbed and enters the systemic circulation. The metabolism of ingested isoflavones varies greatly in human subjects feeding soy products (Xu *et al.*, 1995; Cassidy *et al.*, 1994; Kelly *et al.*, 1993). The bioavailability of daidzein and genistein was investigated by Xu *et al* (1995) by feeding human subjects soymilk as the isoflavone source. The average 24-h urinary recoveries of daidzein and genistein were approximately 21% and 9%, respectively. Total fecal excretion

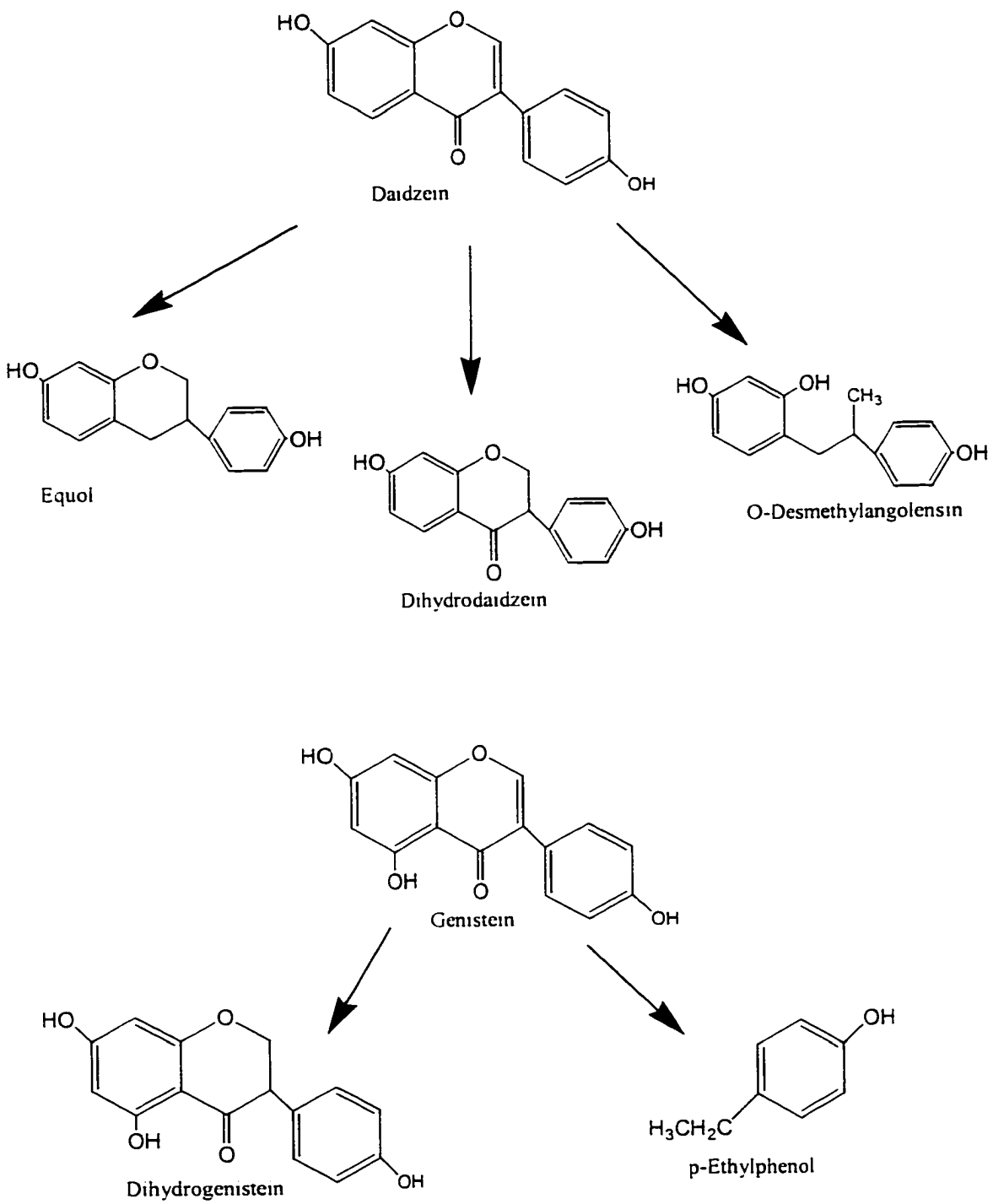


Figure 6. Metabolisms of daidzein and genistein in sheep.

of isoflavones was only 1-2% of the ingested amount. Urinary recovery of daidzein was significantly greater than that of genistein and daidzein is thought to be more bioavailable than genistein. The lower molecular weight and greater water solubility of daidzein compared with genistein might be one of the reason that daidzein is more readily excreted in urine than is genistein (Xu *et al.*, 1995). Significant bacterial degradation of isoflavones might explain the relative low recoveries of daidzein and genistein in urine and feces. Twenty-four hrs after dosing, the concentrations of daidzein and genistein in both plasma and urine were only at trace levels, which demonstrated the rapid metabolism of daidzein and genistein once ingested. The bioavailability of glycitein was compared with daidzein and genistein by feeding 4.5 μmole/kg total isoflavones from soymilk and soygerm to 7 females and 7 males (Zhang *et al.* 1998). Average 48 hrs urinary recoveries of glycitein, daidzein and genistein were approximately 55%, 46% and 29%, which suggested that glycitein is more bioavailable than daidzein and genistein.

Isoflavones undergo urinary as well as biliary excretion in rats (Axelson and Setchell, 1981) and humans (Rondelli *et al.*, 1991). The intestinal uptake, biliary excretion of genistein was investigated in rats by using 4-^{14}C-genistein (Sfakianos *et al.*, 1997). 4-^{14}C-genistein, when infused into the duodenum, was rapidly absorbed from the intestine, taken up by the liver and excreted into the bile as 7-O-β-glucuronide conjugate. The total recovery of ^{14}C-radioactivity over the next four hours in bile was 70 –80% of the administered dose. When rats were placed in metabolic cages, and urine and feces were collected for 120 h after dosing, the total ^{14}C-genistein excreted in urine and feces was 21% and 2%, respectively. Sfakianos *et al* (1997) demonstrated that genistein undergoes an efficient enterohepatic circulation. As a result, genistein may accumulate within the enterohepatic circuit and may

be excreted with a long half-life. They suggested that the measurement of urinary genistein during the first 48 h after administration of genistein by most researchers might underestimate genistein's bioavailability.

King *et al* (1998) investigated the plasma and urinary kinetics of daidzein and genistein in humans by feeding them a single soy meal. Isoflavone concentrations in plasma rose slowly and reached maximum values of 3.14 μM at 7.42 h for daidzein and 4.09 μM at 8.42 h for genistein. The rate of urinary excretion of daidzein was greater than that of genistein throughout of the postmeal period, with mean recoveries of 62% and 22% for daidzein and genistein, respectively. However, the ratio of the areas under the plasma concentration versus time curves for daidzein and genistein was equal to the ratio of the concentrations of the respective isoflavones in the soy meal. They concluded that the bioavailability of daidzein and genistein are similar, even though daidzein had higher urinary excretion.

The absorption and excretion of the soy isoflavone genistein and daidzein have been investigated by King *et al* (1996) in rats given a single oral dose of genistein (20 mg/kg body weight) or an equivalent dose of its glucoside forms, as an isoflavone-rich soy extract. Plasma concentration at 2 h after dosing was approximately 11 μM in genistein-treated rats compared with 5 μM in soy extract-treated rats, but there were no differences after 8 hrs. This suggests that the extent of absorption of genistein is similar for genistein and its glucoside forms. The fecal excretion of genistein and daidzein over the 48 h post-dosing collection period was 22% and 6% respectively. The large difference between fecal excretion of genistein and daidzein may reflect the greater bioavailability of daidzein

compared with genistein. The isoflavone concentrations achieved in rats were comparable to that in humans (Xu *et al* 1995; King *et al* 1998).

In vitro anaerobic incubation of daidzein and genistein with human feces showed that daidzein completely disappeared after 72 h and genistein after 18 h. The loss of isoflavones seemed to follow first-order kinetics, with the half-life of daidzein and genistein estimated at 7.5 and 3.3 h, respectively (Xu *et al.*, 1995). Human intestinal bacteria can metabolize and degrade isoflavones as well as liberate isoflavone aglycones. The bacteria in the gastrointestinal tract play an important role in determining the magnitude and pattern of isoflavone bioavailability (Xu *et al.*, 1995). The extensive metabolism and degradation of isoflavones by intestinal microflora prohibit their resorption from lower bowel and this might explain the strong association between high fecal isoflavones and greater total urinary recovery of isoflavones. Also the shorter half-life of genistein than daidzein might help to explain why less genistein could be absorbed than daidzein. In humans, the concentrations of excreted metabolites can vary many orders of magnitude from individual to individual (Kelly *et al.*, 1993; Xu *et al.*, 1995), and likely the result of different human gut bacteria.

G. Are Isoflavones Cancer Preventive?

Epidemiological studies report that the consumption of soy is associated with a lower risk of breast, colon and prostate cancer (Lee *et al.*, 1991; Messina *et al.*, 1994; Barnes *et al.*, 1994; Parker *et al.*, 1996). The prevalence of breast cancer is much higher in Western countries than in Asian countries. Age-adjusted death rates from breast cancer range from 20 to 26 deaths per 100,000 for Northern Europe and North America, but are only 4.9 and 6.6 deaths per 100,000 in China and Japan (Parker *et al.*, 1996). Western diets usually do not

contain soy products, as a result, they are almost completely lacking in isoflavones. In contrast, Asian diets contain substantial amount of soy products, which are rich sources of isoflavones. Urinary isoflavone excretion was 20-30 times greater among Japanese women and men consuming typical traditional diets compared with the values from women in the United States, and this urinary isoflavone excretion is the result of soy product consumption (Adlercreutz *et al.*, 1991). In a case-control study, Ingram *et al.* (1997) showed that increased excretion of isoflavones is associated with a substantial reduction (30%) in breast cancer risk.

The antioxidant, estrogenic and antiestrogenic activities all have been postulated as mechanisms for soy isoflavones' ability to lower cancer risks. In addition, isoflavones have other interesting biological activities, which may be critical in their health-enhancing properties. Genistein has been shown to be a specific inhibitor of tyrosine-specific protein kinases (Akiyama *et al.*, 1986). Tyrosine kinase phosphorylates tyrosine residues on proteins involved in signal transduction events in both normal and tumor cells. Tyrosine-specific protein kinase activity is known to be associated with oncogene products of the retroviral *src* gene family. This activity is also associated with the cellular receptors for several growth factors such as epidermal growth factor, platelet-derived growth factor, insulin and insulin-like growth factor (Akiyama *et al.*, 1986). Thus, a tyrosine kinase inhibitor like genistein could be an antitumor agent. Genistein is capable of inhibiting the growth of a wide range of both human and rodent cancer cell lines with IC_{50} values ranging from 5 to 40 μM (Messina *et al.*, 1994). Genistein has also been shown to inhibit the activity of DNA topoisomerases (Okura *et al.*, 1988). Topoisomerases alter the linking number of DNA by catalyzing the cleavage of one or both strands of the DNA, passage of a segment of DNA through this

break, then releasing the DNA break. Topoisomerases are very important in the process of replication, transcription and other DNA processes.

By using noncytotoxic concentrations of agents that promote terminal differentiation of human tumor cells is one of the major strategies in fighting cancer. Genistein has been shown to be an inducer of human HL-60 tumor cell differentiation in a dose-dependent manner (Constantinou and Huberman, 1995). Genistein has been shown to have the ability to induce *in vitro* differentiation of mouse erythroleukemia cells at low concentrations of about 20 μM (Watanabe *et al.*, 1991). Makishima *et al.* (1991) reported that genistein has the ability to induce human ML-1, HL-60 and U937 leukemia cells to undergo granulocytic differentiation.

Soybean isoflavone extract, with total normalized isoflavone 1 mmol or 2 mmol/kg diet, suppressed hepatocarcinogenesis in female Fisher 344/N rats as evidenced by the decreased volume percentage of gamma-glutamyltransferase (GGT)- and placental glutathine S-transferase (PGST)-positive altered hepatic foci (AHF) after 3 months of feeding, which were initiated by diethylnitrosamine (15 mg/kg body wt) and promoted by phenobarbital (500 mg/kg diet) (Lee *et al.*, 1995).

Barnes *et al.* (1990) showed that rats consuming a soy-based diet develop fewer mammary tumors following the administration of carcinogen N-methylnitosourea and 7,12-dimethylbenz-anthracene than rats on isocaloric diets without soy. They claimed that the hypothesis about Bowman-Birk inhibitors as anticarcinogens (Troll *et al.*, 1984) are incorrect because they did not observed different effects of autoclaved soy and non-autoclaved soy in suppressing cancer development. However, Bowman-Birk inhibitors in soy have very

unique structure and could not be destroyed by normal autoclave treatment. The possibility of Bowman-Birk inhibitor in soy as anticarcinogen is still under investigation.

Genistein has been shown to alter the ontogeny of mammary gland development and protect against chemically induced mammary cancer in rats. The mechanism of this chemoprevention is probably *via* alteration of early mammary gland maturation (Lamartiniere *et al.*, 1998). In this study, female Sprague-Dawley CD rats were treated *via* subcutaneous injection on day 2, 4, and 6 postpartum (neonatally) with 5 mg genistein/rat or equivalent volume of the vehicle, dimethylsulfoxide (DMSO). In the second study, female rats were injected on day 16, 18 and 20 postpartum (prepubertally) with 0.5 mg genistein/g body weight or an equivalent volume of DMSO. Animals treated with genistein developed a lower incidence of dimethylbenzanthracene (DMBA)-induced mammary tumors compared to the control group. The numbers of tumors/animal were reduced by approximately 50% in both studies. Rats exposed neonatally to genistein, but not prepubertally to genistein, had significantly increased mean time to tumor detection compared to vehicle-treated animals. Neonatal and prepubertal genistein treatments resulted in increased mammary gland sizes in 21- to 22-day-old female rats compared to vehicle-treated rats.

Angiogenesis refers to new blood vessel growth, and the formation of new blood vessels is critical for the growth of tumor. Genistein was shown to inhibit *in vitro* angiogenesis (Fotsis *et al.*, 1995). Bovine microvascular endothelial (BME) cells seeded on the surface of collagen gels invade the gels when exposed to basic fibroblast growth factor (BFGF) and form capillary like tubes beneath the gel surface. Genistein at 200 μM, when added together BFGF, inhibited BME culture's ability to invade the gels and generate capillary like structures. Genistein inhibited BFGF-induced invasion of BME cells with a

half-maximal effect at approximately 150 μM concentration. However this concentration is way above the physiological feasible genistein concentration, and whether or not this inhibitory effect of genistein on angiogenesis may help to suppress the growth of cancer cells through anti-angiogenesis needs further investigation.

Several studies have looked at the effects of soy isoflavone intake on experimental liver cancer or markers of liver cancer. Daidzein and genistein at 1 mmole/kg diet suppressed 35 μmol/kg diet fumonisin B1 (FB1)-promoted rat hepatocarcinogenesis, but showed little or no effects on a 70 μmol/kg diet of FB1 (Lu *et al.*, 1998). Mokhtar *et al.* (1988) reported that in rats administered dibutylamine and nitrite, the severity of liver dysplasia was reduced and liver tumors were inhibited in soy-based diet fed animals compared with control which have no soy in the diet.

Soy isoflavones have been evaluated regarding their ability to suppress skin cancer. In a mouse skin tumor model, the dimethyl sulfoxide extracts of soybean hypocotyls (hypocotyls have higher concentration of isoflavones than whole soybeans) were shown to have strong inhibitory effect on tumor formation (Zaizen *et al.*, 1997). In the same study, the extract as well as daidzein have been shown to have inhibitory effect on Epstein-Barr virus early antigen activation induced by 12-O-tetradecanoylphorbol-13-acetate.

Isoflavones have been shown to stimulate natural killer (NK) cells activity (Zhang 1997), and this may be another mechanism for their anticarcinogenic effects because NK cells represent a subset of lymphocyte with the ability to spontaneously kill tumor or virus-infected cells. NK cells play essential roles in immune surveillance against tumors and infectious agents and the activity of NK cells has been correlated with the ability to fight tumors. A decrease in NK activity has been found to be associated with the development and

progression of cancer (Whiteside and Herberman, 1989). Soy isoflavone extracts can significantly increase NK cell activity in rats. After rats were fed 240-mg isoflavones/kg diet for 10 days, liver associated NK cell activity was two fold higher than that in the control group (Cunnick and Hendrich, unpublished data). The effect of daidzein on immune function in mice was investigated by Zhang *et al* (1997). Swiss mice were fed daidzein at various doses for 7 days. At high doses 20 and 40 mg/kg body weight, daidzein exerted a stimulatory effect on nonspecific immunity. The phagocytic response of peritoneal macrophages and thymus weight was increased in a dose-dependent manner. Activation of humoral and cell-mediated immunity was observed.

Adlercreutz (1998) has summarized the possible mechanisms of isoflavone and other phytoestrogen's anticancer effects: (1) Inhibition of tyrosine and other kinases; (2) Stimulation of production of sex hormone binding globulin; (3) Estrogenic and antiestrogenic activity, binding to estrogen nuclear type-II binding sites; (4) Inhibition of angiogenesis and tumor invasion; (5) Antioxidant activity; (6) Prolongation of menstrual cycle; (7) Inhibition of proliferation and stimulation of differentiation of tumor cells; (8) Inhibition of steroid enzymes, e.g. Aromatase, 17β-hydroxysteroid dehydrogenases; (9) Decrease of sensitivity of breast cells to toxic compounds before puberty. Although no firm conclusions can be reached at this time, the correlation between isoflavone consumption and lower risk of certain types of cancer is clear and the mechanisms of the observed anticancer effects are still under investigation.

H. Soy Protein Is Hypocholesterolemic; What About Soy Isoflavones?

The cholesterol-lowering effects of soy protein as compared with animal protein (casein) consumption have been recognized in animals for more than 80 years. Carroll (1991) reviewed the evidence of the hypocholesterolemic effect of soy protein. Numerous studies conducted by different researchers have found that adding soy protein to the diet, or replacing casein with soy protein, lower plasma cholesterol levels in animals as well as in humans. Hamsters, rats, mice, rabbit and monkeys are the models that have been used to evaluate the hypocholesterolemic effects of soy protein. Monkeys have similar cholesterol profiles and metabolisms as humans, however, because they are expensive to obtain and maintain, not many studies have been performed on monkeys. The rat, in spite of marked differences in cholesterol profile and metabolism from human, has provided important information about cholesterol and lipoprotein metabolism. Spady and Dietschy (1983) observed that some aspects of cholesterol metabolism in hamster are quite similar to those observed in humans. More recently, hamsters were used as models to study cholesterol and lipoprotein metabolism. The rabbit is a species very susceptible to diet-induced hypercholesterolemia and has been widely used in cholesterol study (Carroll *et al* 1975; Terpstra *et al* 1983).

Sirtori *et al.* (1985) reported that substitution of soy protein for animal protein in the diet of very hypercholesterolemic human subjects led to a marked 20 to 30% reduction in serum total and LDL cholesterol levels. The intake of 25 g of soy protein, with or without soybean fiber, significantly lowered total cholesterol levels in men with elevated plasma cholesterol concentrations (Bakhit *et al.* 1994). However, in most of the human studies, the number of participants was limited and the results lacked statistical power. Anderson *et al.*

(1995) conducted a meta-analysis, which combined 29 studies using human subjects. They found that in most of the studies, the intake of energy, fat, saturated fat and cholesterol was similar for the subjects ingesting control and soy-containing diets and the average intake of soy protein was 47g/day. The ingestion of soy protein compared with casein was associated with the following changes in plasma lipid levels: (1) a 9.3% decrease in total cholesterol levels ($P<0.05$); (2) a 12.9% decrease in LDL cholesterol levels ($P<0.05$); (3) a 10.5% drop in triglyceride levels ($P<0.05$); (4) the changes in total and LDL cholesterol levels were directly related to initial serum cholesterol concentrations ($P<0.01$); and (5) HDL cholesterol levels were not significantly different from control with a 2.4% increase.

The mechanisms for the hypocholesterolemic effects of soy protein consumption in animals and humans are not known. There are several different factors in soy proteins that may be responsible to the hypocholesterolemic effect. Carroll *et al.* (1995) suggested that the active hypocholesterolemic component in soy may be the amino acid pattern and peptide structure of soy protein. Setchell *et al.* (1984) suggest that soy estrogens may contribute to the cholesterol-lowering effects of soy protein. Manzoni *et al.* (1998) suggested that the ability of different soy globulins to up-regulate hepatic LDL uptake and clearance might play critical role in soy protein's hypocholesterolemic effects. In their study, the LDL receptor activation has been observed in a human hepatoma cell line (Hep G2) exposed to the 7S globulin from soy. In addition, soy saponins and dietary fibers in soy have been proposed to have the ability to lowering plasma cholesterol levels (Potter, 1995).

The first evidence linked isoflavones to hypocholesterolemic effects was provided by Siddiqui (1975) where they reported that crude biochanin A and formononetin extract, when added to casein-based high fat and cholesterol-enriched diet at 1.5 g/kg diet, significantly

lowered the serum cholesterol level (35%) when compared with the group with no isoflavones in albino rats.

Anthony *et al* (1995) reported that, in male and female peripubertal rhesus monkeys, soy protein with intact isoflavones (1.27 mg genistein and 0.47 mg daidzein per gram of protein, 200 g protein/kg diet) significantly lowered plasma total and LDL-cholesterol levels compared with isoflavone depleted soy protein where 90% of the isoflavones were removed.

Balmir *et al.* (1996) reported that an ethanol and acetone extract of soy flour, which contains 60% isoflavones, when added to casein diets (40, 90 mg total isoflavones/kg diet), significantly reduced the plasma cholesterol levels in both rats and hamsters. Soy proteins, with or without isoflavones, both decreased serum cholesterol levels compared with casein control group. However, there were no differences between these two different soy protein groups.

Tovar-Palacio *et al.* (1997) reported that soy protein lowered plasma cholesterol levels in the gerbil. But when isoflavones concentrates were added to the soy protein groups at three different doses of 2.1, 3.6 or 6.2 mg isoflavones / g protein, they did not reduce cholesterol levels further than soy protein alone. However, these diets did reduce cholesterol levels significantly compared with casein control groups.

All these studies provide indirect evidence that isoflavones may be the factors that contribute to soy's hypocholesterolemic effects. However, because all the isoflavone extracts used in the experiments contain other compounds from soy, such as soy saponins and phenolic acids, the role of isoflavones on serum cholesterol levels is not resolved at this time. However, the studies by Balmir *et al* (1996) did prove that soy protein without isoflavones retains its ability to lower serum cholesterol levels.

Studies in rats have shown that soy protein compared with casein increase fecal excretion of bile acids (Nagata *et al.*, 1982). Hepatic cholesterol metabolism then shifts to provide cholesterol for enhanced bile acid synthesis. As a result, increasing amount of cholesterol will be removed from blood via the apo B/E receptor and the serum cholesterol levels decrease (Beynen, 1990). Other possible mechanisms include (1) increased thyroid hormone levels (Balmir *et al.*, 1996), so that the lipolysis and fatty acid utilization might be stimulated; (2) suppression of lipogenic enzyme gene expression (Iritani *et al.*, 1995), as results, the synthesis of fatty acids is suppressed; or (3) increased LDL receptor activity (Lovati et al. 1998). Baum *et al* (1998) reported that levels of mRNA for LDL receptors are greater in mononuclear cells from humans fed isolated soy protein compared with milk protein. The increased level and activity of LDL receptor will increase the clearance of cholesterol from blood and lower serum cholesterol levels would be achieved. The correlation between isoflavone intake and lower level of serum cholesterol is getting more and more supportive evidence and the mechanisms for isoflavones' effects on cholesterol level are to be clarified in the future.

I. Soy Isoflavones: Cure for Postmenopausal Osteoporosis?

As the life expectancy continues to rise, osteoporosis is becoming a more important aspect of health care costs. Osteoporosis is associated with ovarian hormone deficiency following the menopause and is the predominant cause of age-related bone loss (Gruber *et al.*, 1984). Estrogen replacement therapy is the most effective method to reduce postmenopausal bone loss, but it is accompanied with side effects such as the increased risk of endometrial cancer (Genant *et al.*, 1989). Reports have indicated that ipriflavone (7-

isopropylisoflavone), a synthetic isoflavonoid derivative, is very effective in preventing bone loss in experimental osteoporosis (Benvenuti *et al.*, 1991; Yamazaki, 1986). At dose of 600 mg/day, ipriflavone prevented both postmenopausal and senile osteoporosis (Agnusdei *et al.*, 1992; Nakamura *et al.*, 1992). Interestingly, one of ipriflavone's metabolites is daidzein (Brandi, 1993). Because of isoflavone's estrogenic activity, they might be effective in preventing bone loss caused by estrogen deficiency.

Arjmandi *et al* (1996) tested the ability of soy protein isolate to prevent bone loss in an ovariectomized rat model of osteoporosis. The soy protein (227 g/kg diet) as well as 17 β-estradiol (10 μg/kg body weight/day) gave significantly higher bone density in ovariectomized rats compared to the ovariectomized rats receiving diets without soy or estradiol. The biochemical markers of bone turnover, alkaline phosphatase and tertrate-resistant acid phosphatase, were significantly lower in soy group than control. They concluded that soy protein is effective in preventing bone loss due to ovarian hormone deficiency and suggested that the isoflavones in soy protein may be the major factors. In a recent study, Blair *et al.* (1996) have shown that genistein administered in the diet can significantly increase the mass of weight bearing bones compared to control in ovariectomized rats. Draper *et al* (1997) reported that coumestrol, zearalanol reduced bone loss and bone resorption in ovariectomized rats, a model of postmenopausal bone loss. Ishida *et al* (1998) reported that daidzin and genistin, at doses of 50 mg/kg body weight/day, both prevented the bone loss in ovariectomized rats fed a calcium-deficient diet.

The ovariectomized, lactating rat model has been used to investigate the skeletal effects of genistein over a 14-day period (Anderson *et al.*, 1998). Three doses, 05.mg/rat/day, 1.6 mg/rat/day and 5 mg/rat/day, were given to rats following

ovariectomization (OVX). A positive control group received premarin, an estrogen. The OVX rats receiving no estrogen or genistein (control) on a low-calcium diet loses slightly more than 50% of its bone mineral mass during the first two week of lactation. However, the estrogen and genistein significantly reduced the bone mineral mass loss compared to the control rats. Genistein may act through osteoblastic cells to inhibit osteoclastic bone resorption and the effects may be mediated by binding to estrogen receptors or by altering cellular activity of tyrosine kinase. Genistein may both stimulate the maturation of osteoblast precursors and act on mature osteoblasts to increase the secretion of cytokines, cytokines may inhibit osteoclastic activity and thus reduce bone resorption (Anderson *et al.*, 1998).

J. Isoflavones and Alcohol Intake

Kudzu (*Pueraria lobata*) is one of the earliest medicinal plants used in traditional Chinese medicine as antidipsotropic (antialcohol abuse) agents (Keung and Vellee, 1998). Interestingly, daidzein was found at 950 μg/g in kudzu (Kaufman *et al.*, 1997). Keung *et al* (1993) reported that daidzin is a potent, reversible and selective inhibitor of human mitochondrial aldehyde dehydrogenase (ALDH). At low concentration of 0.1 μM, daidzin appeared to inhibit human ALDH competitively with respect to acetaldehyde. In 1997, they reported that an i.p. dose of daidzin or daidzein (70 meq per hamster per day) suppressed the alcohol intake by 32% and 62% respectively of Syrian golden hamsters. There is a direct relationship between ALDH activity and the body's ability to metabolism alcohol, by inhibiting ALDH activity, the appetite of alcohol intake may be suppressed.

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SOY ISOFLAVONE ANALYSIS: QUALITY CONTROL AND NEW INTERNAL STANDARD

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Abstract

Development of a database for soy isoflavone in foods requires accurate and precise evaluation of different food matrices. To evaluate accuracy, we estimated recoveries of both internal and external standards in 5 different soy foods weekly. Standards are evaluated daily for system quality assurance. To evaluate sample precision, we analyzed soybeans and soymilk for within-day precision and over 4 days for day-to-day precision bimonthly. Coefficients of variation (cv) should be ≤ 8 %. We have validated our methods for single and multiple recovery levels using our new internal standard, 2,4,4'-trihydroxydeoxybenzoin (THB), and external standards, daidzein, genistein and genistin. Levels of 12 isoflavone isomers, three aglycones (daidzein, genistein, glycitein) and nine glucosides (daidzin, genistin, glycitin; acetyl-daidzin, -genistin,-glycitin; malonyl-daidzin, -genistin, -glycitin) have been measured in a variety of soybeans and soy foods. Extraction methods vary

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depending on soy food type. The HPLC conditions for soy isoflavone analysis has been improved, leading to a good separation with a short analysis time- 60 minutes per sample. A data bank of concentration and distribution of isoflavones in different soybean products was assembled by HPLC analysis. A wide range of isoflavone concentrations, from < 50 μg/g to > 20,000 μg/g, was found in different soy products. The glucoside forms are almost 2X the molecular weight of the aglycones, the reported isoflavone levels should be normalized to the aglycone mass (or an isoflavanoid equivalent) rather than a simple sum of all isomers.

KEYWORDS: quality control, internal standard, 2,4,4'-trihydroxydeoxybenzoin (THB), HPLC assay, soy isoflavone.

Introduction

Dietary intake of soy has been associated with lower incidence of hormonally-dependent and -independent cancer (1). Soy foods have been associated with lower risk of cardiovascular disease (2). Isoflavones may directly inhibit bone resorption (3). In animal studies, isoflavones demonstrated anti-cancer capabilities and inhibited the growth of human breast and prostatic cancer cells in culture (4). Recent studies have proposed that isoflavones may be the factor responsible for the cholesterol lowering property (5) of some soy foods. To evaluate the potential of the isoflavones as a dietary health-enhancing compound, the amounts available in typical soy foods and in soybeans must be quantified. Soybeans contain high amounts of the isoflavones (1-3 mg/g), daidzein, genistein and glycitein (6). High performance liquid chromatography (HPLC) has already been shown to be a powerful

method to analyze the isoflavones. Most previous studies of soy isoflavones have focused on daidzein and genistein with little attention to glycitein. Glycitein accounts for about 5-10% of the total isoflavones in soy foods and may have some unique biological activities. We are assembling a data bank of the concentrations of 12 isoflavone isomers, three aglycones (daidzein (Dein); genistein (Gein); glycitein (Glei)) and nine glucosides (daidzin (Din); genistin (Gin); glycitin (Glin) ; acetyl-daidzin (ADin); acetyl-genistin (AGin); acetyl-glycitin (Aglin); malonyl-daidzin (MDin); malonyl-genistin (MGin); and malonyl-glycitin (MGlin)) in soy foods (Figure 1). The distribution of different forms of isoflavones represents a history of the processing of these foods. Heat processing, enzymatic hydrolysis, and fermentation can significantly alter the isomer distribution of the three isoflavones (7). For example, the amount of the malonyl forms, which are very heat sensitive, will be quite different between raw and heat-treated soybeans.

Materials and Methods

Apparatus. Samples were analyzed on a Beckman System Gold chromatography with an autosampler Model 507, a dual pump Model 126 , a photodiode array detector (PDA) Model 168 and an IBM 486 computer with Beckman Gold system HPLC data processing software(version 8 , 1993). All HPLC units are from Beckman Instruments, Fullerton, CA.

Materials. Chemicals used in the synthesis of THB are resocinol, 4-hydroxyphenylacetic acid , boron trifluoride etherate (BF_3Et_2O), N,N-Dimethyl formamide (DMF) and methanesulfonyl chloride, all purchased from Sigma Chemical Co. (St. Louis, MO). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used. HPLC-

grade acetonitrile (ACN) and methanol used in HPLC analysis were purchased from Fisher Scientific (Fair Lawn, NJ). All the soy ingredients and processed soybean products reported here were purchased locally, except soygerm, which was generously provided by Schouten Industries, Inc.(Minneapolis, MN).

Synthesis of 2,4,4'-trihydroxydeoxybenzoin (THB) (8). Resorcinol (2.9 g) was added to a mixture containing 4-hydroxyphenylacetic acid (2g) and boron trifluoride etherate (4.5 mL). The mixture was refluxed for 10 min., cooled and treated with 30 mL saturated NaOAc and 15 mL $NaHCO_3$ respectively. The yellow precipitate was filtered and washed with water, then washed with $CHCl_3$, dried and give yellow crystals. Redissolved crystals were chromatographed through Sephadex LH-20 (Pharmacia LKB Biotech. Inc., Piscataway, NJ) column with 50% ethanol as the mobile phase. A single peak was collected giving high purity THB (>99%) for use as internal standard.

Isoflavone standards. Seven isoflavone standards were used in calibration curves including Dein, Gein, Glei, Din, Gin, Glin, AGin (Table 1). Gein was synthesized in this laboratory according to Chang et al (8). Dein was synthesized in this laboratory with modification of Chang's method: BF_3Et_2O (4 mL) was added to a beaker containing 200 mg THB in 10 mL DMF, the reaction mixture was heated in microwave oven (Kenmore U88) for 30 s using medium energy, then 4 mL of methanesulfonyl chloride was added to the mixture. The mixture was heated again in microwave for 70 s using medium energy. Four hundred mL of cold water was added to the reaction mixture, giving a light yellow precipitate. The precipitate was washed with water and recrystallized from methanol. Gin, Din and AGin were available from previous work in this laboratory (6). Glin was purified

according to Farmakalidis and Murphy (9). Glei was purified from soy germ by using the following method. Soygerm was hydrolyzed in 0.1 N HCl at 98°C for 2 hour, extracted with ACN and filtered through No.42 filter paper. The filtrate was dried by using a rotary evaporator at 50°C. The residue was dissolved in 100% ethanol and applied to a Sephadex LH-20 column with 50% ethanol as the eluent. The glycitein peak was collected and freeze-dried. The identification and purity of glycitein was confirmed by HPLC retention time, UV spectral analysis, melting point and mass spectrum.

Calibration curves and calculation of food levels. Isoflavone stock solutions were prepared by dissolving pure standards in 80% methanol to give a concentration around 400 μg/mL. An aliquot of the solution was then diluted for an absorbance in the range of 0.6 - 0.8. The concentrations of the stock solutions were calculated by absorbance at the wavelength with maximum absorption ($\lambda_{max.}$) using molar extinction coefficients (ε) (10). Purity of the standards was calculated based on the percentage of the peak area in the HPLC chromatograph. The final stock concentration of each individual standard was adjusted for the purity of the standards.

Standard curves were obtained for most isoflavones with high linearity ($r > 0.995$) by plotting the standard concentration as a function of peak area obtained from HPLC analysis of 20 μL injections. The concentrations of standard solutions were carefully chosen in order to cover the possible isoflavone contents based on the previous work in our lab. For the isoflavones without a pure standard (MGin, MDin, MGlin, ADin, AGlin), we adjusted the standard curves based on the molecular weight differences.

We used standard curves to calculate the concentrations of isoflavones in food items. The results were expressed as μg isoflavone / g food with consideration for recoveries.

Isoflavone extraction and HPLC assay. All samples were freeze-dried except for the dry soy ingredients and the instant soy beverage. Two grams of dried, finely ground sample was placed in a 125-mL screw top Erlenmeyer flask and 10 mL of ACN , 2 mL of 0.1N HCl (6) and 5 mL of water were added and stirred for two hours at room temperature. Extracts were filtered through Whatman No. 42 filter paper. The filtrate was taken to dryness on a rotatory evaporator (BÜchi, Brinkmann, Westbury, NY) at < 30°C. The residue was dissolved in 10 mL 80% HPLC-grade methanol in water. A aliquot of sample was filtered through a 0.45-μm PTFE filter unit (poly-tetrafluoroethylene) (Alltech. Associates inc., Deerfield, IL) and analyzed by HPLC. A YMC-pack ODS-AM-303 column (5μm, 25 cm x 4.6 mm) was used. A linear HPLC gradient was composed of (A) 0.1% glacial acetic acid in water and (B) 0.1% glacial acetic acid in ACN. The chromatography method (6) has been modified with 33% less analysis time for each sample. Following injection of 20 μl of sample, solvent B was maintained at 15% for 5 min, then increased to 29% in 31 min, after that increased to 35% in 8 min and returned to 15% at the end of 45 min. The solvent flow rate was 1 mL/min at the first 5 min, then increased to 1.5 mL/min in 0.5 min and maintained for 39 min, then returned to 1 mL/min. A Beckman model 168 photodiode array detector monitored absorbance from 200 to 350 nm. UV spectra were recorded and peak areas were integrated by Beckman Gold system software (version 8, 1993).

Recovery study using internal and external standards. Two hundred μL of internal standard THB (400 μg/mL in methanol) and different amounts (from 50 μL to 700 μL dependent upon the sample) of external standards (Gein (1600 μg/mL), Dein (800 μg/mL), and Gin (600μg/mL)) in methanol solution were added to 2 g of dry sample. The samples were mixed thoroughly, then left at room temperature until methanol had evaporated. The extraction and analysis methods were the same as above.

Statistical Analysis. All samples were run with two to four replications. Statistical analysis including linear regression, average, standard deviation, and coefficients of variation (cv) was calculated by using Microsoft Excel 5.0 statistical functions. Analysis of variance was done by using the SAS package (Version 5.0) developed by the SAS Institute, Inc. (Box 8000, Cary, NC) at $\alpha = 0.05$.

Results and Discussion

Modification of HPLC condition. The HPLC conditions for soy isoflavone analysis (6) gave good separation on 12 isoflavone isomers, with a total analysis time of 90 minutes for one sample. However, using these HPLC conditions, only 10 to 16 samples could be run per day. Therefore, we modified our previous HPLC conditions by programming flow rate and mobile phase gradient as described in Methods. We achieved excellent separation of 12 isoflavone isomers (Figure 2) with a shorter analysis time of 60 minutes. Analysis efficiency was improved 50 % where up to 24 samples can be run per day.

Modification of isoflavone extraction method. The HCl-ACN extraction procedure followed the method described in Murphy (16), which was superior to all other solvent systems in terms of good recovery and less coextractives. During the extraction, the ratio of solvent volume versus food material (mL / gram) was always higher than 6. This method worked well for soybeans. In re-evaluating the extracting process, in addition to the HCl and ACN, we found that additional water gave better recovery (table 2). For most of the foods, 7 ml of water was sufficient to maximize extraction. For some unusual samples, different amount of water may be used to get the best extraction. Most of the soy products have a total isoflavone concentration of 1-3 mg / g. The extraction solvent is, therefore, sufficient for removal of the isoflavones. For other soy products, and in particular, isoflavone supplements, which have >10 mg / g isoflavones, the solvent to sample weight ratio should be adjusted. For example, soygerm has very high isoflavone content (> 10 mg /g) and the normal extraction procedure would underestimate the isoflavone content by 10 to 20%. We found that an adjustment of the solvent to sample weight ratio to 95 (0.2 g sample, 10 mL of ACN , 2mL HCl, and 7 mL of water) resulted in a better extraction of isoflavones.

THB as a reference peak in soy isoflavone assay. During the HPLC analysis of isoflavones, the retention times of the analytes may shift due to several factors, including temperature, flow rate and mobile phase makeup. A reference peak in the chromatography can aid with peak identification. The computer software in HPLC assay in our laboratory measures retention times of analytes relative to the retention time of the reference peak, thus avoiding misidentification of analytes due to retention time shifts of peaks. A good reference peak should not be found in the sample , it should be stable under the analysis conditions and

have a stable retention time and its retention time should be in the range of all the analytes. 2,4,4'-Trihydroxydeoxybenzoin (THB) is the intermediate for the chemical synthesis of daidzein (8). THB has similar structure and polarity to isoflavones(Figure 1). Its retention time does not overlap any of the other peaks in our isoflavone chromatogram. It is not found in natural foods. It is stable and easy to synthesize. The retention time of THB under the HPLC conditions described in Materials and Methods is 36.20 ± 0.40 min. Using these HPLC conditions, the 12 soy isoflavone isomers had different retention times (Figure 2). There is an 'empty' part of the chromatograph (from 32 min to 40 min). THB's retention time falls in this range, does not overlap any of the isoflavones. THB was used as the reference peak to adjust the retention times of all other analytes. By using a reference peak as an anchor, the computer software can assign identification to isoflavone peaks on the basis of their retention times relative to THB and calculate their concentrations from standard curve data.

THB as internal standard. There will be certain losses during the multi-step extraction of isoflavones, and an internal standard should be required for precise isoflavone quantification. An ideal internal standard should be a compound structurally related to the analyte with a similar polarity, but with a retention time that does not overlap with other peaks in the chromatography. Few isoflavone investigators use internal standards to adjust the analyte loss in extraction and analysis (11, 12, 13,14). Most of those compounds that have been used as internal standards were either not structurally related to the analytes, or have retention times which conflict with the isoflavone peaks. Flavone, used by Franke et al(15) as an internal standard, eluted much later than the latest eluting isoflavone - Gein. Using

flavone as internal standard will greatly increase the analysis time for each sample. So for an efficient isoflavone analysis, flavone may not be a good internal standard. We tested THB as an internal standard. To use an internal standard, known mixtures of standard (THB) and analyte are used to construct a standard curve (Figure 3) . When the known amount of internal standard is added to an unknown sample, the calibration curve can be used to find the concentration of unknown. If a standard is added to a sample prior to extraction, then the fraction of standard lost during the extraction is the same as the fraction of sample lost and the ratio of unknown and standard concentration remains the same. We used the internal standard curve method to assay the Gin concentration in five different soy samples (textured vegetable protein (TVP), tofu, soybean, soymilk and tempeh). The results from internal standard method were compatible to the results from HPLC chromatography without using internal standard (The difference is between 3 - 10%). For Dein and Gein, we found that the results from internal standard method were also compatible to the HPLC method, with a difference < 10 %. This indicated that our extraction method works well.

Recovery studies of external standards. Recovery experiments were carried out with Dein, Gein, Gin and THB. The recovery results in table 3 show that daidzein, genistein and genistin gave different recoveries. This is probably because these isoflavones bind differently to the food matrices. The extraction method was designed to optimize the extraction of the glucoside forms of isoflavones because, for most soy products, they account for more than 90 % of the total isoflavones (6). This may explain the lower recovery of the more hydrophobic free forms (Dein and Gein).

Multiple level recovery assay. The use of external standards of isoflavones that are already present in the food, is another way to evaluate multiple levels of recovery. As an example, we added different known amounts of genistin to tofu. A plot of total genistin versus genistin added to the sample yielded a good linear regression line (Figure 4). We can use this external standard recovery curve's Y-intercept as the actual amount of Gin in our food sample. The same evaluations were done in tofu with Dein or Gein. With Dein we obtained a multiple recovery curve of $Y=0.996X + 178.95$, $R^2=0.9885$. With Gein, we obtained a curve of $Y= 1.0143X + 279.67$, $R^2=0.9976$.

Precision of the analysis. We analyzed two soy foods, Vinton soybeans and freeze-dried soymilk with different analyte concentrations every month with four replications per day to determine the precision of our procedure. Table 4 presents the results from Vinton soybeans. The within day cv was 4% and between day cv was less than 8% . We obtained within day cv < 5% and between day cv < 10% for soymilk. These samples are good representatives of typical soy foods. The small CVs indicated good precision in our analytical procedure.

Food levels. By using methods described in this paper, some representative results of soy foods analyzed are presented in Table 5. These foods include soy ingredients (soybeans, TVP, soy isolate), traditional soy foods (roasted soybeans, tofu, tempeh, fried tofu, miso), second-generation soy foods (soy hot dog) and soy supplement product (Soygerm). We recalculated the results with adjustment for recoveries. For aglycones, we used the average recovery of Dein and Gein, 90%. For all the glucoside forms, we used the recovery of Gin, 97%. Since different lots of soy products than previous report were analyzed by the modified

extraction method and reported by adjustment for recovery, there appears to be considerable variation in isoflavone levels from lot to lot of same products which was not unexpected. The normalized concentrations of each individual isoflavone (daidzein, genistein and glycitein) are reported. These values are calculated by multiplying the mass of each individual isoflavone form by the ratio of its aglycone molecular weight / molecular weight of individual form before summing. For example, Gin amounts would be multiplied by 270 / 432 to give free Gein represented by Gin. The total isoflavone in each food sample is not the simple sum of the mass of the 12 isomers. The isomers will be hydrolyzed to free forms in the gut prior to absorption. Thus, normalized results give a picture of the "effective" isoflavone concentration in food.

Acknowledgements

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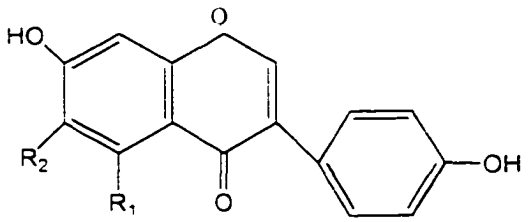
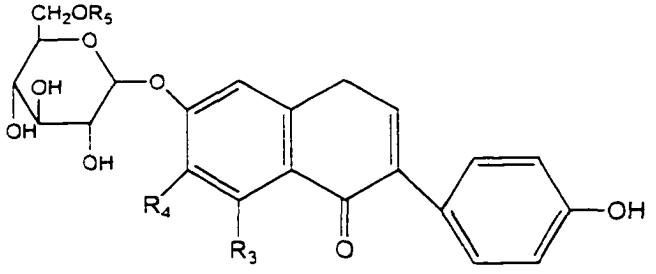
Aglycones



R1	R2	Compound
H	H	Daidzein
OH	H	Genistein
H	OCH3	Glycitein

Glucosidic forms



R3	R4	R5	Compounds
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH3	H	Glycitin
H	H	COCH3	Acetyldaidin
OH	H	COCH3	Acetylgenistin
H	OCH3	COCH3	Acetylglycitin
H	H	COCH2COOH	Malonyldaidzin
OH	H	COCH2COOH	Malonylgenistin
H	OCH3	COCH2COOH	Malonylglycitin

2,4,4'-trihydroxydeoxybenzoin (THB)

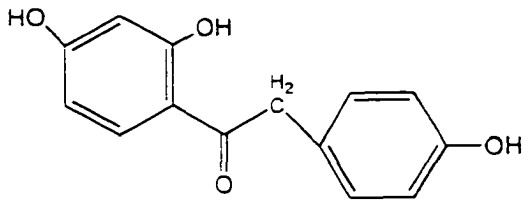


Figure 1. Chemical structures of 12 isoflavone isomers and internal standard THB.

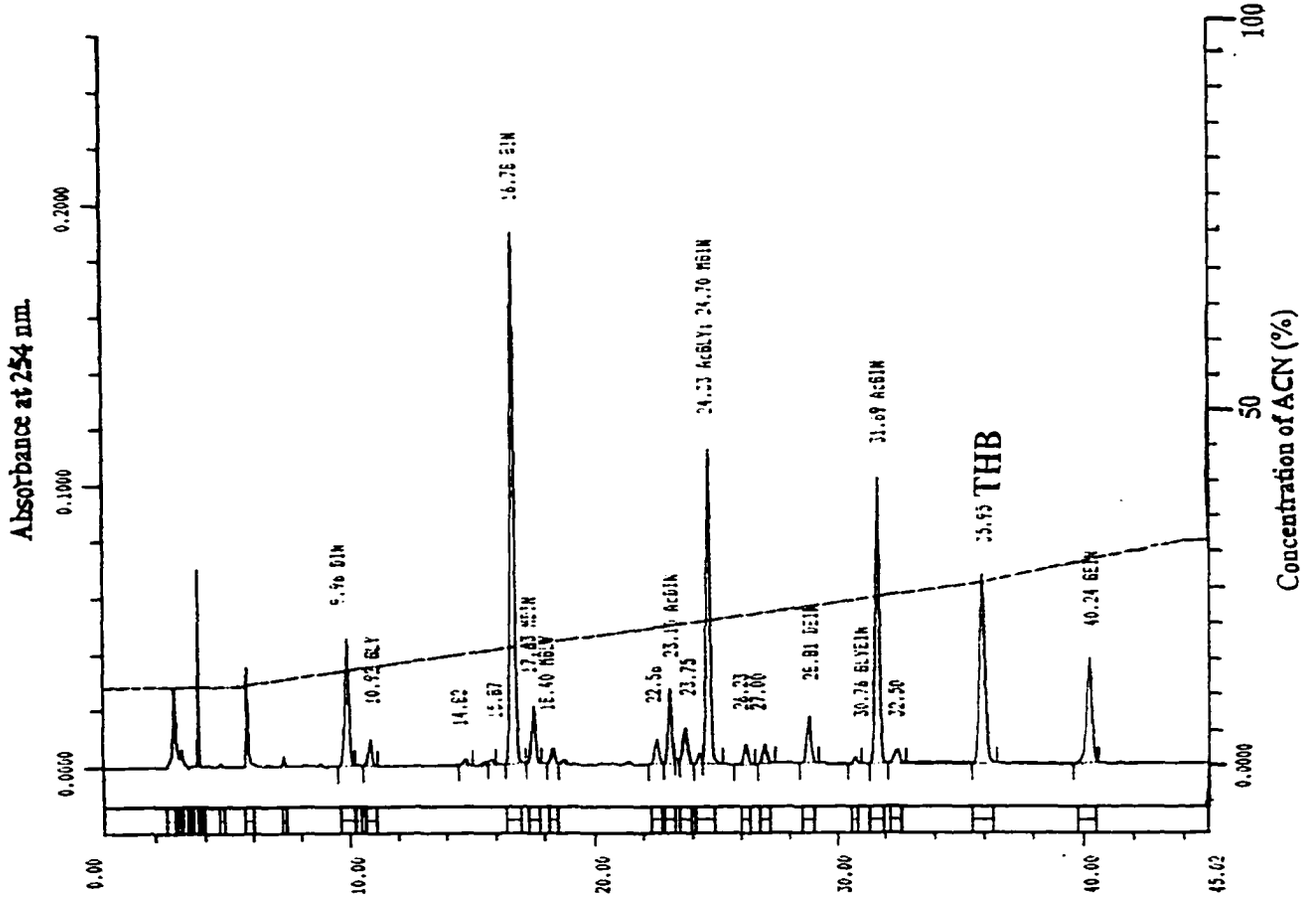


Figure 2. HPLC chromatograph of isoflavone isomers and THB in isolated soy protein.

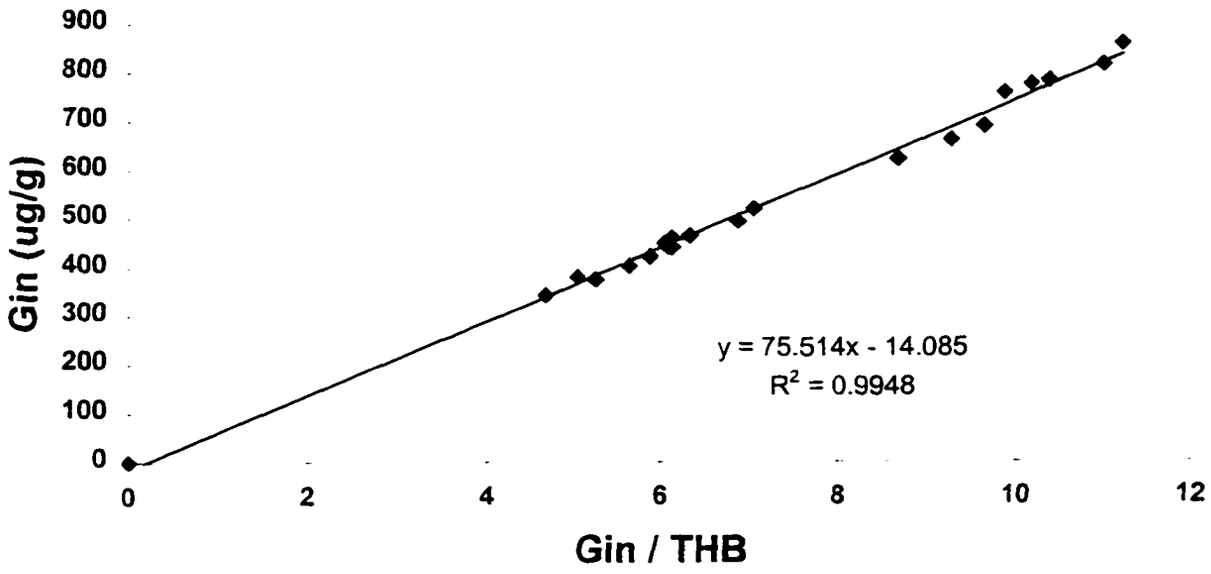


Figure 3. Calibration curve of Gin/THB from five food matices: TVP, tofu, soybeans, soymilk and tempeh.

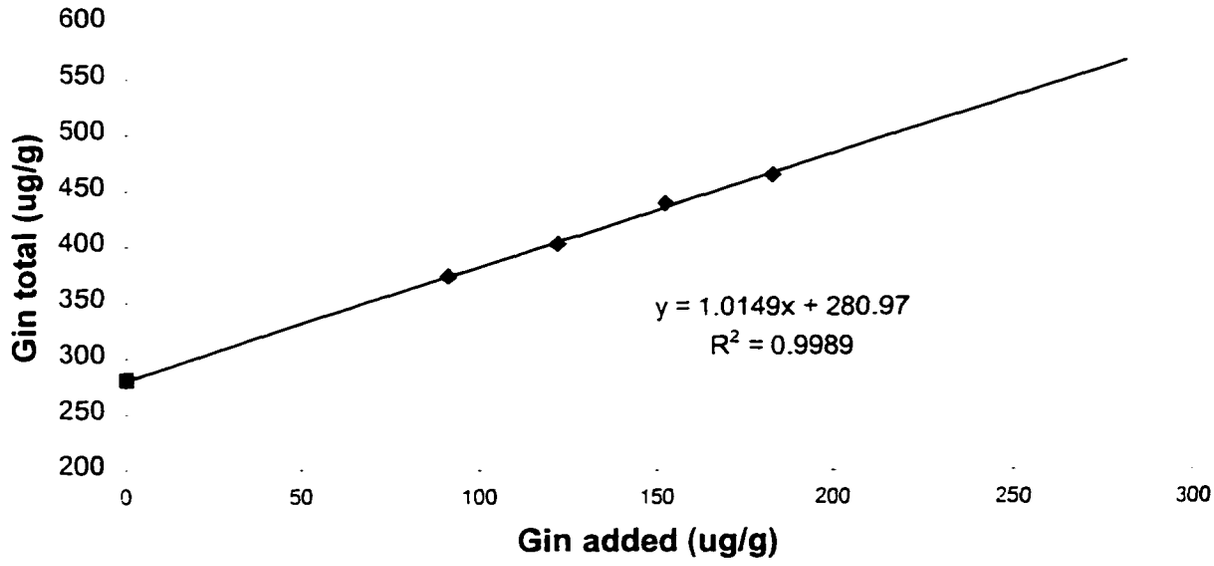


Figure 4. Genistein as an external standard in dry tofu.

Table 1. Standard curves of isoflavones

Compound	Source	λ_{max} (nm)	Molecular weight	Extinction coefficient (ε)	working range (μg / g)
Dein	synthesized by T. Song	249	254	26915	5 to 200
Din	purified by HJ. Wang	249	416	26915	5 to 200
ADin	adjusted from Din	256	458	31622	
MDin	adjusted from Din	258	522	18197	
Gein	synthesized by T. Song	263	270	31622	5 to 200
Gin	purified by HJ. Wang	263	432	31622	5 to 200
AGin	adjusted from Gin	261	474	32358	
MGin	purified by HJ. Wang	260	518	16331	5 to 100
Glein	purified by T. Song	256	284	22387	5 to 100
Glin	purified by T. Song	259	446	26303	5 to 100
AGlin	adjusted from Glin	260	488	26303	
MGlin	adjusted from Glin	260	532	26303	

Table 2. Comparison of isoflavone extraction with water added to the extraction solvent

Food sample	Water added (mL)	replications (n)	Normalized total Dein (μg/g) ± S.D	Normalized total Gein (μg/g) ± S.D	Normalized total Glei (μg/g) ± S.D
ISP[a]	0	2	248 ± 15	695 ± 139	70 ± 7
ISP[a]	3	2	376 ± 15	801 ± 29	80 ± 21
ISP[a]	5	4	417 ± 49	844 ± 70	90 ± 20
ISP[a]	7	3	439 ± 83	890 ± 129	97 ±32
ISP[a]	10	3	445 ± 18	919 ± 32	103 ± 4
Tofu	0	2	316 ± 7	589 ± 11	55 ± 8
Tofu	3	2	431 ± 7	759 ± 17	73 ± 3
Tofu	5	4	455 ± 21	783 ± 28	75 ± 2
Tofu	7	3	485 ± 20	847 ± 36	79 ± 3
Tofu	10	3	504 ± 3	841 ± 2	79 ± 1
Vinton Soybeans[b]	0	5	362 ± 113	533 ± 114	55 ± 9
Vinton Soybeans[b]	3	8	662 ± 213	803 ± 178	85 ± 19
Vinton Soybeans[b]	5	6	815 ± 46	923 ± 33	97 ± 5
Vinton Soybeans[b]	7	3	850 ± 41	959 ± 22	106 ± 5
Vinton Soybeans[b]	10	3	778 ± 43	886 ± 59	90 ± 4

a. ISP = Isolated soybean protein;

b. 1994 crop.

Table 3. Recovery of analytes and internal standard from spiked samples

FOOD	Compound							
	Daidzein		Genistein		Genistin		THB	
	Recovery (mean ± S.D.) (%)	cv (%)	Recovery (mean ± S.D.) (%)	cv (%)	Recovery (mean ± S.D.) (%)	cv (%)	Recovery (mean ± S.D.) (%)	cv (%)
TVP	91 ± 10	11.1	91 ± 6	7.4	98 ± 5	6.1	97 ± 3	2.9
Soymilk	89 ± 7	9.5	92 ± 7	8.3	99 ± 6	6.7	98 ± 4	4.6
Soybean	74 ± 9	11.8	93 ± 9	10.0	98 ± 2	2.4	101 ± 4	3.7
Tofu	91 ± 7	8.1	89 ± 8	9.7	94 ± 4	4.4	97 ± 5	5.1
Tempeh	90 ± 7	8.8	90 ± 7	8.6	97 ± 5	5.3	97 ± 4	4.5

n = 10

Table 4. Precision of analysis with the proposed method for isoflavones from Vinton 81[a] soybean

Compound	Mean (μg / g)	Coefficient of variation (%)	
		Within assay	Between assay
Daidzein	19.8 ± 1.4	1.7	7.2
Daidzin	17.5 ± 0.8	3.2	4.9
Acetyl-Daidzin	0.0		
Malonyl-Daidzin	1290.0 ± 54.2	1.7	4.2
Genistein	18.3 ± 0.8	1.5	6.1
Genistin	221.3 ± 17.1	3.1	7.7
Acetyl-Genistin	27.4 ± 1.0	2.0	3.6
Malonyl-Genistin	1324.8 ± 100.0	2.2	7.6
Glycitein	0.0		
Glycitin	51.6 ± 2.8	3.6	5.6
Acetyl-Glycitin	0.0		
Malonyl-Glycitin	127.1 ± 10.0	2.6	7.9

a. 1994 crop.

Table 5. Isoflavone contents (μg / g) of several soy foods

Product	Aglycone			Glucoside			Malonyl			Acetyl			Normalized			Total[c]
	Dein	Gein	Glei	Din	Gin	Glin	Din	Gin	Glin	Din	Gin	Glin	Dein	Gein	Glei	
Vinton 81[a,d]	21	22	0	163	178	50	1364	1467	137	0	29	0	811	914	105	1830
TVP[a]	13	31	26	524	660	150	95	197	63	193	334	93	488	737	210	1435
Roasted soybeans[b]	44	77	58	474	568	70	46	65	74	40	71	54	379	506	174	1059
Tofu[a,e]	116	140	26	453	562	130	753	788	131	54	66	0	806	939	179	1924
Fried Tofu[b,e]	63	90	18	340	523	87	338	410	60	85	110	0	490	693	106	1289
Roasted soyflour[b]	18	16	13	470	584	62	956	885	46	1643	1375	56	1696	1626	109	3431
Tempeh[a,e]	318	518	31	117	346	34	404	750	34	68	76	65	633	1168	109	1910
Miso[a]	61	39	11	157	122	37	1180	979	217	0	0	0	759	625	150	1534
Soy milk[a,e]	18	19	10	410	710	65	690	871	39	22	820	89	632	1384	124	2140
Soy hot dog[b]	9	18	9	36	69	16	13	43	16	0	4	15	38	86	36	160
Soygerm[b]	303	114	684	5208	1664	6505	3466	857	3313	10830	2616	3952	11204	3092	8905	23201
lsd.	7	6	6	38	18	57	28	25	12	57	284	32	98	38	63	157

a Data from three replications; b. Data from two replications; c. Adjusted for molecular weight; d. 1994 crop.

e. Data based on dry samples weight.

lsd. Least significant difference

ESTROGENIC ACTIVITY OF GLYCITEIN, A SOY ISOFLAVONE

A paper submitted to the Journal of Agricultural and Food Chemistry

Tong T. Song[1], Suzanne Hendrich[1] and Patricia A. Murphy[1,2]

Abstract

Glycitein (4',7-dihydroxy- 6 -methoxyisoflavone) accounts for 5-10% of the total isoflavones in soy products. The biological activity of this compound has not been reported to date, although numerous studies have been performed with the other soy isoflavones, daidzein and genistein. Glycitein was isolated from soy germ to 99% purity. Weaning female B6D2F1 mice were dosed with glycitein (3 mg/day), genistein (3 mg/day) and diethylstilbestrol (DES) (0.03 μg/day) in 5% Tween 80 by gavage for 4 days. Control group received equal volume of 5% Tween 80 solution daily. The uterine weight increased 150% with glycitein ($p < 0.001$), 50% with genistein ($p<0.001$) and 60% with DES ($p<0.001$) compared with the control group. DES, 17β-estradiol and three isoflavones (daidzein, genistein and glycitein) were examined for their competitive binding abilities with 17β-(^{3}H) estradiol to the estrogen receptor proteins of the B6D2F1 mouse uterine cytosol. The concentrations of each compound required to displace 50% of the (^{3}H) estradiol at 5 nM in the competitive binding assay were DES, 1.15 nM, 17β-estradiol, 1.09 nM, genistein, 0.22 μM, daidzein, 4.00 μM and glycitein, 3.94 μM. These data indicated that glycitein has weak estrogenic activity, comparable to the other soy isoflavones, but much lower than that of DES and 17β-estradiol.

Key words: Isoflavone, Glycitein, Genistein, Daidzein, Phytoestrogen, Estrogenic activity.

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Introduction

Estrogens play important hormonal roles among all vertebrates. Animal estrogens are exclusively steroidal compounds, and the principal physiological estrogen in most species is 17β-estradiol. Many plants produce compounds that possess estrogenic activity in animals and are, thus called phytoestrogens. These phenolic compounds have some structural similarity to the mammalian estrogen, 17β-estradiol. A common structural characteristic of these compounds is the presence of a phenolic ring, a prerequisite for the binding to the estrogen receptor (Figure 1).

Soybeans contain the highest concentrations of isoflavones, at 1-3 mg/g, as daidzein, genistein, glycitein and their corresponding glucosides, of foods consumed by humans. These soy isoflavones may have some important health-enhancing properties such as prevention of certain cancers (Barnes et al. 1991), lowering the risk of cardiovascular diseases (Anderson et al. 1995) and improvement of bone health (Bahram et al. 1996). The estrogenic activities of daidzein, genistein, and their glucoside forms, genistin and daidzin, were 100,000 to 500,000 times lower than that of diethylstilbestrol (DES) (Farmakalidis and Murphy, 1985). Their estrogenic activity may play an important role in their health-enhancing properties. Genistein and daidzein account for the major portion of isoflavones in soy foods and have been the focus of numerous studies. No biological studies on glycitein have been reported to date. However, glycitein accounts for 5 to 10% of the total isoflavones in soy foods, and may be as high as 40% in some soy supplements. Therefore, it is important to evaluate the

biological activity of glycitein. Since glycitein has a similar structure to genistein and daidzein, we hypothesize that it will have estrogenic activity comparable to them.

There are a number of methods to assess estrogenic activity. Reel et al. (1996) proposed a test array for potential estrogenic activity. These methods can be grouped into four categories: (1) estrogen receptor binding, (2) estrogen receptor-dependent transcriptional expression, (3) reproductive tract response, and (4) non-reproductive-tract target tissue response. It is preferable to perform more than one assay to confirm the results. Estrogen receptor binding studies with soy isoflavones, genistein and daidzein, demonstrated they had the abilities to bind to estrogen receptors from different species including mice, rats and sheep (Verdeal et al. 1980). The binding affinities of these isoflavones were much lower than that of estradiol and DES. Farmakalidis and Murphy (1985) evaluated the estrogenic activity of genistin and daidzin, the isoflavone glucosides, and showed that the glucosides had equal estrogenic activity to the aglycones on a molar basis. The mouse uterine enlargement assay has been the standard *in vivo* method to evaluate estrogenic activity (Bickoff et al. 1962), and, since it is performed in an intact animal, the effects of absorption, metabolism, serum binding and pharmacokinetics are taken into account.

Materials and Methods

Chemicals. DES, Tween 80, 17-β-(2,4-^{3}H) estradiol (23 mCi / mmol), 17-β estradiol and dextran-coated charcoal (DCC) were obtained from Sigma Chemical Co. (St. Louis, MO).

ACS scintillation fluid was purchased from Fisher Scientific Inc. Glycitein was purified by the following method: 10 g soygerm (generously donated by Schouten USA, Inc., Minneapolis, MN) was hydrolyzed in 100 mL 0.1 N HCl at 98°C for 2 hours, extracted with acetonitrile and filtered through Whatman No. 42 filter paper. The filtrate was dried by a rotary evaporator at 50 °C. The residue was dissolved in 80% ethanol and applied to a Sephadex LH-20 column (2.5 cm x 50 cm) with 50% ethanol as the eluent. The glycitein peak was collected and freeze-dried. Genistein was purified from soybeans using the same procedure as glycitein. Daidzein was chemically synthesized according to Song et al (1998). The identification and purity of glycitein, daidzein and genistein were confirmed by HPLC, ultraviolet (UV) spectral analysis, melting point and mass spectrum analysis. Glycitein, daidzein and genistein were analyzed by HPLC on a Beckman System Gold chromatography system including a Model 507 autosampler, a Model 126 dual pump, a Model 168 photodiode array detector and an IBM 486 computer with Beckman Gold system HPLC data processing software (version 8, 1993) according to Murphy et al. (1997). A YMC-pack ODS-AM-303 column (5 μm, 25 cm x 4.6 mm) (YMC Inc. Wilmington, NC) was used. UV spectral analysis was performed according to Marbry et al. (1970) using a Beckman DU 7400 spectrophotometer. The melting point was measured with a Perkin Elmer 7 series differential scanning calorimeter (DSC) (Perkin Elmer Inc. Norwalk, CT). Mass spectrum analysis, using chemical ionization, was performed on a Finnigan model TSQ-700 mass spectrometer (Finningan Inc. Piscataway, NJ).

Animals and treatments. The mice uterine enlargement assay was performed according to Farmakalidis and Murphy (1985). Inbred B6D2F1 mice were obtained from Harlan Sprague-Dawley (Madison, WI). Each dam was housed separately with her female pups (14 days old on arrival), and the mice received AIN-93 M diet (Reeves et al., 1993) and water. Pups were weaned at 21 days of age. The mice were randomly assigned to treatment groups (control, DES, genistein and glycitein) of 20 mice. An AIN 93-G diet and water were provided. The diet contained no soy isoflavones. Genistein, DES and glycitein were suspended in 5% Tween 80 and were administered to the mice by gavage needle in four daily doses of 0.1 ml suspension / day beginning on the day after weaning for 4 days. The total doses were 0.12 μg DES / mouse or 12 mg genistein or glycitein / mouse. The control group was given 0.1 ml of 5% Tween 80 for four days. At twenty-four hours after the last dose, mice were weighed and sacrificed under CO_2. Their uteri were dissected out, and the wet weights were obtained immediately. Mouse uteri were homogenized, and cytosols were prepared for estrogen receptor binding assay as described below. The blood from genistein and glycitein groups was collected by using cardiac puncture. Plasma was prepared by centrifugation at 5000 g for 10 min at 4°C and kept frozen until analysis of plasma isoflavones. The animal experimental protocol was approved by Iowa State University Animal Use Committee.

Tissue handling and estrogen receptor assay. The estrogen-binding assay was modified from Verdeal et al. (1980). Mouse uteri were homogenized (Brinkmann Instruments, Rexdale, Canada) in 10 mM Tris and 1 mM EDTA, pH 7.4 buffer (1:10 wt/vol). The cytosol

fractions were obtained by centrifugation of the homogenate at 100,000 g for 1 hour. Total binding was determined by adding 0.2 ml each of 1 mM EDTA, pH 7.4 buffer, (^{3}H) estradiol (1.5 ng / ml in 1 mM EDTA pH 7.4 buffer), and cytosol fraction. Non-specific binding was determined by replacing the 1 mM EDTA buffer with 0.2 ml of 17β-estradiol (1500 ng / ml in the 1 mM EDTA, pH 7.4 buffer). Blanks contained 0.4 ml of 1 mM EDTA buffer and 0.2 ml (^{3}H) estradiol. Competitive binding was determined by replacing the 0.2 ml buffer in the total binding mixture with 0.2 ml of solution containing the different estrogens at appropriate concentrations in 1 mM EDTA, pH 7.4 buffer. Duplicates were performed for each estrogen at each concentration. Samples were incubated at room temperature (23°C) for two hours. One half ml of 2.5% DCC in 1 mM EDTA buffer was added to all mixtures after incubation. The samples were mixed, incubated for 15 min, and centrifuged at 1000 g for 10 min. After centrifugation, the supernatants were decanted into scintillation vials containing 10 ml ACS scintillation fluid. The samples were counted for 10 minutes on a Packard liquid scintillation analyzer model 1900TR (Packard Instrument Co., Downers Grove, IL).

Plasma isoflavone analysis: Plasma samples of the genistein and of the glycitein group were combined to give one pooled sample for each treatment. Two ml of 0.2 M sodium acetate pH 5.5 buffer, 100 μL of β-glucouronidase/sulfatase and 20 μL of 4 mg/ml internal standard, 2,4,4'-trihydroxydeoxybenzoin (THB) (Song et al 1998), were added to 2 ml of plasma sample. The mixture was incubated at 37°C for 16 hours. Six ml of methanol was

added to the mixture, mixed well and centrifuged at 10,000 g for 20 min. Eight ml of supernatant was brought to dryness and dissolved in 400 µl of 80% methanol. After centrifugation, 20 µL of sample was taken for isoflavone analysis in our HPLC system. The HPLC conditions were the same as in identification of genistein and glycitein stated above. The minimal detection level by UV detector at 254 nm for genistein and glycitein was 0.5 µg/ml or 1.9 µM in injection solution.

Statistical analysis. The uterine enlargement data were analyzed by using a one-way classification analysis of a completely randomized design (SAS version 6, SAS Institute Inc. Box 8000, Cary, NC) at $\alpha = 0.05$.

Results and Discussions

The purity of glycitein and genistein was confirmed by HPLC, UV spectral analysis, melting point and mass spectrum analysis. The HPLC chromatography of glycitein showed one single peak and the purity of glycitein was confirmed by peak area percentage and peak purity program to be > 99%. Naim et al.(1973) first isolated glycitein from soybeans and reported UV spectral data of glycitein. The UV spectral data for our glycitein were: λ_{max} (methanol) 257, 319 nm; λ_{max} (sodium methoxide) 259, 344 nm; λ_{max} ($AlCl_3$) 257, 317 nm; λ_{max} ($AlCl_3$ - HCl) 257, 317 nm; λ_{max} (sodium acetate) 255, 346 nm; λ_{max} (sodium acetate-H_3BO_3) 255, 320 nm. These data were the same as reported by Naim et al. (1973). The

molecular weight of our glycitein from the mass spectrum analysis was 284, which matched the mass reported by Naim et al (1970) and the calculated molecular weight of glycitein. The melting point of glycitein measured by DSC was 337 - 339 °C. This melting point differed from that reported by Naim et al. (1973) of 311 - 313 °C. Recently, Nogradi and Szollosy (1995) chemically synthesized glycitein and reported a melting point of 337 - 339 °C. The purity of genistein was confirmed to be greater than 99% by using the similar method for glycitein.

In our preliminary study, we used chemically synthesized genistein (Chang et al., 1994) as one of the positive controls and did not obtain any estrogenic response from the mice uterine enlargement assay. However, we did get estrogenic responses from glycitein and DES. We evaluated the purity of the synthesized genistein and found that it contained a contaminant which co-eluted in our analytical HPLC system with authentic genistein. A broad melting point thermogram indicating a mixture of genistein and the contaminant was detected by DSC analysis. To avoid this problem, we purified the genistein from soybeans for the present study.

The uterine enlargement assay yielded significant differences ($p < 0.001$) in uterine weights among treatments (Table 1). The glycitein, genistein and DES group had 150%, 50% and 60% greater uterine weight, respectively, compared with the control. The relative estrogenic potencies of DES, genistein and glycitein were 100,000, 1, and 3, respectively (Table 1). The relative estrogenic potencies of DES, glycitein, and genistein were estimated based on the doses required to produce the same increase of uterine weights. The relative

estrogenic potencies of genistein and DES from the present study were similar to the results of Farmakalidis and Murphy (1985), where the relative molar potencies of DES, genistein, genistin and daidzin were 100,000, 1, 1 and 0.26, respectively. The present study demonstrated that the estrogenic potency of glycitein in mice uterine enlargement assay was three times higher than that of genistein, but, as hypothesized, was much lower than that of DES.

It is generally assumed that nonsteroidal estrogens exert their stimulatory effect on the estrogen receptor by binding to the same site as that occupied by steroidal estrogens such as 17β-estradiol. Our competitive binding study results are shown in figure 2 where increasing concentrations of unlabeled competitors displace bound (^{3}H) estradiol. These results confirm that glycitein has the ability to bind to the estrogen receptor. DES had much higher binding affinity compared with that of the three isoflavones. The relative affinities of these compounds for the mice estrogen receptor were calculated by dividing the CB_{50} of unlabeled 17β-estradiol by the CB_{50} of competitor and then multiplying by 100 (table 2). These data reconfirmed that both DES and estradiol had much greater binding affinity to estrogen receptor than the three isoflavones. Among the three isoflavones, genistein had the greatest estrogen receptor binding affinity. The *in vitro* estrogen receptor binding data in this study was comparable to the previous results summarized by Verdeal et al. (1980). They reported that genistein had binding affinity roughly 100 times lower than 17β-estradiol, and daidzein had binding affinity 10 to 15 times lower than genistein.

The plasma extraction and analysis recovery was 85% for internal standard THB. The recovery-corrected plasma isoflavone concentration for genistein-treated and glycitein-treated group pooled sample was 0.42 μM and 0.72 μM, respectively. Xu et al. (1994) and King et al. (1995) have reported in humans and in rats that 4 to 6 hours after isoflavone dosing, daidzein and genistein reached maximal concentrations in plasma, then decreased thereafter. At 24 hours after dosing, there were only trace concentrations of daidzein and genistein in the plasmas. Since we collected blood 24 hours after the last dose of isoflavones, the low plasma isoflavone concentrations may be the result of rapid excretion of isoflavones after dosing.

Glycitein gave a much lower *in vitro* binding affinity compared to genistein in the estrogen receptor-binding assay. However, it gave higher estrogenic response in the *in vivo* mice uterine enlargement assay. This could be explained by the probable higher bioavailability of glycitein compared to genistein in mice. In a human isoflavone metabolic study, a higher bioavailability of glycitein than genistein was demonstrated (Zhang, Hendrich and Murphy, unpublished data). This may be the case in mice as well. Another possible explanation is that genistein may be metabolized to certain compounds that have lower or no estrogenic activity. Shutt et al (1970) reported that genistein could be metabolized to p-ethylphenol, which is not an estrogenic compound. It is possible that glycitein may be metabolized to certain compounds with greater estrogenic potency than the parent compound.

Daidzein has been reported to be a less effective estrogen than genistein when fed to mice (Bickoff et al. 1962). Our present study demonstrated that glycitein is a stronger

estrogen in mice uterine enlargement assay compared to genistein. Of the three soy isoflavone aglycones, it appears that glycitein has the highest estrogenic potency in the *in vivo* estrogen assay, although it has relatively lower binding affinity to estrogen receptor than genistein. The *in vitro* binding affinity to estrogen receptor is not the only factor determining the *in vivo* potency of the estrogen. There are other factors, such as absorption and metabolism, which would also be important.

This study has demonstrated the *in vivo* estrogenic activity and the *in vitro* binding affinity to estrogen receptors of glycitein. These data reveal that, although glycitein accounts for about 10% of the total isoflavones in soy foods, its biological potency should not be neglected. Development of soybean and soy food isoflavone databases cannot ignore glycitein and its glucosides. Additionally, the bioavailability, metabolism and other health protective aspects of glycitein and its glucosides need to be explored.

Acknowledgments

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Table 1. Estrogenic activities of soy isoflavones in mice

Treatment			Uterine weight (mg)	Body weight (g)		Relative Potency[a]
Compound	Total dose (mg)	No. of mice	Mean ± SEM	Initial	Final	
Control	0	20	17.7± 0.9[c]	8.1	11.6	------
DES	$0.12x10^{-3}$	20	28.5± 1.1[b]	8.1	11.7	100,000
Glycitein	12	20	44.6± 3.1[a]	8.3	12.1	3
Genistein	12	20	26.4±1.0[b]	8.2	11.7	1

Uterine weight means bearing different letters are significantly different, $p<0.05$.

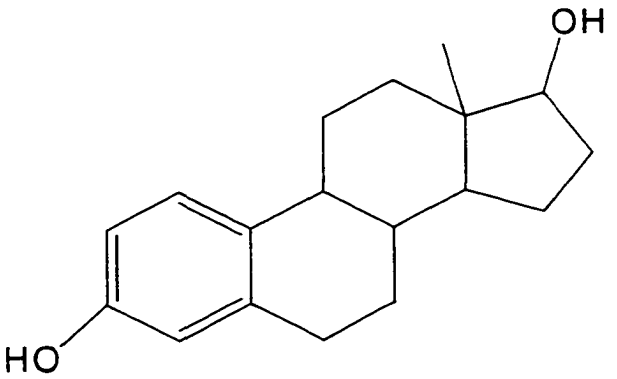
a. The relative potency was calculated based on the doses of estrogens required to produce a 10-mg increase of uterine weight.

Table 2. Relative affinities of estrogens for estrogen receptors [a]

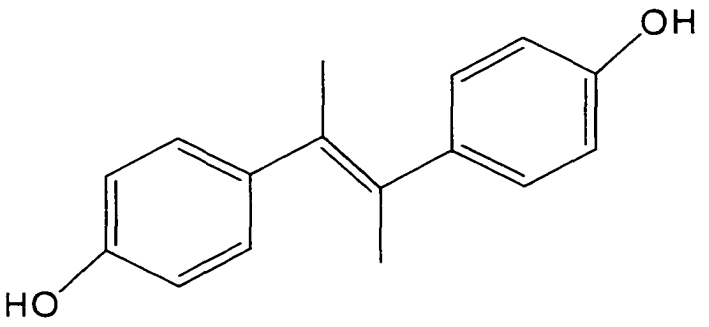
Compounds	CB_{50} (μM)	Relative affinity
17β-estradiol	0.00109	100
DES	0.00115	95
Genistein	0.223	0.49
Daidzein	4.00	0.027
Glycitein	3.94	0.028

[a]. Based on the molar concentrations (CB_{50}) required to displace 50% of the (^3H) estradiol. n=2.

Estrodiol



Diethylstilbestrol



Soy isoflavones	R1	R2
Daidzein	H	H
Genistein	H	OH
Glycitein	OCH3	H

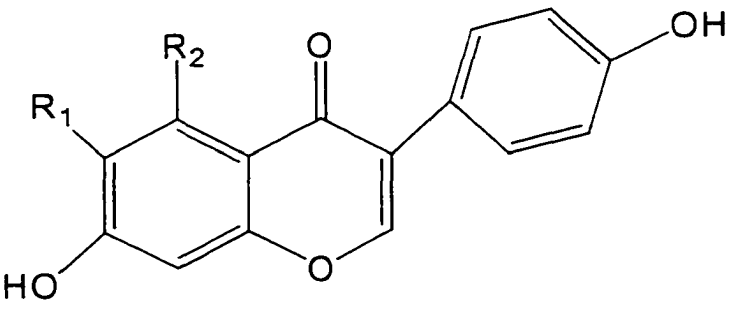


Figure 1. Chemical structures of soy isoflavones, 17β-estradiol and diethystilbestrol.

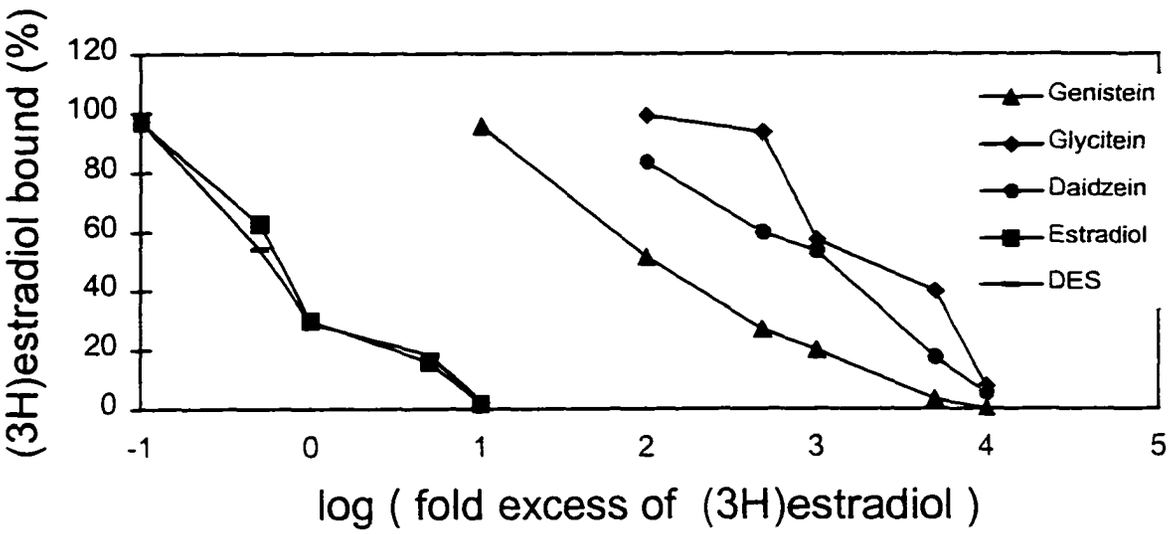


Figure 2. Competitive binding analysis of isoflavones. Cytosol from mice uteri were incubated with 1.5 ng/ml 17β-(^{3}H)estradiol in the presence of increasing concentrations of unlabeled estrogens. n=2.

SOY PROTEIN, WITH OR WITHOUT ISOFLAVONES, AND DAIDZEIN LOWER PLASMA CHOLESTEROL LEVELS IN GOLDEN SYRIAN HAMSTERS

A paper to be submitted to the Journal of Nutrition

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Abstract

The plasma cholesterol lowering effects of dietary isolated soy protein (ISP), isoflavone-depleted ISP (ISP (-)), daidzein or soygerm were investigated using Golden Syrian hamsters. Sixty males and 60 females were randomly assigned to six different groups (n=10) and fed for 10 weeks. The six experimental diets were casein control, ISP, ISP(-), daidzein, soygerm or soygerm extract to give a total isoflavone content of 1.3 mmol/kg diet (except for casein and ISP(-) groups). ISP (-) diet had 0.013 mmol isoflavone / kg diet. All hamsters fed ISP, ISP (-), daidzein, soygerm and soygerm extract had significantly lower plasma total cholesterol (16% to 28% lower), LDL cholesterol (15 to 50% lower) and LDL/HDL ratios compared with the control group ($p<0.01$). For male hamsters, there were no differences among treatments in plasma HDL concentrations. For females, ISP and ISP (-) groups had significantly higher HDL levels (20 to 30% higher) ($p<0.01$) than the casein-based treatments. There were no differences in triglyceride levels among treatments for both genders. These data suggested that soy protein, with or without isoflavones, the soy isoflavone daidzein and soygerm have cholesterol-lowering effects in hamsters.

Key words: Isoflavones, daidzein, soygerm, soy protein hamsters, cholesterol, cholesterol-lowering effects.

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Introduction

An elevated plasma level of LDL-cholesterol represents a major risk factor for the development of atherosclerosis (Newman *et al*, 1986). While dietary intake of cholesterol and saturated fat raise the plasma LDL-cholesterol levels, there are a number of dietary factors, such as dietary fiber or soy protein, that can counteract these effects. Consumption of soy protein, in place of animal protein, has been reported to decrease serum total and LDL-cholesterol concentrations in humans (Mercer *et al.*, 1987; Gooderham *et al.*, 1996 and Bakhit *et al.*, 1994) and in animals including rats, hamsters, guinea pigs, monkeys and baboons (Terpstra *et al.*, 1991; Potter *et al.*, 1996; Campbell *et al.* 1995)). A meta-analysis of the effects of soy protein intake on serum lipid levels (Anderson *et al* 1995) showed a significant relationship between soy intake and lower levels of total and LDL- cholesterol and lower risk of coronary heart disease, one of the leading causes of death in the United States. However, the components of soy responsible for these cholesterol lowering effects have not been determined.

The amino acid composition of soy protein was investigated for its effects on plasma lipids. Huff *et al* (1977) reported that soy protein or an enzymatic hydrolysate of soy protein compared with casein lowered plasma total cholesterol and LDL-cholesterol levels in rabbits. Soybean protein amino acid reconstitutions were not as effective in lowering plasma cholesterol as the intact protein. Casein amino acid reconstitution induced a similar effect on plasma cholesterol levels as the intact casein (Huff and Carroll, 1980). Several reports have suggested that other non-protein components in soy, including soy isoflavones, soy saponins, dietary fiber and soy phenolic acids may be the possible factors for soy protein's hypocholesterolemic effect (Carroll, 1991; Setchell, 1985; Anthony *et al.*, 1995).

Setchell (1985) suggests that isoflavones, the soy phytoestrogens, may contribute to the cholesterol-lowering effect. Soybeans and soy foods are the major source of isoflavones in human diets, as daidzein, genistein, glycitein and their glycoside forms, at concentrations from 0.25 to 3 mg/g (Wang and Murphy, 1994). Soy isoflavones have weak estrogenic activities (Bickoff *et al.*, 1962; Farmakalidis and Murphy, 1985; Verdeal *et al.*, 1980; Song *et al.*, 1998). Soy isoflavones may have effects similar to other estrogens. The administration of oral estrogens or the synthetic anti-estrogen, tamoxifen, decreased total serum cholesterol and LDL-cholesterol levels in postmenopausal women (Walsh *et al.*, 1991; Love *et al.*, 1991). Lovati *et al* (1991) reported that an ethanol extract from soy protein reduced serum LDL-cholesterol levels and increased LDL receptor activity in mice. The ethanol extract contained soy isoflavones and soy saponins. Anthony *et al.* (1995) tried to address the question of whether isoflavones are responsible for the cholesterol-lowering effects of soy protein. They showed that an isoflavone-rich soy protein diet significantly lowered the total (15% to 20%) and LDL (30% to 40%) cholesterol levels in both male and female colony-born Rhesus monkeys when compared with ethanol-extracted soy protein from which more than 90% of the isoflavones had been extracted. Recent studies by Balmir *et al.* (1996) showed that soy isoflavone extracts, which contained 50-60% isoflavones, when added to diets with casein as protein source, significantly lowered the total (10% to 20%) and LDL (20 to 30%) cholesterol levels in rats and hamsters compared with the casein control group. These data are consent with the hypothesis that the observed hypocholesterolemic effect of soy protein is due to the soy isoflavones. However, none of these studies used purified isoflavones. The ethanol-acetone extracts from soy proteins contain, in addition to isoflavones, other ethanol-acetone soluble compounds including soy saponins and soy

phenolic acids. Soy saponins are a group of phytochemicals at concentrations comparable to isoflavones and have been associated with a hypocholesterolemic effect (Potter *et al.*, 1993).

We hypothesize that soy isoflavones are the major hypocholesterolemic factor in soy. To test this hypothesis, we fed purified isoflavones to hamsters. Golden Syrian hamsters were selected because there are several general similarities in cholesterol metabolism between hamsters and humans, including a low basal rate of hepatic cholesterol synthesis and a comparable bile acid pool composition (Spady and Dietschy, 1985; Spady *et al* 1985; Bravo *et al.*, 1994). In addition, hamsters fed a diet enriched with cholesterol and saturated fat manifest substantial increase in their plasma total and LDL-cholesterol levels, thus, making these species a good model to assess cholesterol metabolism (Terpstra *et al.*, 1991).

Materials and methods

Chemicals and Diets. The isoflavone, daidzein, was chemically synthesized according to Song *et al.* (1998). The purity of daidzein, determined by HPLC chromatograph peak area percentage and Beckman Gold HPLC System peak purity software, was >98%. Soygerm was generously donated by Schouten USA, Inc. (Minneapolis, MN). Isolated soy protein (ISP) was purchased from Protein Technology International (St. Louis, MO). The soygerm extract was prepared by using ethanol and acetone extraction according to Balmir *et al.* (1996). The isoflavone- free isolated soy protein (ISP (-)) was prepared by extracting ISP with 70% ethanol at weight to solvent ratio of 1 g ISP: 4 ml of aqueous ethanol four times. The isoflavone contents in ISP, soygerm, soygerm extract and ISP (-) were analyzed by using HPLC method (Murphy *et al.*, 1997). The molar concentrations of daidzein and total isoflavones were presented in Table 1.

The control diet for hamsters was based on Terpstra et al (1991). There were six different diet treatments: casein control, ISP, ISP (-), daidzein, soygerm or soygerm extract. Daidzein, soygerm and soygerm extract diets were casein-based. The diets were formulated to contain the amount of daidzein, soygerm, or soygerm extract, which would equal the total molar isoflavone contents in ISP, 1.3 mmol/kg diet. Experimental diet composition is shown in Table 2. Cholesterol at 0.1% level was formulated into the diet. Rice flour was used as carbohydrate source to replace cornstarch in normal rodent diet because rice flour essentially eliminates "wet tail" disease, a form of chronic diarrhea and accompanying high rate of mortality for hamsters (Terpstra *et al.*, 1991).

Animals. The animal experimental protocol was approved by Iowa State University Animal Use Committee.

Sixty male and 60 female Golden Syrian hamsters, 6-8 week old, weighing between 100 and 110 g were obtained from Charles River Breeding Laboratories (St. Constant, Canada). They were housed individually in a temperature-controlled room (23°C) with a 12-h light: dark cycle. Males and females were kept in separate rooms. Upon arrival, hamsters were fed a powdered AIN 93 M diet for one week to acclimate animals to the facility and powdered diets. Hamsters were randomly assigned to one of the six diet treatments with10 male and 10 female animals per treatment. Hamsters had free access to food and water during the 10-week experimental period. At the end of the feeding period, diets were withdrawn from hamsters 14-16 hrs before they were sacrificed under CO_2. Blood was collected by using cardiac puncture. Plasmas were prepared by centrifugation at 5000 g for 10 min at 4°C and kept frozen at -20°C until analysis.

Plasma lipid analysis: Plasma total cholesterol, HDL cholesterol and triglyceride (TAG) concentrations were measured by using Sigma diagnostics kits (St Louis, MO). LDL cholesterol was calculated by subtraction of HDL cholesterol from total cholesterol and represented LDL+IDL+VLDL cholesterol.

Plasma isoflavone analysis: Plasma samples were combined within each group to give two pooled samples for each treatment. Two ml of 0.2 M acetate buffer (pH 5.5), 100 μl of β-glucouronidase/sulfatase and 10 μl of 4 mg/ml internal standard 2,4,4'-trihydroxydeoxybenzoin (THB) (Song *et al.*, 1998) were added to 2 ml of plasma sample. The mixture was incubated at 37°C for 16 hours. Six ml of methanol was added to the mixture, which was mixed well and centrifuged at 10,000g for 20 min. Eight ml of supernatant was brought to dryness and redissolved in 400 μl of 80% methanol. After centrifugation, 20 μl of sample was taken for isoflavone analysis. A Beckman System Gold chromatograph with a Model 507 autosampler, a Model 126 dual pump, a Model 168 photodiode array detector, and an IBM 486 computer using Beckman System Gold HPLC data processing software (version 8, 1993) was used. A YMC-pack ODS-AM-303 analytical column (5 μm, 25 cm x 4.6 mm) was used for the chromatographic separation. A linear gradient was composed of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile). After injection of 20 μl sample, the system was maintained at 15% B for 5 min, then increased to 29% in 31 min, and then to 35% in 8 min. The system was recycled to 15% at the end of 45 min. The flow rate was 1.0 ml/min for the first 5 min, then increased to 1.5 ml/min for the next 40 min, and returned to 1 ml/min for recycle. The minimal detection level for daidzein, genistein and glycitein was 1.6 μM in the injection

solution by UV detector at 254 nm. The recovery of the extraction and analysis based on the internal standard THB was 85%, and the recovery-adjusted plasma isoflavone concentrations were reported.

Statistical analysis: All data were analyzed by using two-factor ANOVA (SAS version 6.03, SAS Institute, Cary, NC). Since there were no interactions between gender and treatments, all analyses were redone separately for each gender by one-way ANOVA. Differences between treatments were determined by the least significant difference test. A α of 0.05 was used to indicate statistically significant differences.

Results

The powdered diets were well accepted by hamsters throughout the experiment. Food intakes were 7.3± 0.4 g/day and 7.6 ±0.4 g /day for males and females, respectively. Final body weights were 142±10 g and 154±10 g for male and female hamsters, respectively. The weight gain was 40±3 g for males and 52±4 g for females. There were no final weight or food intake differences among dietary treatment groups for females. For males, there were no food intake differences, however, the casein+daidzein diet treatment had 10% lower final body weight ($p<0.05$) compared with other treatment groups.

Two-way ANOVA showed that there no interactions between diets and genders on total cholesterol, HDL cholesterol, LDL cholesterol and TAG levels. Therefore, we analyzed the results for males and females separately and the results were summarized in Table 3 for male hamsters and in Table 4 for female hamsters. Total cholesterol, LDL-cholesterol levels and LDL/HDL ratios were significantly lower ($p<0.01$) in the ISP, ISP (-), daidzein, soygerm, and soygerm extract diet treatment groups compared with the casein control diet in

both males and females. There were no differences among treatments in HDL cholesterol levels in males. However, ISP and ISP (-) groups had 30% higher HDL cholesterol levels ($p<0.01$) compared with the casein-fed hamsters in females. There were no differences in TAG levels among treatments for both genders.

Plasma isoflavone levels: The plasma isoflavone levels (μM) were summarized in Table 5. Daidzein, genistein, glycitein and equol, the metabolite of daidzein, were detected in most of the male hamster plasma samples but only in a few of the female samples, ranging from 0.4 to 3.6 μM.

Discussion

In a preliminary experiment, we used pure chemically synthesized isoflavones, daidzein and genistein, to examine their possible cholesterol-lowering effects in male C57BL/6 mice. A 0.1% cholesterol-enriched, 20% fat, casein-based diet was used. The isoflavone levels in the diets were: 0.6 mmole or 1.2 mmole daidzein/kg diet, 0.6 mmole or 1.2 mmole genistein/kg diet, or 0.6 mmole daidzein +0.6 mmole genistein/kg diet. This preliminary data showed that either daidzein or genistein at 1.2 mmole/kg diet, or 0.6 mmole daidzein + 0.6 mmole genistein/kg diet, appeared to lower total plasma cholesterol levels by 8 to 10%, but were not different from the control ($p>0.1$). The LDL-cholesterol levels were significantly reduced by the 1.2 mmole daidzein /kg diet and the 0.6 mmole daidzein + 0.6 mmole genistein /kg diet ($p<0.01$). There were no significant differences among dietary groups in HDL cholesterol and TAG levels. These preliminary data suggested the possible activity of daidzein in lowering plasma cholesterol levels, especially LDL cholesterol.

The hamster has been identified as the rodent species most closely related to humans

in lipoprotein metabolism (Spady and Dietschy, 1985; Bravo *et al* (1994) and has been suggested as a better animal model to study cholesterol metabolism than the mouse or rat. Terpstra et al. (1991) reported that the LDL fraction of the lipoproteins in hamsters fed 0.1% cholesterol-enriched diet was around 40-50% of the total cholesterol. However, in the mouse or rat, the LDL portion only represented 20-30% of the total cholesterol (Balmir et al., 1996). The data from our preliminary study suggested that the decrease in total cholesterol levels by the daidzein dietary treatment in mice was due principally to the decrease in the LDL portion. In order to confirm this hypothesis that isoflavone daidzein can decrease total cholesterol, and especially, the LDL portion, it may be better to use hamsters as the model. In the present study, we chose hamsters as the animal model to examine the cholesterol-lowering effect of daidzein.

The data from the study reported here suggested that the dietary protein source and the dietary soy isoflavones influenced the plasma cholesterol levels in hamsters. Daidzein, in a casein-based diet, significantly reduced total cholesterol, LDL-cholesterol levels and the LDL/HDL ratios. This study confirmed our hypothesis that the soy isoflavone, daidzein, was a major factor contributing to soy protein's cholesterol-lowering effects.

Decreases in plasma total and LDL-cholesterol levels were observed in hamsters fed isoflavone-intact soy protein compared with the casein control group. However, the removal of isoflavones from soy protein gave the similar plasma cholesterol lowering effects. These results were similar to that of Balmir *et al.* (1996) where soy protein, with or without soy isoflavones, had the ability to lower plasma cholesterol levels in rats. These data suggested that, in addition to isoflavones, there were other factors in soy protein capable of producing cholesterol-lowering effects. Our finding of the similar cholesterol lowering effects of ISP

and ISP (-) suggested that the cholesterol-lowering effects can not be attributed to isoflavones alone. Additionally, the cholesterol lowering effects of soy protein or soy isoflavones appeared to reach a plateau. This plateau was observed by Tovar-Palacio *et al* (1998) where they reported that soy protein lowered plasma cholesterol levels in the gerbil compared with the casein control. But when isoflavone extracts were added to the soy protein diet at three different doses of 8.0, 13.8 or 23.8 μmol isoflavone / g protein, they did not reduce cholesterol levels further than soy protein alone. However, these diets significantly reduced cholesterol levels compared with the casein control group. They suggested that the cholesterol-lowering effects might have reached a plateau or maximum. In the current study, both the soy protein level and isoflavone level may have caused plasma cholesterol lowering to reach a plateau level. There was no additive effect for dietary soy protein and soy isoflavones. If lower soy protein levels or lower isoflavone levels were used, the additive effects may be revealed

Soygerm, the hypocotyl portion of the soybean seed, is a naturally concentrated soy isoflavone product. Soygerm has a unique isoflavone profile, with daidzein accounting for 50% of the total isoflavones, glycitein for 35%, and genistein only for about 15%. This profile is very different from intact soybeans, where genistein accounts for 50% of the total isoflavones and glycitein for only 5 to 10%. The total isoflavone concentration in soygerm is 20-30 mg/g, much higher than soybeans or typical soy foods at 0.25 to 3 mg/g. Our study demonstrated the same magnitude hypocholesterolemic effect of soygerm and soygerm extract as for ISP, ISP (-) or daidzein.

Dietary soy protein has been reported to enhance bile acid excretion compared with casein in rabbits and rats (Potter, 1996). The hepatic cholesterol metabolism shifts to provide

cholesterol for enhanced bile acid synthesis and the rate of cholesterol removal from blood was increased, resulting in plasma cholesterol level reduction. Nagata *et al* (1982) reported increases in total, neutral and acidic fecal bile acids and decreases in plasma total and LDL-cholesterol levels in rats fed soy protein.

The cholesterol lowering effects observed in the present study were mainly due to the decrease in the LDL-cholesterol. The steady state plasma LDL-cholesterol concentrations have been determined by four separate variables: the maximal rate of receptor-mediated uptake of LDL-cholesterol by the tissues, the rate of LDL-cholesterol production, the affinity of the LDL molecule for its receptor and the rate of LDL-cholesterol uptake through an LDL-receptor independent process (Meddings and Dietschy, 1987). Soy protein and soy isoflavones may influence the plasma LDL-cholesterol levels through any one of these factors. Lovati *et al* (1998) reported that β-conglycinin (7S globulin) from soy has the ability to up-regulate LDL receptor activity in human hepatoma cell line culture. Their results suggested that soy protein might increase the clearance of LDL-cholesterol by increasing the activity of LDL receptor. Kirk *et al* (1998) reported that dietary soy protein with isoflavones (1.33 mmol/kg diet) reduced plasma LDL-cholesterol levels (30%) in C57BL/6 mice compared with the isoflavone-depleted soy protein. In the same study, they found no cholesterol lowering effects of isoflavone-containing soy protein in the LDL receptor-deficient mice. This data suggested that the influence of isoflavones on LDL-cholesterol levels were not mediated through the LDL-receptor independent pathway. Instead, soy isoflavones might lower LDL-cholesterol by increasing the activity of LDL receptor, and thus, the clearance of LDL-cholesterol. The possible effects of isoflavones on LDL-cholesterol production, which usually occurs with the intake of diet enriched with saturated

fat and cholesterol, should be investigated. Additionally, how isoflavones may affect the LDL receptor activity is very important in elucidating the mechanisms of isoflavone's cholesterol-lowering effects.

We detected all three isoflavones and equol in the plasma samples, ranging from 0.4 to 3.6 μM, which were similar to the levels detected in human plasma (Xu *et al.*, 1994) and rat plasma (King, *et al.*, 1995). We found both daidzein and its metabolite-equol in plasma of daidzein-treated groups, which suggested that hamsters had the ability to metabolize daidzein to equol. While isoflavones were detected in most of the male hamster plasma samples, only a few females had detectable plasma isoflavone levels. This suggested that there might be a gender difference in isoflavone absorption and metabolism in hamsters.

The plasma cholesterol-lowering activity for the other isoflavones, genistein and glycitein, as well as the combinations of these three isoflavones, needs to be examined. The possible cholesterol lowering effects of soy isoflavones suggests there is potential to use these soy components as dietary cholesterol lowering agents.

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Table 1. Isoflavone concentrations[3] in different diet components.

Components	Daidzein (μmol / g)	Genistein (μmol / g)	Glycitein (μmol / g)	Isoflavone content [1] (μmol / g)
ISP (Supro-670 [2])	1.9	2.9	0.4	5.2
Soygerm	36.3	9.9	25.6	71.8
Soygerm extract	336.2	82.2	310.4	728.8
ISP (-)	0.015	0.02	0.015	0.05

1. The total mole concentration of isoflavone was calculated as the sum of mole concentrations of daidzein, genistein and glycitein.
2. Isolated soy protein Supro-670 was purchased from Protein Technologies International (St. Louis, MO).
3. Minimum detection level was 1 μmole /g sample.

Table 2. Composition of experimental diets (g / kg)

Ingredient	Casein	ISP	ISP (-)	Daidzein	Soygerm	Soygerm extract
Casein[1]	250	0	0	250	250	250
ISP	0	250	250	0	0	0
Coconut oil	100	100	100	100	100	100
Safflower oil	20	20	20	20	20	20
Soybean oil	40	40	40	40	40	40
Cellulose	75	75	75	75	75	75
Wheat bran	75	75	75	75	75	75
Choline chloride	3	3	3	3	3	3
Vitamin mix [2]	10	10	10	10	10	10
Mineral mix [3]	35	35	35	35	35	35
Potassium bicarbonate	20	20	20	20	20	20
Cholesterol	1	1	1	1	1	1
Rice flour[4]	371	371	371	370.67	353	369.22
Daidzein	0	0	0	0.33	0	0
Soygerm	0	0	0	0	18	0
Soygerm extract	0	0	0	0	0	1.78

1. Vita-free casein (Harlan/Teklad, Madison, WI)
2. Vitamin mixture #400160 (Harlan/Teklad, Madison, WI)
3. Mineral mixture #170910 (Harlan/Teklad, Madison, WI)
4. Rice flour (Bioserve, Frenchtown, NJ).

Table 3. Plasma cholesterol levels (mmole/L) in male hamsters[1,2] fed casein- or ISP-based diets with or without soy isoflavones

	Casein	ISP	ISP (-)	Dadizein	Soy germ	Germ extract
Total	6.22±0.33[a]	5.18±0.30[b]	4.93±0.29[b]	4.74±0.31[b]	4.83±0.31[b]	4.77±0.24[b]
HDL	3.01±0.18[a]	2.71±0.20[ab]	2.79±0.15[ab]	2.50±0.16[b]	2.81±0.17[ab]	2.55±0.12[ab]
LDL[3]	3.21±0.23[a]	2.47±0.16[b]	2.14±0.13[b]	2.24±0.17[b]	2.02±0.17[b]	2.22±0.17[b]
LDL/HDL	1.10±0.11[a]	0.95±0.06[ab]	0.77±0.0.02[bc]	0.87±0.03[bc]	0.72±0.03[c]	0.88±0.06[bc]
Triglyceride	2.88±0.23[a]	2.50±0.18[a]	2.44±0.28[a]	2.67±0.17[a]	2.74±0.18[a]	2.56±0.22[a]

1. Values represent means ± SEM, n=10;
2. Within a row, means with different superscripts are different (P<0.05);
3. Represents the VLDL+IDL+LDL fractions (by difference: Total - HDL).

Table 4. Plasma cholesterol level (mmole/L) in female hamsters[1,2] fed Casein- or ISP-based diets with or without soy isoflavones

	Casein	ISP	ISP (-)	Daidzein	Soy germ	Germ extract
Total	5.61±0.27^{a}	4.73±0.31^{bc}	4.91±0.35^{ab}	4.37±0.25^{bc}	4.08±0.30^{c}	4.28±0.23^{bc}
HDL	2.10±0.09^{b}	2.70±0.21^{ab}	2.82±0.35^{a}	2.18±0.10^{b}	2.32±0.25^{ab}	2.13±0.16^{ab}
LDL[3]	3.51±0.28^{a}	2.03±0.21^{b}	2.09±0.25^{b}	2.19±0.11^{b}	1.77±0.21^{b}	2.16±0.11^{b}
LDL/HDL	1.72±0.18^{a}	0.81±0.11^{b}	0.84±0.11^{b}	1.01±0.04^{b}	0.83±0.11^{b}	1.05±0.08^{b}
TAG	1.53±0.18^{a}	1.12±0.12^{ab}	1.05±0.17^{b}	1.26±0.14^{ab}	1.25±0.18^{ab}	1.10±0.11^{ab}

1. Values represent means ± SEM, n=10;
2. Within a row, means with different superscripts are different ($P<0.05$);
3. Represents the VLDL+IDL+LDL fractions (by differences: Total - HDL).

Table 5. Plasma isoflavone concentrations in hamsters (μM)[1,2]

Male	ISP	ISP (-)	Daidzein	Soygerm	Germ Extract
Daidzein	1.4	-	0.4	2.3	-
Glycitein	1.2	-	-	1.7	-
Genistein	0.8	-	-	0.8	3.6
Equol	-	-	1.6	0.5	-
Total	3.4	-	2.0	5.3	3.6

Female	ISP	ISP (-)	Daidzein	Soygerm	Germ Extract
Daidzein	-	-	0.6	-	-
Glycitein	-	-	-	-	-
Genistein	-	-	-	-	3.6
Equol	-	-	4.0	-	-
Total	-	-	4.6	-	3.6

1. = not detected.

2. Minimal detection level: 0.4 μM in plasma sample.

GENERAL CONCLUSIONS

Soy isoflavones have attracted attention recently because these compounds have been associated with certain health beneficial effects such as prevention of cancer, lowering blood cholesterol levels and reduction of postmenopausal bone loss. Isoflavones have some important biological activities, including estrogenic, antiestrogenic, antioxidant and protein tyrosine kinase-inhibiting activities.

Because of these important beneficial activities, the consumption of isoflavone-containing foods is increasing dramatically. A good database is needed to provide public dietary information on soy isoflavones. There have been considerable efforts to quantify the isoflavone content in various food products. To perform an accurate and precise analysis, a good internal standard is very critical. However, to date, there has not been a good internal standard for soy isoflavone analysis. 2,4,4'-Trihydroxydeoxybenzoin (THB), the new internal standard we discovered for isoflavone analysis, has been demonstrated to be an excellent internal standard in the analysis. THB gives us the opportunity to do a better quality control job in isoflavone analysis and will be widely used in future isoflavone analysis, both in food and biological samples. A soy isoflavone database in the most widely used soy foods in U.S. has already been developed in our lab and will be available to the public in the near future.

The estrogenic activity of daidzein and genistein had attracted great attention and may play significant roles in isoflavone's health beneficial effects. However, for the third isoflavone is soy, glycitein, no biological activities have been reported. Our study demonstrated that glycitein had estrogenic activity 3 times higher than genistein in the *in vivo*

mice uterine enlargement assay, but much lower than DES. Glycitein has the ability to bind to estrogen receptor in the *in vitro* estrogen receptor-binding assay. In addition to the demonstrated estrogenic activity of daidzein and genistein, we now have a more complete picture of all soy isoflavones' estrogenic activity. These estrogen-like activities may be very important in isoflavones' health protective activities. Although glycitein only accounts for 5 to 10% of the total isoflavones in soybeans, its biological activity and possible health enhancing activities should not be neglected. It will be interesting to test the antioxidant activity of glycitein, as well as its ability to inhibit the protein tyrosine kinase activity. In addition, the effects of glycitein on the growth cancer cells need to be investigated.

Soy protein's hypocholesterolemic effects have been reported in animal models as well as in human subjects. The factors that are responsible for this cholesterol lowering effects are still under investigation. Soy protein itself, soy saponins, soy phenolic acids, as well as soy isoflavones, have been proposed to be hypocholesterolemic. The hypocholesterolemic activity of daidzein proved in our study is the first direct evidence showing the relationship between soy isoflavones and soy's cholesterol lowering effects. The ability of isoflavone-free soy protein to lower plasma cholesterol in hamsters suggested that there were more than one active factor that are responsible for soy protein's reported hypocholesterolemic effects. Future studies need to be done to elucidate the mechanisms of isoflavone's cholesterol lowering effects. It will be very interesting to test the cholesterol lowering effects of genistein, glycitein or different combinations of isoflavones. How these isoflavones interact with the important steps in cholesterol metabolisms, including cholesterol synthesis and clearance, will be another interesting issue to look at.

The evidence to support isoflavone's beneficial effects is accumulating. People are more aware of these compounds in soy and the consumption of isoflavone-containing foods in rising. On important issue to be addressed is the possible side or toxic effects of these chemicals. Few studies have been performed to investigate the toxicity of soy isoflavones. Because of their estrogen-like activity, it is very important to investigate the possibilities of isoflavones to disturb the balance of human endocrine system. A thorough toxicology study on isoflavones, combine with a good isoflavone database in foods, will help us to establish a safe guideline for the intake of these phytochemicals.

In order to fully understand the biological activities, the beneficial effects, as well as the possible toxic effects of isoflavones, a stable and inexpensive source of pure isoflavones is needed. Chemical synthesis of isoflavones is a practical way to obtain large amount of pure isoflavones. Chang et al. (1994) reported a simple procedure to synthesize genistein. Our modified method to chemically synthesize daidzein provides us a simple, fast method to produce large quantities of pure daidzein at relative low costs. However, no simple and high yield method to synthesize glycitein is available. Based on the synthesis of daidzein and genistein, it is very likely that a similar protocol may be developed. Cyclizing of the corresponding deoxybenzoin of glycitein to obtain pure glycitein seems promising and needs future investigation.

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IMAGE EVALUATION
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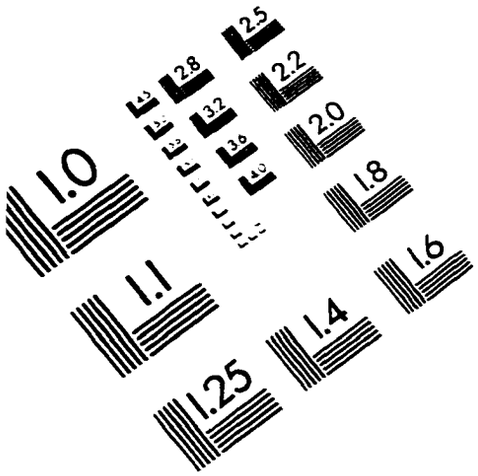

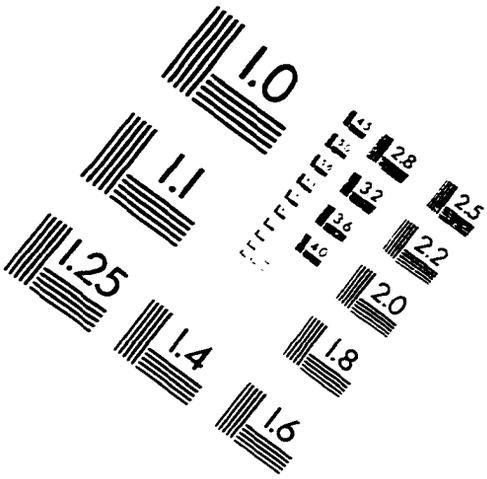

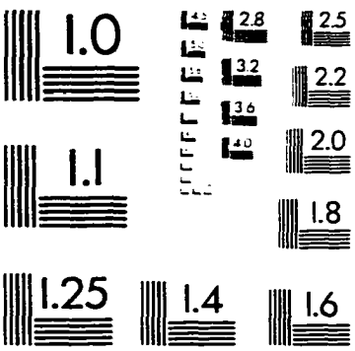

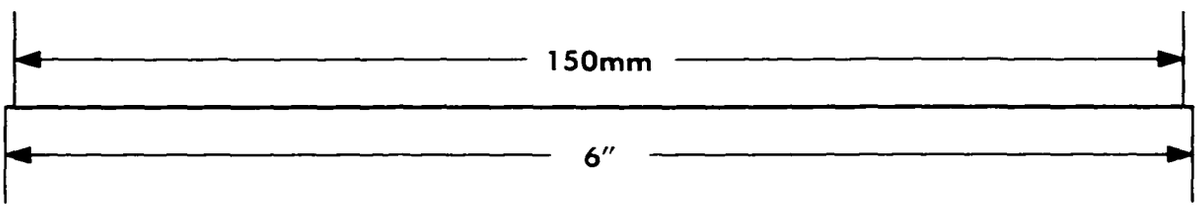

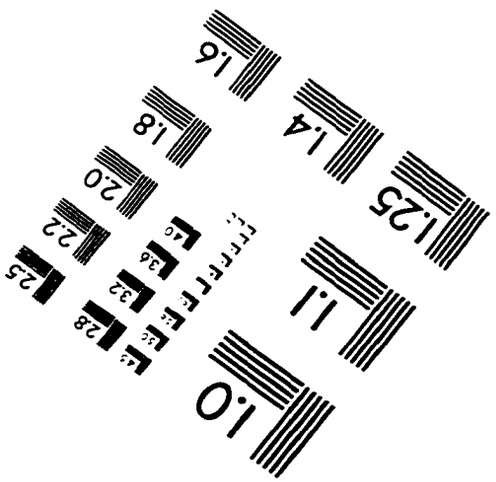

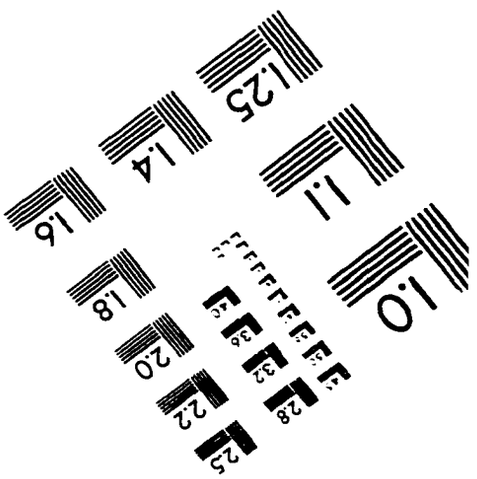

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