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Effects of [beta]-conglycinin, soy isoflavones, and group B soyasaponins on plasma lipid concentrations

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Effects of β -conglycinin, soy isoflavones, and group B soyasaponins
on plasma lipid concentrations

by

Sun-Ok Lee

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Nutrition

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For the Major Program

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ABSTRACT

Consumption of soy and soy products is associated with reduction in risk factors for heart disease, especially lowering plasma concentrations of lipoproteins and cholesterol. It is important to determine which soy protein-associated components including β -conglycinin, isoflavones, or group B soyasaponins, improve plasma lipid concentrations for decreasing cardiovascular disease risk.

The effects on plasma lipid concentrations of β -conglycinin, one of the two major storage protein in soybeans, containing high and low levels of isoflavones (213 μmol v. 22 $\mu\text{mol/person/day}$) and soyasaponins (276 μmol v. 23 $\mu\text{mol/person/day}$) and the mechanism of β -conglycinin to affect plasma lipid levels were investigated in mildly hypercholesterolemic women. A significant reduction of plasma total and LDL cholesterol occurred after 14 and 28 days during ingestion of β -conglycinin but only when it contained high levels of isoflavones and soyasaponins. A slight but nonsignificant increase in excretion of fecal bile acids and neutral sterols may have contributed to these changes. This study suggests that isoflavones and soyasaponins may be needed companions for β -conglycinin to exert a cholesterol lowering effect.

The beneficial effects of daidzein, genistein, or glycitein (0.9 mmol/kg diet, purity > 97%) on plasma lipid concentrations were investigated in female Golden Syrian hamsters fed these compounds for 4 weeks. Hamsters fed glycitein had significantly lower plasma total and non-HDL cholesterol levels by 15% and 24%, respectively, compared with those fed casein ($P < 0.05$). The percentage of urinary recovery of each isoflavone was glycitein > daidzein > genistein (32.2% > 4.6% > 2.2%, $P < 0.05$). These results suggest that glycitein's

greater cholesterol-lowering effect was due to greater bioavailability, as reflected in urinary recovery of glycitein compared with the other purified isoflavones.

An animal study was conducted to determine if group B soyasaponins affect plasma lipid concentrations by increasing excretion of fecal bile acids and neutral sterols, to investigate the relationship between group B soyasaponin metabolite and plasma lipid concentrations, and to identify group B soyasaponin metabolites. Compared with hamsters fed casein, hamsters fed group B soyasaponins significantly lower plasma total cholesterol (by 20%), non-HDL cholesterol (by 33%), and triglycerides (by 18%). Cholesterol-lowering was probably by a mechanism involving greater excretion of fecal bile acids and neutral sterols ($P < 0.05$). Two fecal soyasaponin metabolite excretion phenotypes (high v. low) were observed. The high producers of soyasaponin metabolite showed significantly lower total/HDL cholesterol ratio compared with the low producers (1.38 ± 0.7 v. 1.59 ± 0.13 ; $P < 0.03$). Greater production of group B soyasaponin metabolite in hamsters was associated with improved plasma lipid profile.

These findings suggest that both soy isoflavones and soyasaponins in nutritionally relevant concentrations contribute to the cholesterol-lowering effects of soy foods and soy protein ingredients, and might be useful as purified supplements for cholesterol-lowering as well.

GENERAL INTRODUCTION

A. Introduction

Atherosclerotic cardiovascular disease (CVD), principally coronary heart disease (CHD) and stroke, was the leading cause of death in the United States in 2001, accounting for more than 38% of all deaths (American Heart Association, 2004). Several clinical and experimental studies have shown the cardiovascular protective properties of soy components including soy protein itself, isoflavones, and soyasaponins (Anderson et al., 1995; Duranti et al., 2004; Song et al., 2003; Crouse et al., 1999; Fukui et al., 2002).

It is well-known that a soy protein based diet improves plasma lipid status of humans and animals. A meta-analysis showed a significant relationship between soy consumption and decreased plasma total and LDL cholesterol and reduced risk of coronary heart disease (Anderson et al., 1995). β -conglycinin, one of the two major storage protein in soybeans, lowered plasma total cholesterol and triglyceride concentrations in rats compared with casein (Aoyama et al., 2001; Duranti et al., 2004).

Considerable evidence suggests that alcohol-extractable components of soy protein are responsible for the hypocholesterolemic effects. Soy isoflavones are largely extracted from soy protein using alcohol. Isolated soy protein (25 g) with 62 mg isoflavones significantly reduced plasma total and LDL cholesterol concentrations in moderately hypercholesterolemic men and women (Crouse et al., 1999). Daidzein lowered plasma total and non-HDL cholesterol concentrations in hamsters compared with casein (Song et al., 2003). Soyasaponins are another alcohol extractable component of soy protein and have been considered to have a role in the hypocholesterolemic effect of soy. Isoflavone-depleted soy

protein isolate, but containing 49% of soyasaponins, lowered plasma total cholesterol in rats (Fukui et al., 2002). A diet containing 1% soyasaponins decreased hepatic and plasma total cholesterol in rats (Oakenfull et al., 1984).

Many previous studies used an isolated soy protein extract rich in isoflavones and soyasaponins (Clarkson et al., 2001; Song et al., 2003) or an alcohol-washed isolated soy protein deficient in isoflavones and soyasaponins (Song et al., 2003; Tovar-Palacio et al., 1998) to investigate the cholesterol-lowering effects of isoflavones. Also, the contents of soyasaponins were not determined in these studies. Therefore, it is necessary to investigate which soy components are responsible for its cholesterol-lowering effects.

B. Objectives of current research

A great deal of attention has been focused on the health benefits of using soy and soy products for their hypocholesterolemic and anti-atherosclerotic effects. The overall objective of my doctoral research was to determine which components of soy, including soy protein itself, isoflavones, or soyasaponins, improve plasma lipid concentrations, a key cardiovascular disease risk factor. Our central hypothesis states that the isoflavones and soyasaponins in association with β -conglycinin or isoflavones (daidzein, genistein, or glycitein) and soyasaponins, separate from β -conglycinin are responsible for the beneficial effects of soy on plasma lipid concentrations.

To test the hypothesis, the proposed aims of my research were: 1. to examine the effect of β -conglycinin containing low or high levels of isoflavones and soyasaponins on plasma lipid concentrations and to investigate the mechanism of soy protein that affect plasma cholesterol concentrations by monitoring the excretion of fecal bile acids and neutral

sterols in mildly hypercholesterolemic women; 2. to determine if the cholesterol-lowering effects are due to daidzein, genistein, or glycitein by measuring plasma lipid concentrations in female Golden Syrian hamsters fed these compounds and the association between the urinary recovery of each isoflavone and plasma lipid levels; 3. to determine if group B soyasaponins lower plasma lipid levels by a mechanism involving greater excretion of fecal bile acids and neutral sterols in female hamsters, to investigate the relationship between group B soyasaponin metabolite and plasma lipid concentrations, and to identify group B soyasaponin metabolites.

The information obtained in this study will help to clarify the bioactive components of soy protein responsible for the cholesterol-lowering effects and to understand the possible mechanisms of the hypocholesterolemic effects.

C. Dissertation organization

This dissertation consists of a literature review and three papers. The first paper, “Beta-conglycinin deficient in soy isoflavones and soyasaponins did not lower plasma concentrations of lipoproteins and cholesterol over time in mildly hypercholesterolemic women”, will be submitted to the Journal of Nutrition. The second paper, “The isoflavone glycitein lowered plasma cholesterol in female Golden Syrian hamsters”, will be submitted to Experimental Biology and Medicine. The third paper, “Group B soyasaponins lower plasma cholesterol and increase fecal bile acids in female Golden Syrian hamsters”, will be submitted to Experimental Biology and Medicine. The papers are written in the format of the journals to which they will be submitted. A general conclusion is included following the three papers.

LITERATURE REVIEW

A. Introduction to soy protein

It has been suggested that consumption of soy and soy products is associated with certain health-promoting benefits, including hypocholesterolemic activity (Teixeira et al., 2000; Bakhit et al., 1994), antitumor-promoting activity (Bennink et al., 2001; Wu et al., 1998), and reducing postmenopausal bone loss (Ho et al., 1993; Horiuchi et al., 2000).

Soybean, *Glycine max* (L.) Merr., is in the legume family and is rich in high quality protein. Soybeans contain 40% protein, 20% oil, 17% cellulose, 7% sugars, 6% ash, 5% crude fiber, and 5% miscellaneous.

Soy protein is produced from whole ground soybeans by multiple processes that remove the oil and fiber to produce high protein powder. The two major storage proteins in soybeans are β -conglycinin and glycinin corresponding to 7S and 11S globulins, respectively (Koshiyama 1965; Thanh et al., 1975 and 1976; Derbyshire et al., 1976; Nagano et al., 1992; Shewry 1995; Shutov et al., 1996; Wu et al., 2000). The protein contents of β -conglycinin and glycinin fractions were about 98% and 92% on a purified dry basis, respectively (Wu et al., 2000). In addition to protein, β -conglycinin and glycinin fractions contain other naturally occurring soy components, such as isoflavones and soyasaponins that have biologically active properties.

Soy protein is commonly consumed as a component of soy foods such as tofu, soymilk, soy nuts, soy yogurt, textured vegetable protein, vegetable-based soy burgers, miso, tempeh, and soy sauce. The estimated consumption of soy products in Asian countries is about 20 to 100 g/day (Wu et al., 1998; Nagata et al., 2003), compared with 3 to 10 g/day in

the United States (Kirk et al., 1999).

B. Introduction to soy isoflavones

Many studies have demonstrated the health protective properties of consuming soy isoflavones, including prevention of cancer (Lamartiniere et al., 1995; Pollard and Luckert, 1997), reducing bone loss (Ho et al., 2001; Alekel et al., 2000), and lowering plasma cholesterol concentrations (Crouse et al., 1999; Merz-Demlow et al., 2000; Wangen et al., 2001).

Soy isoflavones are diphenolic phytoestrogens found in soybeans and soy-derived foods. The basic structure of isoflavones is two aromatic rings (A and B ring) linked by a three carbon aliphatic chain to form a pyran ring (C ring) (Figure 1). The B ring of isoflavone is joined at position 3 on the C ring. The soybean isoflavone aglucones include daidzein, genistein, and glycitein (Figure 1). Nine β -glucoside forms are daidzin, genistin, glycitin, 6''-O-acetyldaidzin, 6''-O-acetylgenistin, 6''-O-acetylglycitin, 6''-O-malonyldaidzin, 6''-O-malonylgenistin, and 6''-O-malonylglycitin (Figure 2). Both acetylation and malonylation of isoflavone glucosides occurs at position 7 of the A ring. These glucoside conjugate forms of isoflavones are found primarily in soybeans.

In the USDA-Iowa State University Isoflavone Database (1999), the isoflavone contents of soybeans are about 1 to 3 mg/g. Isolated soy protein contains isoflavones ranging from 0.5 to 2 mg/g depending on the processing method. Soy products such as tofu, soymilk, and miso provide about 0.2 to 0.5 mg of isoflavones/g fresh weight. Daily isoflavone intake in Asia varies among countries, ranging from 15 to 55 mg/day (Seow et al., 1998; Chen et al., 1999; Wakai et al., 1999; Arai et al., 2000; Nagata et al., 2000; Kim and Kwon, 2001;

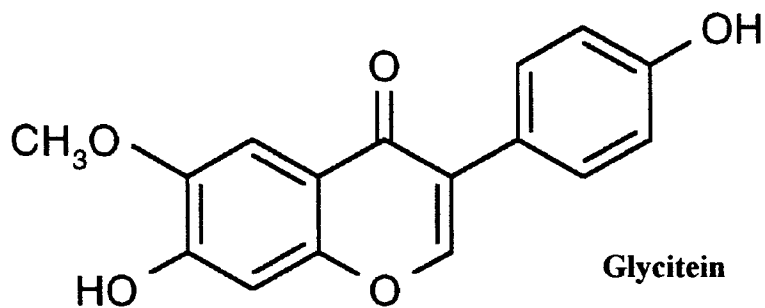
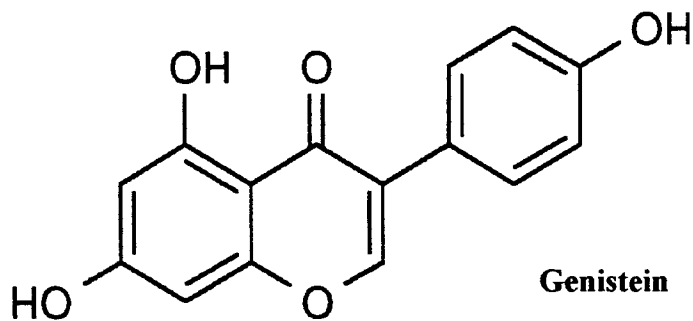
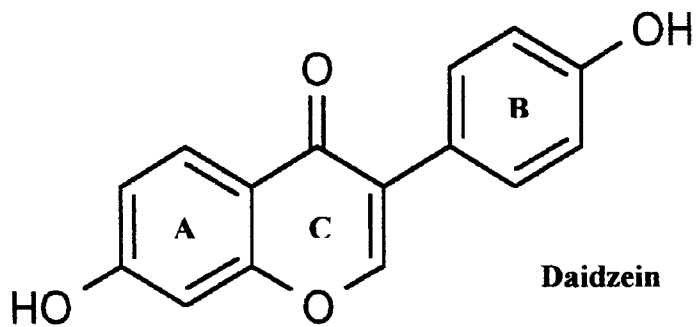
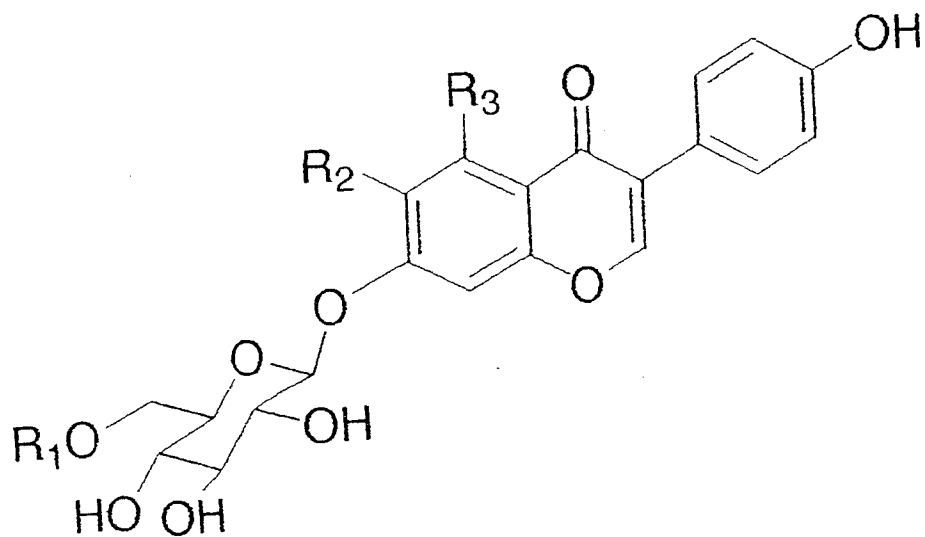


Figure 1. Chemical structure of soy isoflavone aglucone forms



	R1	R2	R3
Daidzin	H	H	H
6'-O-Malonyl daidzin	COCH ₂ COOH	H	H
6'-O-Acetyl daidzin	COCH ₃	H	H
Genistin	H	H	OH
6'-O-Malonyl genistin	COCH ₂ COOH	H	OH
6'-O-Acetyl genistin	COCH ₃	H	OH
Glycitin	H	OCH ₃	H
6'-O-Malonyl glycitin	COCH ₂ COOH	OCH ₃	H
6'-O-Acetyl glycitin	COCH ₃	OCH ₃	H

Figure 2. Chemical structures of isoflavone glucosidic forms

Somekawa et al., 2001). The estimated daily isoflavone intake was from 0.1 to 0.24 mg/d in postmenopausal women in the United States (de Kleijn et al., 2001).

B.1 Analysis of isoflavones in biological fluids

Since dietary soy isoflavones have several health-promoting effects, analysis of the isoflavone content in human bodily fluids may be helpful to determine the role of soy isoflavones in preventing chronic disease. Interest in analytical methods, including chromatographic assay and immunoassay, for analysis of isoflavones has risen dramatically and been reviewed recently (Hendrich, 2002; Wang et al., 2002). Improved methods of detection of the isoflavones and isoflavone metabolites in biological fluids and tissues are important because isoflavone metabolism and bioavailability are key to understanding and assessing the potential health effects of isoflavones.

Gas chromatography-mass spectrometry (GC-MS) method is particularly useful for the low concentrations of isoflavones and isoflavone metabolites in blood, feces, and urine. However, it is a very labor-intensive method even though this method offers very high chromatographic resolution. In 1984, the contents of isoflavones and their metabolites in human urine were first quantified by using gas chromatography (Axelson et al., 1984; Bannwart et al., 1984). Adlercreutz et al. (1991) developed a method to determine isoflavonoids and ligands and identify genistein in human urine by GC-MS using ^{14}C -estrone as the internal standard.

High performance liquid chromatography (HPLC) also allows the identification, confirmation, and quantitation of the physical properties of each isoflavone and its metabolite. The reverse-phase HPLC-ultraviolet absorbance (UV) detection method was

developed by Lundh et al. (1988) to detect isoflavone aglucons in bovine urine. Xu et al. (1994) applied a method of Lundh et al. (1988) to human feces, plasma, and urine. Franke and colleagues (1995 and 1998) also used reverse-phase HPLC method to analyze isoflavone metabolites, equol and O-desmethylangolensin, in human fluids. Fluorescence detection is more sensitive and specific than UV absorption. Electrochemical detection is a useful method for determining phytoestrogens that contain electroactive compounds, such as phenolic groups. Electrochemical detection is more sensitive than UV detection for HPLC system. Gamache et al. (1998) analyzed phytoestrogens in plasma, urine, and tissue samples by using a HPLC system with eight electrochemical detection channels.

Immunoassay methods, including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and time-resolved fluoroimmunoassay (TR-FIA), are non-chromatographic methods and provide a high degree of specificity and rapid screening of large numbers of samples. A RIA method has high sensitivity but involves radiation and a cross-reaction with structurally related compounds (Lapcik et al., 1997 and 1998). An ELISA method is a non-isotopic immunoassay but the sensitivity of this method is about 100 times less than the RIA method. TR-FIA has been suggested as a powerful method for analysis of isoflavones in plasma and urine samples (Adlercreutz et al., 1998; Uehara et al., 2000; Stumpf et al., 2000). The advantages of this method are lack of radiation and reagent stability in comparison with RIA, and high sensitivity over ELISA.

In terms of expense, equipment, supplies, and labor, the HPLC-UV detection or HPLC with electrochemical detection method seems to be adequate for analysis of isoflavones and their metabolites.

B.2 Metabolism and bioavailability of isoflavones

The predominant isoflavones in soy products are mostly the glucoside forms of daidzein, genistein, and glycitein (Wang and Murphy, 1994; Murphy and Hendrich, 2002). The glucoside forms may undergo chemical hydrolysis by gastric acid or enzymatic hydrolysis by mammalian intestinal microorganisms, resulting in the removal of the sugar moiety to yield aglucones. Following absorption of these aglucones from intestinal lumen, these compounds are readily metabolized to glucuronide and sulfate conjugates in the intestinal mucosa and liver (Sfakianos et al., 1997; Barnes et al., 1998; Coldham et al., 1999). A large portion of these conjugates is excreted in bile. Then, these conjugates are reconverted to aglucone forms and are reabsorbed and further degraded to different metabolites by gut microorganisms or excreted in the urine. Equol and *O*-desmethylangolensin (ODMA) via their respective intermediates, dehydroequol and dihydrodaidzein, respectively, and *cis*-4-equol have been identified as the major metabolites of daidzein (Kelly et al., 1993; Heinonen et al., 1999; Bayer et al., 2001). Genistein can be metabolized to 6'-hydroxy-*O*-desmethylangolensin via the intermediate dihydrogenistein (Joannou et al., 1995) and *p*-ethylphenol (Barnes et al., 1998; Goldwyn et al., 2000). Gut microbial metabolites of glycitein have been tentatively identified in human urine as 5'-OH-*O*-desmethylangolensin and 5'-methoxy-*O*-desmethylangolensin (Heinonen et al., 2000). Investigation of metabolic pathways and metabolites of soy isoflavones may be helpful to understand the potential health protective properties of isoflavones. The gut microorganisms that are involved in metabolic pathways and the biological activities of these metabolites should be further investigated.

Bioavailability refers to the proportion of a certain substance absorbed and reaching the target tissues. The bioavailability of soy isoflavones is influenced by many factors, including endogenous digestive and biotransformation enzymes and activities of gut microflora. Thus, understanding the bioavailability of isoflavones is important to assess the potential health effects of isoflavones. Different chemical structure of isoflavones may affect the metabolic fate and bioavailability. Setchell et al. (2002) investigated the intestinal absorption of isoflavone glucosides in healthy premenopausal women who ingested either 50 mg of daidzin or genistin or 250 mL soymilk. Only aglucone forms, daidzein or genistein, were detected in plasma. The results suggested that hydrolysis of the sugar moiety was necessary for the absorption of isoflavone glucosides and increased bioavailability of soy isoflavones. Setchell et al. (2001) found that the total amount of isoflavones in the plasma over time (AUC, area under curve) was not different between isoflavone aglucone and glucosides forms, suggesting that isoflavone glucosides just needed more time to reach the maximum plasma isoflavone concentrations than aglucone forms because of hydrolysis of the sugar.

The metabolic response to the ingested isoflavones varies in human subjects eating soy products (Franke et al., 1994; Xu et al., 1994 and 1995; Kelly et al., 1995; King and Bursill 1998; Watanabe et al., 1998; Zhang et al., 1999). Xu et al. (1994) investigated the bioavailability of daidzein and genistein in human subjects consuming soymilk. The fecal recovery of isoflavones was only 1 to 2% of the ingested dose. The average 24 h urinary recoveries of daidzein and genistein were approximately 21% and 9%, respectively. The data indicated that daidzein is more bioavailable than genistein, as reflected in urinary recovery of daidzein compared with that of genistein. Zhang et al. (1999) investigated the bioavailability

of glycitein in comparison with daidzein and genistein. Seven females and seven males consumed approximately 71 mg and 72 mg total aglucone isoflavones/day (approximately 0.56 mmol and 0.57 mmol/kg diet) from soymilk and soy germ. The average 48 h urinary recoveries of daidzein, glycitein, and genistein were approximately 52%, 47%, and 37%, respectively, suggesting that bioavailability is daidzein = glycitein > genistein. The different recoveries of each isoflavone in feces or urine may be explained by the extensive metabolism or degradation of isoflavones by intestinal microorganisms.

Single-dose studies have been conducted to investigate the plasma and urinary kinetics of daidzein and genistein in human (Watanabe et al., 1998; King and Bursill, 1998). Watanabe et al. (1998) reported that isoflavone concentrations in plasma reached maximum values of 1.56 $\mu\text{mol/L}$ at 6 h for daidzein and 2.44 $\mu\text{mol/L}$ at 6 h for genistein. The rate of urinary excretion of daidzein was greater than that of genistein following intake of 60 g of kinako (baked soy powder; approximately 56 mg total aglucone isoflavones/d), with mean recoveries of 36% and 18% for daidzein and genistein, respectively. King and Bursill (1998) found that concentrations of plasma daidzein and genistein rose slowly and reached maximum values of 3.14 $\mu\text{mol/L}$ at 7.4 h and 4.09 $\mu\text{mol/L}$ at 8.4 h, respectively. The greater proportion of the ingested isoflavones excreted as daidzein (62%) than genistein (22%) throughout the postmeal period. However, King and Bursill concluded that the bioavailability of daidzein and genistein are similar because the ratio of the areas under the plasma content versus time curves for daidzein and genistein was equal to the ratio of the concentrations of the respective isoflavones in the soybean flour-based meal.

Xu et al. (1995) investigated fecal isoflavone disappearance. Intestinal half-lives of daidzein and genistein were estimated at 7.5 and 3.3 h, respectively, suggesting that more

rapid disappearance of genistein may be due to lower apparent absorption of genistein compared with that of daidzein. Zheng et al. (2003) reported that women who showed low fecal genistein disappearance rate and rapid gut transit time had greater genistein bioavailability, as reflected in urinary recovery of genistein compared with women who had high genistein disappearance rate and long gut transit time. Hendrich et al. (1998) showed three different isoflavone disappearance phenotypes in humans and a negative correlation between plasma isoflavone concentrations and isoflavone disappearance rate constant in young adult men. These findings suggested that individual variability in metabolic response to isoflavones may be due to differences in the relative ability of gut microorganism to degrade isoflavones.

B.3 Toxicity of isoflavones

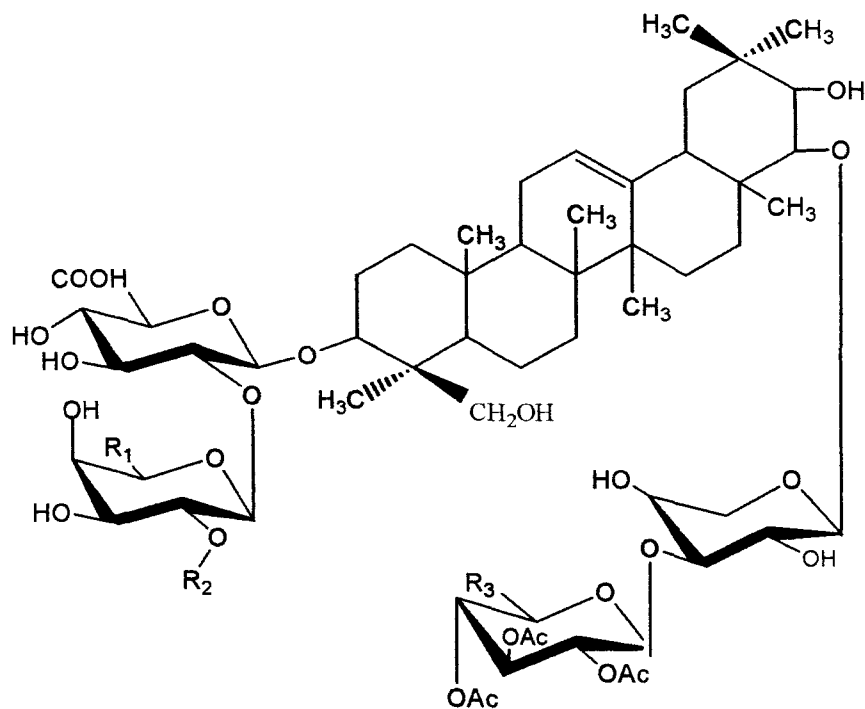
The potential toxicity of soy isoflavones must be considered and addressed. A variety of feeding or intervention studies have indicated that oral exposure to soy isoflavones does not produce toxic effects (Anastasia et al., 1990; National Cancer Institute, 1996; Murrill et al., 1996; Rao et al., 1997; Flynn et al., 2000; Arjmandi et al., 1998a and 1998b; Lamartiniere et al., 1998; Casanova et al., 1999; Picherit et al., 2000; You et al., 2002). However, fewer studies have reported the subchronic and chronic toxicities of soy isoflavones. Whereas soy protein isolate with isoflavones (approximately 1.6 mmol/kg diet)(Arjmandi et al., 1998a and 1998b) or genistein alone (500 µg/g body weight)(Murrill et al., 1996) are not uterotrophic, subcutaneous administration of genistein (150-500 mg/kg body weight/d) showed short-term uterotrophic effects in rats and mice (Brown and Lamartiniere, 2000; Ishimi et al., 2000). Song et al. (1999) reported that female B6D2F1 mice dosed with glycitein (3 mg/day) by

gavage for 4 days showed a uterophic effect. Consumption of 60 g soy containing 45 mg isoflavones for 2 weeks in women with benign and malignant breast disease stimulated breast tissue proliferation (McMichael-Phillips et al., 1998). However, isoflavone-rich soy protein isolate did not induce proliferation in endometrial and mammary tissue in surgically postmenopausal female macaques (Foth and Cline, 1998). Existing observational and epidemiologic studies support the safety of isoflavones as typically consumed in diets based on soy or soy containing products. However, more research on the safety of soy isoflavone intake should be done.

C. Introduction to soyasaponins

Saponins are widely distributed and have been identified more than a thousand different types in a variety of plants (Hostettmann and Marston, 1995). Legumes are rich sources of dietary saponins.

Soyasaponins are the triterpenoid glycosides naturally occurring in a variety of leguminous plants such as soybeans, alfalfa, cowpeas, lentils, runner beans, and kidney beans (Price et al., 1986; Tsukamoto et al., 1995; Ruiz et al., 1996; Kinjo et al., 1998; Oleszek et al., 1998). Soyasaponins have been reported to possess the health benefits including hypocholesterolemic (Oakenfull et al., 1984; Sidhu and Oakenfull, 1986), anticarcinogenic (Rowlands et al., 2002; Gurfinkel and Rao, 2003), and antimutagenic (Berhow et al., 2000) activities. Soybean saponins are classified into group A and B on the basis of their aglycone parts, soyasapogenol A and soyasapogenol B, respectively (Figure 3). The primary saponins in soybeans are the bisdesmosidic group A soyasaponins with two different polysaccharides and the monodesmosidic group B soyasaponins with one di- or tri-saccharide chain. Ireland



	R1	R2	R3
Soyasaponin Aa	CH ₂ OH	β-D-Glc	H
Soyasaponin Ab	CH ₂ OH	β-D-Glc	CH ₂ OAc
Soyasaponin Ac	CH ₂ OH	α-L-Rha	CH ₂ OAc
Soyasaponin Ad	H	β-D-Glc	CH ₂ OAc
Soyasaponin Ae	CH ₂ OH	H	H
Soyasaponin Af	CH ₂ OH	H	CH ₂ OAc
Soyasaponin Ag	H	H	H
Soyasaponin Ah	H	H	CH ₂ OAc

Figure 4. Chemical structures of group A soyasaponins (Yoshiki *et al.* 1998)
 Glc: glucopyranosyl; Rha: rhamnopyranosyl; Ac: acetyl

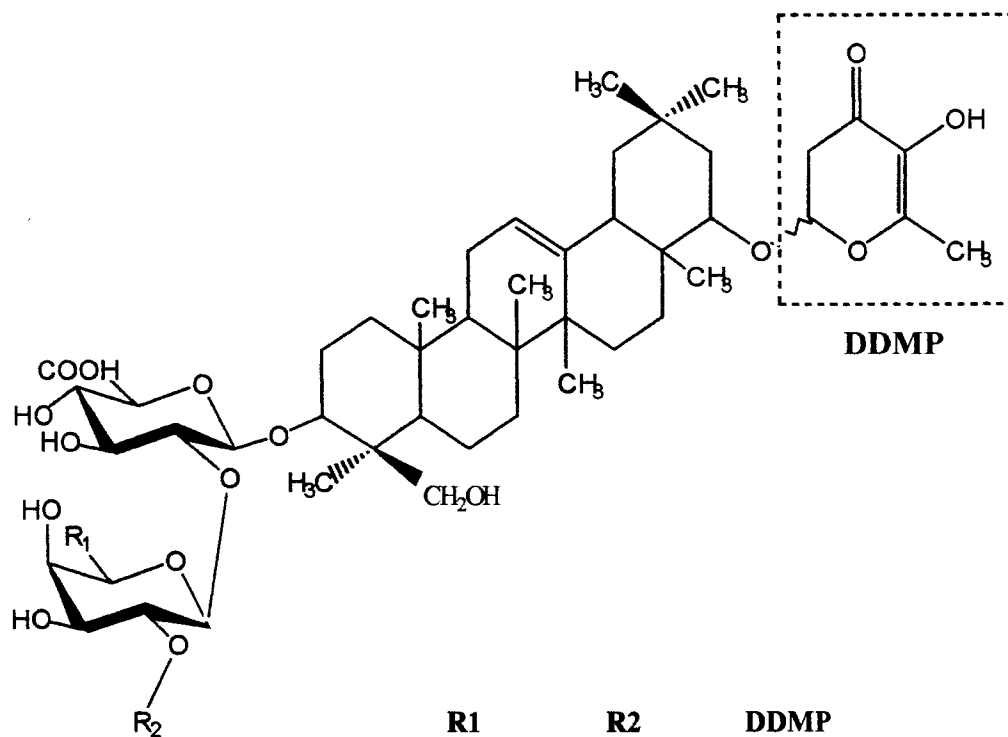


Figure 5. Chemical structures of group B soyasaponins (Kudou *et al.* 1994)
Rha: rhamnopyranosyl; **Glc:** glucopyranosyl;
DDMP: 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one
Y: yes; **N:** no.

soyasaponin V, I, and II, of soy products such as commercial soy burgers and meatless franks are about 0.5 to 1.8 $\mu\text{mol/g}$ and 0.02 to 0.3 $\mu\text{mol/g}$, respectively.

C.1 Analysis of soyasaponins

Analytical methods have been developed for the separation, identification, and quantification of soyasaponins from soybean and soy products, including HPLC with UV (Ireland et al., 1986; Shiraiwa et al., 1991; Kudou et al., 1993; Hu et al., 2002) or evaporative light scattering detector (ELSD) (Rupasinghe et al., 2003), liquid chromatography (LC) combined with thermospray mass spectrometry (Fuzzati et al., 1997) or electrospray ionization mass spectrometry (Gu et al., 2002; Berhow et al., 2002; Dalluge et al., 2003).

A method for detection of the soyasaponins and their metabolites in biological fluids was developed by Hu et al. (2004a), based on HPLC combined with UV detection. Hu et al. (2004a) found two metabolites of soyasaponin I, soyasapogenol B and soyasapognin III, *in vitro* fermentation system (Figure 6). In an *in vivo* study, only soyasapogenol B was detected in human feces but no soyasaponins or their metabolites were detected in 24-h urine samples. To determine and assess the potential health effects of soyasaponins, improved methods for detection of the soyasaponins and their metabolites in biological fluids must be developed.

C.2 Metabolism and bioavailability of soyasaponins

Not many of the biological activities associated with soyasaponins have been investigated because of the lack of availability of large quantities of purified soyasaponins and efficient methods for the detection and identification of soyasaponins and their

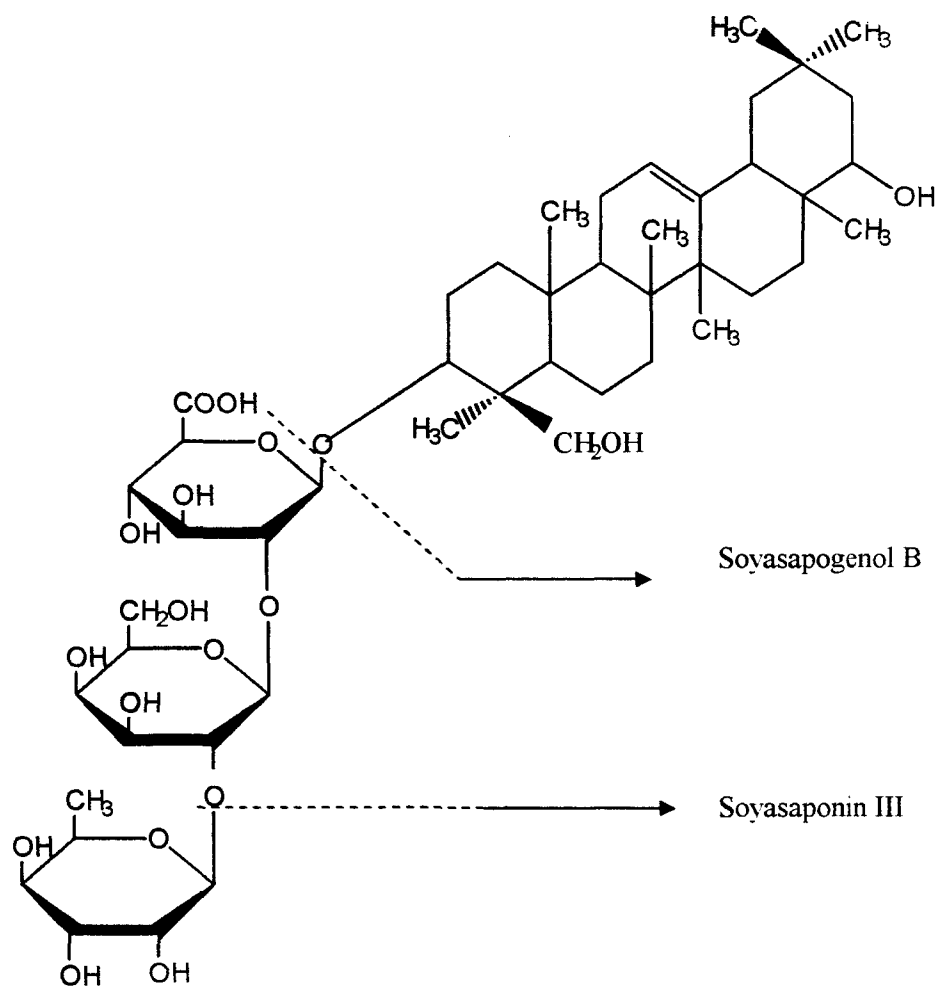


Figure 6. Structures of soyasaponin I and its metabolites, soyasaponin III and soyasapogenol B (Hu et al. 2003)

metabolites.

It has been considered that soyasaponins are poorly absorbed in the digestive tract. Thus, soyasaponins may be either excreted unchanged or metabolized in the gut. In *in vivo* experiments, soyasaponins or soyasapogenols (aglycone forms) were not detected in the blood of rats, mice, or chicks (Gestetner et al., 1968), suggesting that ingested soybean saponins may not be absorbed into the blood stream as soyasaponins or soyasapogenols. Hu et al. (2004b) reported that soyasapogenol B but no group B soyasaponins was detected in human feces after a single dose of soy extract containing 434 μmol of group B soyasaponins. Neither soyasapogenol B nor soyasaponin metabolites were found in a 24 h urine samples. Using an *in vitro* system, both soyasaponins and soyasapogenols were identified when the contents of the cecum and colon were incubated with soybean saponin extract for 3 hours at 37°C (Gestetner et al., 1968). The sugars, glucose, arabinose, galactose, rhamnose, and glucuronic acid, liberated by the soybean saponins-hydrolyzing enzymes (nonspecific glycosidases) purified from the cecal microorganisms of rats. Gurfinkel and Rao (2003) reported that colonic microflora readily hydrolyzed the group A and group B soyasaponins to soyasapogenol A and soyasapogenol B *in vitro*. Hu et al. (in press, b) found that two gut microbial metabolites of soyasaponin I, soyasaponin III, and soyasapogenol B, were detected using an *in vitro* fermentation system. These results suggest that soybean saponins were hydrolyzed to soyasapogenols, partially hydrolyzed forms, and sugars by mammalian gut microbial enzymes. Rowland et al. (1970) and Hawksworth et al. (1971) found that human intestinal bacteria have glycosidase and β -glucuronidase activities and may hydrolyze soybean saponins to yield aglycones or partially hydrolyzed forms in the human intestinal tract. It was confirmed by Hu et al. (2004a) that only soyasapogenol B (8.4% fecal recovery

of ingested dose of group B soyasaponins) was detected in human feces. More detailed information under *in vivo* conditions is needed to elucidate and understand the fate of soybean saponins in animals and humans.

C.3 Toxicity of soyasaponins

A dose of up to 0.5 mg soyasaponin I/plate in the Ames mutagenicity assay was reported not to produce mutagenic effects (Czeczot et al., 1994). After cultured rat hepatocytes were exposed to CCl₄ for 1 hour, soyasaponin I showed antihepatotoxic activity to inhibit the elevation of glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) activities, except at the highest dose (Miyao et al., 1998). Hu et al. (2004a) investigated the cytotoxicity of soyasaponin I and soyasapogenol B by measuring cell viability in a human colon cancer CaCo2 cell. Compared with control, soyasaponin I did not show an apparent toxic effect at concentrations up to 3 mM, while soyasapogenol B at 1 mM or higher concentrations significantly decreased cell viability. Several *in vitro* studies showed the interaction between soyasaponins and nutrients. Crude soybean soyasaponins increased resistance of bovine serum albumin (BSA) to chymotrypsin hydrolysis (Ikeda et al., 1996), suppressed chymotrypsin hydrolysis of soy proteins (Shimoyamada et al., 1998), and protease hydrolysis of β -lactoglobulins. Another nutritional problem of dietary saponins is that of decreasing mineral absorption. Southon et al. (1988a and b) reported that rats fed a diet with 2% of *gypsophila* saponins or alfalfa saponins showed greater fecal excretion of minerals, while a diet containing a similar level of soyasaponin I did not affect zinc (Zn) and iron (Fe) absorption. More studies are needed to investigate the safety of soyasaponins.

D. Introduction to atherosclerotic cardiovascular disease

Atherosclerotic cardiovascular disease (CVD), principally coronary heart disease (CHD) and stroke, was the leading cause of death in the United States in 2001, accounting for more than 38% of all deaths (1 in every 2.6 deaths) (AHA 2004). Coronary heart disease and stroke caused nearly 669,000 and 282,000, respectively. In 2004, the direct and indirect cost of heart disease and stroke is about \$133.2 billion and \$53.6 billion, respectively (AHA 2004).

Atherosclerosis is a process of thickening and hardening of the arteries and causes nearly three-fourths of all deaths from CVD. Elevated blood cholesterol, specifically those of total and low-density lipoprotein (LDL) cholesterol, or decreased high-density lipoprotein (HDL) cholesterol concentrations, are associated with the development of atherosclerotic CVD.

E. Link between soy components and atherosclerotic cardiovascular disease

Great interest has focused on the cardiovascular protective properties of soy components including soy protein itself, isoflavones, and soyasaponins. Huff et al. (1977) investigated the effects of intact soybean protein or a mixture of amino acids that was identical to the composition of intact soybean protein in lowering plasma cholesterol concentrations. Rabbits fed diets containing a mixture of amino acids did not show as much decrease in plasma cholesterol as those fed the intact protein. The data suggested that some components in intact soybean protein other than the amino acids may play a role in plasma lipid metabolism.

Soy protein-associated components including β -conglycinin and glycinin corresponding to 7S and 11S globulin, respectively, were investigated for their effects on plasma lipid metabolism (Wright, 1985 and 1988, Lovati et al., 1992, 1998 and 2000; Manzoni et al., 2003; Duranti et al., 2004). Peptides from β -conglycinin increased LDL receptor expression in human hepatoma (HepG2) cells while glycinin peptides had no effect (Lovati et al., 1992). Lovati et al. (1998) reported that the soybean 7S globulin (β -conglycinin), particularly the $\alpha + \alpha'$ subunits, upregulated hepatic LDL receptors in HepG2 cells. Duranti et al. (2004) found that purified α' subunit from soybean 7S globulin significantly decreased plasma total cholesterol and triglycerides and increased hepatic β -VLDL receptor expression in rats. These data imply that β -conglycinin may lower plasma cholesterol concentrations, specifically LDL cholesterol, through increased hepatic LDL receptor expression.

Several human and animal studies indicate that alcohol-extractable components of soybean protein are responsible for the hypocholesterolemic and anti-atherogenic effects (Song et al., 2003; Demonty et al., 2002; Blair et al., 2002; Adams et al., 2002; Clarkson et al., 2001; Gardner et al., 2001; Uesugi et al., 2001; Crouse et al., 1999; Peluso et al., 2000; Balmir et al., 1996; Anthony et al., 1996 and 1997, Tovar-Palacio et al., 1998; Wangen et al., 2001; Merz-Demlow et al., 2000). Isoflavones are largely extracted with alcohol from soybean protein. Accumulating evidence indicates that isoflavones reduce CVD risk factors. Soybean saponins are one of major alcohol-extractable components of soybean protein that may affect plasma cholesterol concentrations (Sidhu and Oakenfull, 1986; Oakenfull et al., 1984; Oakenfull and Sidhu, 1990; Potter et al., 1995; Ueda et al., 1996; Fukui et al., 2002). Purified saponins from soybean, soapwort, and quillaja added to the diet (10% by weight of

diet) reduced plasma cholesterol in rats by increasing excretion of bile acids or decreasing cholesterol absorption from the small intestine (Sidhu and Oakenfull, 1986). Fukui et al. (2002) reported that Sprague-Dawley rats fed soy protein isolate, either intact or depleted of isoflavones but containing 49% of soyasaponins in a soy protein isolate-based diet, had significantly lower plasma total cholesterol concentration compared with those fed casein.

F. Effects of soy components on plasma lipids and lipoproteins

Many studies have focused on the health benefits of using soybean and soy products for hypocholesterolemic activity. Total plasma cholesterol above 240 mg/dl, LDL cholesterol above 160 mg/dl, and HDL cholesterol below 40 mg/dl are strong indicators of CVD risk. Every 1% drop in plasma total cholesterol may be associated with a 2% reduction in coronary heart disease incidence (Roussouw and Rifkind, 1990). Therefore, optimal concentrations of plasma lipid and lipoproteins are important in the prevention of atherosclerotic CVD.

F. 1 Hypocholesterolemic effect of soy protein

It is well-known that a soy protein diet improves the blood lipid profiles of humans and animals (Potter et al., 1993; Bakhit et al., 1994; Anderson et al., 1995; Teixeira et al., 2000; Aoyama et al., 2001; Moriyama et al., 2004). A meta-analysis showed a significant relationship between soy consumption and decreased total and LDL cholesterol by 9% and 13%, respectively, and lesser risk of heart disease (Anderson et al., 1995). In 1999, the U.S Food and Drug Administration (FDA) approved a health claim that “diets low in saturated fat and cholesterol that include 25 g of soy protein a day may reduce the risk of heart disease.”

Consumption of 25 g soy protein per day significantly decreased plasma total cholesterol by 7-8% in men with initial total cholesterol concentration > 5.7 mmol/L compared with the casein diets (Bakhit et al., 1994). Furthermore, Teixeira et al. (2000) investigated the minimum amount of soy protein needed to lower plasma lipid concentrations in 81 men (23-74 year of age) with moderate hypercholesterolemia. Compared with casein, consuming as little as 20 g of isolated soy protein significantly reduced plasma total cholesterol, non-HDL cholesterol, and apolipoprotein B concentrations by week 6. Aoyama et al. (2001) reported that a 20% β -conglycinin diet significantly lowered plasma total cholesterol and triglyceride concentrations in male Wistar young and adult rats compared with a 20% casein diet. Male mice fed soybean β -conglycinin for 2 weeks had significantly lower plasma triglyceride concentration compared with those fed casein. The triglyceride-lowering effect of β -conglycinin was mediated by an increase of β -oxidation, suppression of fatty acid synthase, or an increase of fecal triglyceride excretion (Moriyama et al., 2004).

In addition, the cholesterol-lowering effect of the alcohol-washed isolated soy protein has been reported (Balmir et al., 1996; Blair et al., 2002; Song et al., 2003). In the study by Balmir et al. (1996), ethanol-acetone extracted isolated soy protein significantly reduced plasma total and non-HDL cholesterol concentrations in Sprague-Dawley rats compared with casein. In male hamsters, isolated soy protein with isoflavones removed lowered the plasma LDL+VLDL cholesterol concentration compared with casein (Blair et al., 2002). Compared with casein, isolated soy protein lacking isoflavones significantly decreased plasma total and non-HDL cholesterol concentrations in male and female Golden Syrian hamsters (Song et al., 2003). These results indicate that soy protein itself does play a role in the hypocholesterolemic activity.

In view of the growing attention to the mechanisms of the hypocholesterolemic response to soy protein, it was of interest to examine specific soy protein and its related components for their ability to lower plasma lipid concentrations. The 7S globulin and in particular the $\alpha + \alpha'$ subunits up-regulated hepatic LDL receptors in HepG2 cells (Lovati et al., 1998). Manzoni et al. (2003) also showed that the LDL receptor upregulation in HepG2 cells was induced by the α' subunit of 7S globulin. Purified α' subunit from soybean 7S globulin significantly reduced plasma total cholesterol and triglycerides and upregulated hepatic β -VLDL receptor in rats (Duranti et al., 2004). These data suggest that soy protein may affect plasma concentrations of lipoproteins and cholesterol through LDL receptor-dependent pathways. Some investigators have reported that dietary soy protein increased excretion of fecal bile acids and neutral steroids (Beynen, 1990; Schwerin et al., 2002; Wright and Salter 1998; Nagaoka et al., 1999). Wright and Salter (1998) showed that fecal bile acids were 2-fold greater in soy protein-fed hamsters than in hamsters fed a casein diet. There was a significant correlation between soy intake and bile acid excretion. Hepatic cholesterol decreased by 32%, as the amount of soy protein consumed increased from 16.3% to 36.3% soy, suggesting that it is this pool of cholesterol that is used to replace the excreted bile acids.

F. 2 Hypocholesterolemic effect of soy isoflavones

Several animal studies showed that intact soy protein has favorable effects on plasma lipid and lipoprotein concentrations compared with alcohol-washed soy protein (Anthony et al., 1996 and 1997; Kirk et al., 1998; Ni et al., 1999; Clarkson et al., 2001). Anthony et al. (1997) found that male *Cynomolgus* monkeys fed intact isolated soy protein (1.2 mmol total

isoflavones/kg diet) for 14 months had significantly lower plasma total and LDL+VLDL cholesterol levels compared with both casein group and isolated soy protein with the phytoestrogen mostly extracted (0.13 mmol total isoflavones/kg diet). Plasma and liver total cholesterol were significantly lower in ExHC rats fed intact isolated soy protein (providing approximately 1.1 mmol total isoflavones/kg diet) than in those fed ethanol-extracted isolated soy protein (Ni et al., 1999). Clarkson et al. (2001) also reported that both soy protein isolate containing soy phytoestrogens (1.0 mmol total isoflavones/kg diet) and soy phytoestrogens-depleted soy protein isolate with conjugated equine estrogens significantly lowered plasma total and LDL+VLDL cholesterol concentrations in ovariectomized female *Cynomolgus* monkeys compared with soy protein isolate depleted soy phytoestrogens (0.005 mmol total isoflavones/kg diet). Song et al. (2003) found that soygerm extract or daidzein (1.3 mmol total isoflavones/kg diet) significantly decreased plasma total and non-HDL cholesterol levels compared with casein. These findings suggest that an isolated soy protein extract rich in isoflavones or consumption of a high level of isoflavones lowered plasma total and non-HDL cholesterol concentrations.

The relationship between the amount of isoflavones in the diet and the effect on the plasma lipid profiles was investigated in animal models (Balmir et al., 1996; Tovar-Palacio et al., 1998). Balmir et al. (1996) investigated the effects of an alcohol extract of isolated soy protein, which contains isoflavones, on plasma cholesterol concentrations. A significant reduction in plasma LDL cholesterol concentration was observed in male Golden Syrian hamsters fed diets containing intact isolated soy protein (ISP), ISP with added ethanol extract (providing 0.6 mmol total isoflavones/kg diet; ISP+), casein with ethanol extract (providing 0.3 mmol total isoflavones/kg diet; casein+) for 8 weeks compared with those fed casein.

Addition of twice the level of extract to casein (providing 0.6 mmol total isoflavones/kg diet; casein++) resulted in a slight but nonsignificant reduction in plasma total and non-HDL cholesterol concentrations compared with casein. Gerbils fed alcohol-washed isolated soy protein (containing approximately 0.04 mmol/kg diet; ISP) or supplemented with one of three different levels of an alcohol extract of isolated soy protein to ISP (providing approximately 1.5 mmol, 2.6 mmol, or 4.4 mmol total isoflavones/kg diet) had significantly lower plasma total cholesterol, LDL+VLDL cholesterol, and apolipoprotein B concentrations than those fed casein. The addition of isoflavone-containing extract to ISP did not further lower plasma cholesterol levels (Tovar-Palacio et al., 1998). These results suggested the existence of a threshold above which an increase in isoflavone intake had no additional cholesterol-lowering effect.

Several human studies also reported that increasing the level of isoflavones in the diet are more effective in improving plasma lipid and lipoprotein concentrations (Crouse et al., 1999; Merz-Demlow et al., 2000; Wangen et al., 2001; Gardner et al., 2001). Crouse et al. (1999) investigated the effects of feeding 25 g isolated soy protein containing different amounts of isoflavones for 9 weeks in moderately hypercholesterolemic men and women. Consumption of 25 g isolated soy protein with 62 mg of isoflavones (approximately 0.5 mmol/kg diet) significantly reduced plasma total and LDL cholesterol by 4% and 6%, respectively, compared with casein. Plasma LDL cholesterol was significantly decreased in premenopausal women consuming 53 g soy protein isolate with 129 mg isoflavones (approximately 1.0 mmol/kg diet) compared with women consuming soy protein isolate with 10 mg isoflavones (approximately 0.08 mmol/kg diet) (Merz-Demlow et al., 2000). In the study by Wangen et al. (2001), postmenopausal women consuming 63 g of isolated soy

protein containing 132 mg isoflavones (approximately 1.04 mmol/kg diet) had significantly lower plasma LDL cholesterol compared with subjects consuming isolated soy protein with 7 mg isoflavones (approximately 0.06 mmol/kg diet).

Recent studies investigated the effect of adding pure synthetic isoflavones or isolated pure isoflavones from soybean fraction to the casein diet to avoid the confounding effects of other components present in alcohol extract from soy protein (Nogowsky et al., 1998; Uesugi et al., 2001; Demonty et al., 2002; Song et al., 2003). Uesugi et al. (2001) showed that ovariectomized rats fed isolated pure isoflavone glycosides daidzin (25 or 50 mg/kg body weight/d) or genistin (50 mg/kg body weight/d) orally using a stomach tube for 4 weeks had significantly lower plasma total cholesterol and triglyceride concentrations compared with those fed casein. Compared with Sprague-Dawley rats fed casein, rats fed diets containing either intact soy protein isolate (containing 1.6 mmol/kg diet) or casein plus synthetic isoflavones (providing approximately 1.4 mmol/kg diet) had significantly reduced plasma triglycerides (Demonty et al., 2002). Song et al. (2003) reported that plasma total and non-HDL cholesterol concentrations were significantly lowered by 22-24% and 30-38%, respectively, in hamsters fed casein plus pure synthetic daidzein (1.3 mmol/kg diet; purity >98%) compared with those fed casein. These studies suggest that soy isoflavones are responsible, at least in part, for lowering plasma cholesterol concentrations, separate from soy protein.

Potential mechanisms of soy isoflavones in lowering plasma cholesterol concentrations are not clear. Soy isoflavones have similar structure to natural estrogens and can bind estrogen receptors (ER- α and - β), especially ER- β . The loss of estrogen increased plasma total cholesterol, LDL cholesterol, and triglycerides and decreased HDL cholesterol

concentration in women (Jensen, 1992; Gaspard et al., 1995). Jensen (1992) investigated the changes in plasma lipid concentrations after a natural menopause in 170 premenopausal women. After menopause, women significantly increased plasma total (by 6%) and LDL cholesterol (by 8%) and decreased HDL cholesterol (by 7%) concentration. It may be possible that soy isoflavones may affect plasma lipid concentrations through their estrogenic activity. Angelin et al. (1992) reported that hepatic ^{125}I -LDL binding activity (reflecting the expression of the LDL receptor) was increased in the estrogen-treated men compared with control. Kirk et al. (1998) reported that soy protein isolate containing isoflavones at 1.33 mmol/kg diet lowered plasma LDL cholesterol by 30% in C57BL/6 mice compared with soy protein lacking isoflavones. However, no cholesterol-lowering effect by soy protein was observed in LDL receptor-deficient mice. Based on the results from these studies, soy isoflavones may lower LDL cholesterol concentration through increased gene expression and hepatic LDL receptor activity, thereby increasing LDL clearance.

F. 3 Hypocholesterolemic effect of soyasaponins

It has been proposed that soyasaponins, one of the primary phytochemicals associated with soy protein, may play a role in the hypocholesterolemic effect of soy (Oakenfull et al, 1984; Potter et al., 1995; Ueda et al., 1996; Fukui et al., 2002). Compared with Sprague-Dawley rats fed casein, plasma total cholesterol concentration was significantly lowered in rats fed soy protein isolate, either intact or depleted of isoflavones but containing 49% of the amount of saponins present in the soy protein isolate-based diet (Fukui et al., 2002). Hepatic and plasma total cholesterol was significantly decreased in male Wistar rats fed a standard

diet that contained 1% cholesterol plus 1% purified soyasaponins compared with those fed a standard diet with 1% cholesterol (Oakenfull et al., 1984).

The potential mechanisms responsible for the hypocholesterolemic effects of soyasaponins and synthetic saponins have been investigated (Oakenfull et al., 1984; Price et al., 1987; Oakenfull and Sidhu, 1990; Morehouse et al., 1999; Oakenfull, 2001). In the study by Morehouse et al. (1999), the intestinal absorption of cholesterol was significantly decreased in male New Zealand white rabbits fed synthetic saponins, pamaqueside and tiqueside, compared with those fed control. There was a significant relationship between decreased hepatic and plasma total and non-HDL cholesterol concentrations and increased fecal neutral sterol excretion. Oakenfull et al. (1984) found greater fecal excretion of bile acids and neutral sterols in rats fed a diet containing 1% cholesterol and 1% purified soyasaponins. A diet containing crude soyasaponins (10 g/kg diet) decreased the rate of absorption of bile salts by the formation of large mixed micelles with bile acids (Sidhu and Oakenfull, 1986). Two mechanisms of soyasaponins in lowering plasma lipid concentrations were proposed by Oakenfull (2001). One possible mechanism was that soyasaponins may inhibit the intestinal absorption of cholesterol by forming large complexes with cholesterol. Another possible mechanism was that soyasaponins may inhibit the bile acid absorption from the intestine and then increase fecal excretion of bile acids. As a result of inhibiting the intestinal absorption of bile acids, soyasaponins lower plasma total cholesterol by enhancing bile acid synthesis.

G. Role of soy components in other cardiovascular disease risk factors

Soy consumption has been shown to improve cardiovascular health or modify other cardiovascular disease risk factors except plasma lipid profiles including inhibition oxidation of LDL, improvement of arterial function, and reduction of platelet aggregation and serotonin content.

G. 1 LDL oxidation

The oxidized LDL particle is thought to be more atherogenic than the native LDL particle. Soy isoflavones have been reported to inhibit the oxidation of LDL (Kapiotis et al., 1997; Yamakoshi et al., 2000; Jenkins et al., 2000 and 2003) and enhance the resistance of LDL to oxidation (de Whalley et al., 1990; Kanazawa et al., 1995, Tikkanen et al., 1998; Wiseman et al., 2000). Yamakoshi et al. (2000) reported that the atherosclerotic lesion of rabbits fed the isoflavone aglycone-rich extract (providing approximately 1.8 mmol or 5.6 mmol total isoflavones/kg diet) plus 1% cholesterol showed fewer oxidized LDL-positive macrophage-derived foam cells compared with that of rabbits fed 1% cholesterol alone. Hyperlipidemic men and postmenopausal women consuming soy protein foods (33 g soy protein/d) containing 86 mg isoflavones (approximately 0.7 mmol/kg diet) reduced the concentration of the oxidized LDL fraction (Jenkins et al., 2000). Wiseman et al. (2000) found that subjects (19 women and 5 men, aged 19-40 y) who consumed the textured soy protein containing 56 mg isoflavones (approximately 0.42 mmol/kg diet) had longer lag time for copper-ion-induced LDL oxidation compared with subjects who consumed a low level of isoflavones (approximately 0.014 mmol/kg diet). It seems that antioxidant properties of soy isoflavones inhibit the oxidation of LDL and increase oxidative resistance of lipoproteins, thereby exerting an antiatherosclerotic effect.

G. 2 Arterial function

The effects of soy and soy components on arterial function are inconsistent. Constriction of coronary arteries following acetylcholine injection was inhibited in premenopausal macaques fed isolated soy protein containing isoflavones (approximately 1.13 mmol/kg diet) compared with isoflavone-depleted isolated soy protein (containing approximately 0.11 mmol total isoflavones/kg diet). Arteries dilated in macaques fed isolated soy protein containing isoflavones (Honore et al., 1997). Compared with placebo, flow-mediated dilation was not significantly changed in postmenopausal women either consuming 40 g isolated soy protein with 118 mg isoflavones for 3 months (Teede et al., 2001) or taking 80 mg soybean phytoestrogen tablets daily (Simons et al., 2000). Nestel et al. (1997) reported that soy isoflavone tablets (80 mg/d) improved systemic arterial compliance in menopausal and perimenopausal women. It seems that soy isoflavones may affect vascular reactivity, although more evidence is needed to confirm the potential health benefits of using soy and soy components on arterial function.

G. 3 Platelet aggregation and serotonin storage

Reduced platelet aggregation is important in the prevention of CVD. Using an *in vitro* system, platelets obtained from animals fed intact soy protein isolate containing isoflavones showed lower platelet aggregation in response to thrombin and serotonin compared with platelets from those fed alcohol-washed soy protein isolate (Williams and Clarkson 1998). These investigators found that blood flow was significantly less in female rhesus monkeys fed isoflavone-depleted soy protein (providing approximately 0.005 mmol/kg diet) compared

with those fed soy protein with isoflavones (providing approximately 1.0 mmol/kg diet). Helmeste and Tang (1995) reported that uptake of serotonin in platelets was decreased by genistein. These findings suggested that soy isoflavones may play a role in reducing serotonin storage and in inhibiting platelet aggregation.

H. Role of soy components in atherosclerosis

The formation of atherosclerotic lesions is initiated by some endothelial injury. The cellular infiltration and proliferation is caused by injury and then contributes to the advanced lesion formation. Several studies suggested that isolated soy protein with isoflavones reduced atherosclerosis compared with isoflavone-depleted isolated soy protein (Clarkson et al., 2001; Adams et al., 2002; Anthony et al., 1997; Kirk et al., 1998). Thus, the potential mechanisms of soy isoflavones in preventing atherosclerosis may include beneficial effects on plasma lipid concentrations, antioxidant effects, effects on thrombus formation, antiproliferative and antimigratory effects on smooth muscle cells, and maintenance of normal vascular reactivity. Considerable evidence suggests that soy protein with isoflavones has an antiatherosclerotic effect and inhibition of atherosclerosis is likely mediated by lowering plasma lipid concentrations and inhibiting the oxidation of LDL or enhancing the resistance of LDL to oxidation.

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**BETA-CONGLYCININ DEFICIENT IN SOY ISOFLAVONES AND
SOYASAPONINS DID NOT LOWER PLASMA CONCENTRATIONS
OF LIPOPROTEINS AND CHOLESTEROL OVER TIME IN MILDLY
HYPERCHOLESTEROLEMIC WOMEN**

A paper to be submitted to the Journal of Nutrition

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ABSTRACT

To clarify which components of dietary soy protein reduce plasma cholesterol concentrations in hypercholesterolemic individuals, 52 women with low-density lipoprotein (LDL) cholesterol levels ranging from 2.58 to 5.17 mmol/L were randomly assigned to one of three groups for 28 days. Subjects received one muffin per day of the following composition depending upon treatment group: 1) 28 g of casein as the protein source; 2) 28 g of β -conglycinin, a major soybean protein, containing 22 μ mol of isoflavones and 23 μ mol of saponins; or 3) 28 g of β -conglycinin containing 218 μ mol of isoflavones and 276 μ mol of saponins. At days 14 and 28, women fed β -conglycinin containing high levels of isoflavones and saponins had significant decreases in total cholesterol by 6.5% and 6%, respectively, and LDL cholesterol by 9% and 8%, respectively, compared with day 0 ($P < 0.05$). In women fed casein, LDL cholesterol concentration was significantly decreased by 7.5% at day 28 compared with day 0 ($P < 0.05$). No significant effects of dietary treatments on either bile acid or neutral sterol excretion in feces were found. β -conglycinin low in isoflavones and

saponins was ineffective at lowering plasma cholesterol over time in mildly hypercholesterolemic women. This study suggests that isoflavones and saponins seem to be needed companions for β -conglycinin to exert a cholesterol-lowering effect. The components associated with dietary casein that lower LDL cholesterol level remain to be determined.

Keywords: β -conglycinin, saponins, isoflavones

INTRODUCTION

Coronary heart disease (CHD) mortality and morbidity are major health problems in the United States (AHA, 2004). Increased blood cholesterol concentrations, specifically those of total or low-density lipoprotein (LDL) cholesterol, are associated with the development of atherosclerotic cardiovascular disease (CVD) (Law, 1999). Several studies have shown that a diet containing soy protein improves the plasma lipid profile of humans and animals (Potter et al., 1993; Bakhit et al., 1994; Anderson et al., 1995; Teixeira et al., 2000; Aoyama et al., 2001; Moriyama et al., 2004; Duranti et al., 2004). A meta-analysis showed a significant relationship between soy protein consumption and decreased total and LDL cholesterol by 9% and 13%, respectively, and lower risk of heart disease (Anderson et al., 1995). Aoyama et al. (2001) reported that a diet containing 20% β -conglycinin, one of the two storage proteins in soybeans, significantly lowered plasma total cholesterol and triglyceride concentrations in male Wistar young and adult rats compared with a diet containing 20% casein.

The bioactive components of soy protein responsible for the hypocholesterolemic effect have yet to be defined. Currently, there is great interest in phytoestrogenic soy isoflavones, glycitein, genistein, and daidzein, regarding their possible role in lipid metabolism (Anthony et al., 1996 and 1997; Clarkson et al., 2001; Kirk et al., 1998; Honoré

et al., 1997; Ni et al., 1999; Song et al., 2003; Merz-Demlow et al., 2000; Uesugi et al., 2002; Cassidy et al., 1995; Crouse et al., 1999; Wangen et al., 2001). Crouse et al. (1999) investigated the effects of feeding 25 g isolated soy protein containing different amounts of isoflavones for 9 weeks in moderately hypercholesterolemic men and women. Compared with casein, 25 g of isolated soy protein with 62 mg of isoflavone reduced plasma total and LDL cholesterol concentration. In subjects with LDL cholesterol above the median (> 4.24 mmol/L) of the population studied, isolated soy protein with 37 mg of isoflavones also reduced both total and LDL cholesterol compared with casein. Wangen et al. (2001) reported that 63 g of isolated soy protein per day containing 132 mg of isoflavones reduced plasma LDL cholesterol compared with isolated soy protein containing 7 mg of isoflavones in mildly hypercholesterolemic postmenopausal women.

Saponins, another class of phytochemicals associated with soy protein, also have been suggested as a plasma cholesterol-lowering factor (Oakenfull and Sidhu, 1990; Potter et al., 1979; Newman et al., 1958; Fukui et al., 200). Sidhu and Oakenfull (1986) showed that various saponins added to the diet at 10% by weight reduced blood cholesterol in rats by increasing excretion of bile acids or decreasing cholesterol absorption.

Although the hypocholesterolemic effect of soy protein is documented in many species including humans, the mechanism has not been demonstrated conclusively. Possible mechanisms for soy protein-induced changes in blood lipids include a decrease in the intestinal absorption of dietary cholesterol or bile acids (Potter, 1998; Greaves et al., 2000), or changes in the hepatic metabolism of cholesterol and lipoproteins via change in apolipoprotein content or composition (Samman et al., 1989; Huff and Carroll, 1980; Lovati et al., 1992; Duranti et al., 2004). Samman et al. (1989) found that a 57% reduction of LDL

cholesterol concentration in New Zealand white rabbits fed soy protein compared with casein was primarily due to an enhancement in receptor-mediated catabolism of LDL-apo B. In addition, an increase in bile acid and neutral steroid excretion may decrease hepatic cholesterol reserves and increase hepatic LDL receptor activity, thereby decreasing plasma LDL cholesterol concentration (Sitori et al., 1984; Beynen, 1990; Nagaoka et al., 1999; Jaskiewicz et al., 1987; Gaddi et al., 1991). Lower serum total cholesterol in rats fed soy protein peptic hydrolysate, compared to those fed casein tryptic hydrolysate, was associated with increased fecal excretion of total steroids (Nagaoka et al., 1999).

In view of the growing attention to the mechanisms of the hypocholesterolemic response to soy proteins, it was of interest to examine specific soy proteins and related components for their ability to reduce plasma cholesterol concentrations. Peptides from β -conglycinin (7S globulin) up-regulated hepatic LDL receptors in human hepatoma (HepG2) cells while glycinin (11S globulin) peptides had no effect (Lovati et al., 1992). Manzoni et al. (2003) also reported LDL receptor upregulation in HepG2 cells induced by the α' subunit of 7S globulin. Purified α' subunit from soybean 7S globulin significantly reduced plasma total cholesterol and triglycerides and increased liver β -VLDL receptor expression in rats (Duranti et al., 2004). This study was designed to examine the effect of the major soy protein, β -conglycinin, containing high or low levels of isoflavones and saponins on plasma total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides. Mildly hypercholesterolemic women consumed common baked products to determine if β -conglycinin affects circulating cholesterol by increasing excretion of fecal bile acids and neutral sterols.

SUBJECTS AND METHODS

Preparation of β -conglycinin

β -conglycinin, a major soy storage protein, was prepared from defatted soy flakes by scaling up a process developed by Wu et al. (2000). Defatted soy flakes at 90 PDI (protein dispersibility index; Cargill Inc., Minneapolis, MN) or 70 PDI (Archer Daniels Midland, Decatur, IL) were combined with water at a ratio of 1 part flakes : 8 parts water. The pH was adjusted to 8.5 with 2 *N* NaOH and the slurry was stirred for 1 h at 20°C to solubilize the soy protein. The solubilized protein was separated from the insoluble fraction by passing the slurry through a Sharples P660 continuous centrifuge (Alfa Laval Separation Inc., Warminster, PA) using 5700 rpm bowl speed and 4000 rpm backdrive pinion speed. The slurry was fed to the centrifuge using a Moyno-type transfer pump (Electric Pump, model IFFCA SSE SAA, Des Moines, IA) with a 250 rpm pump speed. Sharples centrifuging yielded an insoluble fraction and an extract fraction. The insoluble residue was re-extracted at a ratio of 1 part flakes : 5 parts water, and this slurry was passed through the Sharples centrifuge again. The first extract and resulting second extract were combined, treated with sodium bisulfite (0.98 g/L or ~10 mM SO₂), and adjusted to pH 6.4 using 2 *N* HCl. After chilling overnight at 7°C, the protein solution was centrifuged using an Alfa Laval BTPX 205 disc centrifuge (Alfa Laval Separation Inc., Warminster, PA) at 9800 rpm bowl speed and 420 rpm transfer pump speed. Sodium chloride was added to the supernatant fraction of this centrifuge pass to adjust the ionic strength of the β -conglycinin-containing supernatant fraction to 0.25 M and 2 *N* HCl was added to adjust the pH of the solution to 5.0. The solution was stirred for 1 h at 7°C, then passed through the Alfa Laval centrifuge. To the

supernatant of this centrifuge pass, a volume of water twice the amount of supernatant was added to adjust the ionic strength of the supernatant, and the supernatant was adjusted to pH 4.8 using 2 *N* HCl. After chilling overnight at 7°C, the precipitated β -conglycinin was separated with the Alfa Laval centrifuge as above. The β -conglycinin precipitate was redissolved in an aqueous solution and desalted with a Feed and Bleed Membrane Filtration system (Model SRT-50; North Carolina SRT Inc., Cary, NC) and 30-KD regenerated cellulose membrane (North Carolina SRT Inc., Cary, NC). Diafiltration was performed until an amount of permeate equal to five times the volume of the β -conglycinin precipitate had been collected. The retentate was dried in an Anhydro compact spray-dryer (APV Crepaco Inc., Attleboro Falls, MA) with an air inlet temperature of 180°C and an air outlet temperature of 85°C.

Protein content was determined by measuring the nitrogen content using the micro-Kjeldahl method (AOAC, 1999, methods 988.05 and 960.52). The nitrogen conversion factor used for β -conglycinin was 6.37. The protein content of the β -conglycinin was 85-88%. Purity of the β -conglycinin was > 85% as measured by urea-sodium-dodecylsulfate-polyacrylamide gel in electrophoresis (SDS-PAGE). Protein moisture contents were measured using a forced-air oven (Fischer Scientific Isotemp 750°F, Fischer Scientific, Pittsburgh, PA) and averaged 10-11%.

Extraction of isoflavones and saponins from β -conglycinin

β -conglycinin deficient in isoflavones and saponins was prepared by extracting β -conglycinin, 1 g β -conglycinin : 3 ml of 70% ethanol (food grade) in water. The mixture was

stirred for 2-3 h and decanted. Extraction of β -conglycinin was carried out 5 times to reduce the isoflavone concentration to 20 $\mu\text{g/g}$ of the β -conglycinin fraction. β -conglycinin deficient in isoflavones and saponins was dried using a Hobart convection oven (Hobart Mfg. Co., Troy, OH) at 60°C for 2 h and then was ground to a homogenous powder in a Coffee Mate coffee grinder (Black and Decker Inc., Shelton, CT).

Subjects

Study procedures were approved by the Iowa State University Human Subject Protection Committee. About two hundred women (18-40 y of age) were recruited from the Iowa State University campus and surrounding areas and initially screened their plasma lipid concentrations. The women were interviewed in person and completed detailed questionnaires on health and medical history, typical food intake, and physical activity at the beginning of the study. A total of 72 healthy women selected had LDL cholesterol at screening between 2.58 mmol/L and 5.17 mmol/L. All subjects were non-smokers, did not consume soy products in their usual diets, and did not have diabetes mellitus, or other chronic illness that might affect blood lipid concentrations. This study was conducted with 2 separate cohorts, each of which participated in a 38 days feeding study including a 10 day washout period: cohort 1 during the fall of 1999 (n = 36), cohort 2 during the spring of 2000 (n = 36). For cohorts 1 and 2, the numbers of subjects who dropped out were 15 and 5, respectively. Thirteen subjects who failed the inclusion criteria, by having LDL cholesterol concentrations lower than 2.58 mmol/L, were excluded at day 0. Three subjects failed to comply with required procedures and the other 4 subjects chose to withdraw from the study.

Fifty-two subjects completed the study. All subjects who met the inclusion criteria and wanted to participate signed informed consent forms before they started the study.

Diet and study design

All subjects received detailed dietary instructions to avoid foods known to be rich in soy protein, isoflavones or other phytoestrogens, or saponins, such as soy beverage, tofu, flaxseed, garbanzo beans, bean sprouts, and frozen food products containing soy protein. During the 10 day washout periods, they were instructed to consume a diet composed of < 30% energy from fat (polyunsaturated fat: monounsaturated fat: saturated fat ratio of 10: 10: 10), \leq 15% from protein, \leq 55% from carbohydrate, and 15-20 g dietary fiber/day (step I diet). After this 10 day washout period, subjects were randomly assigned to 3 experimental groups. The study used a double-blind design; subjects did not know to which of the groups they were assigned, nor did the investigator. The women received 28 g protein/d which included 1) casein (New Zealand Milk Products, Inc., Santa Rosa, CA) as the control, 2) β -conglycinin containing 22 μ mol of isoflavones and 23 μ mol of saponins, or 3) β -conglycinin containing 218 μ mol of isoflavones and 276 μ mol of saponins, for 28 days. Test proteins were incorporated into a muffin which the subject received at breakfast 7 d/wk. The micromole concentrations of isoflavones/subject per day (Fig. 1), as daidzein, genistein, and glycitein, and the concentrations of soyasaponins α g, β g, I, and II/subject per day (Fig. 2) were calculated based on analytical data described below. Six different flavors of muffin (apple-cinnamon, banana, pumpkin spice, blueberry, cranberry-orange, and dried fruit) were served during the study. All the participants were required to eat the same flavor each

morning. Their consumption of soy muffin was monitored 5 times/wk and subjects were reminded to consume one muffin in its entirety each weekend day.

Analysis of isoflavone content in muffins

Two grams of dried, finely ground muffin was extracted with 10 mL of acetonitrile and 7 mL of distilled water for 2 hours at room temperature. The extract was filtered through Whatman No. 42 filter paper. The filtrate was collected in a round-bottom flask and dried in a rotary evaporator (Buchler Instrument, Fort Lee, NJ) at $< 30^{\circ}\text{C}$. The dried residues were dissolved in 80% aqueous methanol and made up to 10 mL in the volumetric flask. An aliquot was vortexed and filtered through a 0.45 mm PTFE filter (Alltech Associates Inc., Deerfield, IL) and analyzed by HPLC. The soy isoflavone content in the muffins were measured by HPLC using the method previously described (Murphy et al., 1999).

Analysis of saponin content in muffins

Four grams of dried, ground muffin was extracted by stirring with 100 mL of 70% aqueous ethanol for 2.5 h at room temperature. The extract was filtered through Whatman No. 42 filter paper and evaporated to dryness below 30°C . The dried residues were dissolved in 80% aqueous methanol and made up to 10 mL in the volumetric flask. An aliquot was vortexed and filtered through a 0.45 mm PTFE filter (Alltech Associates Inc., Deerfield, IL) prior to HPLC analysis. The content of saponins in the muffins were determined by HPLC system as previously reported by Hu et al. (2002).

Plasma lipid analysis

Blood samples were collected at day 0 (baseline), day 14, and day 28 during the study after a 12-hr overnight fast. Blood was drawn into tubes containing EDTA, set at room temperature for 30 min to allow coagulation, and centrifuged at 3000 x g for 15 min at 18°C to obtain serum. Serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride were analyzed enzymatically by Quest Diagnostics (Des Moines, IA), a certified clinical laboratory. LDL-cholesterol was calculated by Quest Diagnostics using the Friedewald formula (Friedewald et al., 1972).

Analysis of fecal bile acids and neutral sterols

On day 23 of dietary treatments, all subjects were given two capsules containing carmine red dye marker (first marker) and 24 hours later they received two capsules containing sterilized yellow ceramic beads (second marker). Feces were collected from the time that the red marker appeared in feces until the time that yellow beads appeared.

Quantitation of bile acids and neutral sterols in human feces was performed according to the method of Batta et al. (1999). Two hundred μL of n-butanol which contained hyocholic acid and 5α -cholestane (internal standards) and 50 μL of concentrated hydrochloric acid were added to 15 mg freeze-dried fecal samples and to the standards (10 μg of each bile acid and 20 μg of each neutral sterol). The mixture was heated at 60°C for 4 h and then solvents evaporated at 60°C under N_2 . The butyl esterified bile acids, the neutral sterol standards, and fecal samples were reacted with 100 μL of Sil-prep (trimethyl silylation reagent, Alltech Associates Inc., Deerfield, IL) for 30 min at 55°C. Solvents were evaporated

at 55°C under N₂. Trimethyl silyl ether derivative of fecal samples and standards were resuspended in 200 µL hexane, centrifuged to separate the fecal debris, and injected 2 µL onto the gas-chromatography column.

Analysis was performed using a Varian Chrompak CP-Sil 5 CB Low Bleed/MS fused silica capillary column, 25 M x 0.25 mm ID x 0.25 mm film thickness (Supelco Park, Bellefonte, PA) installed in a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and autosampler. Carrier gas was helium at a flow rate of 1.5 mL/min. Injector temperature was 260°C and detector temperature was 290°C. Peaks were identified by comparing retention times with those of authentic bile acids (hyodeoxycholic acid, deoxycholic acid, hyocholic acid, cholic acid, chenodeoxycholic acid, ursodeoxycholic acid,ursocholic acid, and lithocholic acid) or neutral sterol standards (coprostanol, campesterol, stigmasterol, stigmastanol, cholestanone, cholesterol, cholestenone, cholestane, β-sitosterol, lanosterol, and lathosterol) (Steraloids Inc., Newport, RI). The weight percentage of each bile acid, fatty acid, and neutral sterol was determined by integration of the peak areas.

Other measurements

Typical intake of foods was used to assess energy needs in order to provide each subject with appropriate dietary guidelines. Twenty-four hour food records were collected at baseline, day 14, and day 28. All food records were analyzed for daily nutrient intake using Nutritionist V for Windows, version 1.7 (First Databank, The Hearst Corporation, San Bruno, CA). Physical activity during the previous seven days assessed using the Five-City Project physical activity recall (Sallis et al., 1985). Percentage body fat was assessed by dual-

energy X-ray (QDR-2000+; Hologic, Waltham, MA) after 28 day treatment period. Body weight and body mass index (BMI) were measured at the end of the 28 day feeding study.

Statistical analysis

All statistical analyses were conducted with the Statistical Analysis System (SAS, Release 8.2, SAS Institute Inc, Cary, NC). Values were expressed as means \pm SD. One-way ANOVA was used to determine the relationship between dietary treatments, age, body weight, BMI, physical activity, fecal bile acids and neutral sterols, blood lipid profiles and treatment. Results were considered significant at $P \leq 0.05$. Pairwise comparisons between means were performed using Tukey's procedure. Tukey's procedure was selected because it consecutively controls for type I error.

RESULTS

Body weight, body mass index, physical activity, and body fat content

The final mean body weights were 74.7 ± 22.7 kg, 69.5 ± 8.4 kg, and 71.7 ± 14.7 kg for the groups fed casein, β -conglycinin containing high levels of isoflavones and saponins (β -conglycinin+), and β -conglycinin containing low levels of isoflavones and saponins (β -conglycinin-) diets, respectively, and were not significantly different ($P < 0.05$). There were no significant differences between treatment groups in BMI, physical activity, or percentage body fat ($P < 0.05$) (Table 1).

Dietary intake

Consumption of muffins containing casein or β -conglycinin was well accepted by the subjects. No significant differences were found among the different groups for total fatty acids, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids intakes ($P < 0.05$) (Table 2). Polyunsaturated fatty acid intake increased in all the groups between baseline and both days 14 and 28. There were no significant differences between days 14 and 28 for polyunsaturated fatty acid intake (Table 2). The intakes of other macronutrients did not differ among the groups during the study.

Effects of soy protein on plasma lipids concentrations

At baseline, day 14, or day 28, subjects fed β -conglycinin containing low or high levels of isoflavones and saponins did not have significantly altered plasma lipids, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, and ratio of total cholesterol to HDL cholesterol, compared with the group fed casein (Table 3). At days 14 and 28, subjects fed β -conglycinin containing high levels of isoflavones and saponins had significantly lower total plasma cholesterol (6.5% and 6%, respectively) and LDL cholesterol (9% and 8%, respectively) compared with day 0 ($P < 0.05$) (Table 3). In subjects fed casein, LDL cholesterol was significantly decreased by 7.7% at day 28 compared with day 0 ($P < 0.05$) (Table 3).

When compared with day 0 (baseline), the ratio of total to HDL cholesterol was significantly lower at days 14 and 28 (7.8% and 7.3%, respectively) in women who consumed β -conglycinin containing high levels of isoflavones and saponins ($P < 0.05$) (Table 3). The ratio of total to HDL cholesterol was significantly decreased by 7.8% at day 14 compared with day 0 in women who consumed casein ($P < 0.05$) (Table 3).

Fecal bile acids and neutral sterols

There were no significant effects of the dietary treatments on either bile acid or neutral sterol outputs ($P < 0.05$) (Fig.3). The mean value of bile acid and neutral sterol outputs tended to be greater in the soy protein groups compared with the casein group.

DISCUSSION

The amount of soy protein per day (28 g) fed in this study was similar to the qualifying amount of soy protein (25 g) for the Food and Drug Administration (FDA)-approved health claim that soy protein lowers blood cholesterol (1999). In this study, feeding β -conglycinin containing low or high levels of isoflavones and saponins did not significantly alter plasma lipids, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, or ratio of total to HDL cholesterol, compared with the group fed casein at any time point. Although the majority of the literature shows that the plasma cholesterol lowering associated with soy protein intake occurs mainly in the LDL fraction (Teixeira et al., 2000; Sirtori et al., 1984; Carroll 1991; Erdman and Fordyce 1989), our results showed the lack of effect of soy protein on plasma total cholesterol and LDL cholesterol, in agreement with other human studies (Teede et al., 2001; Dewell et al., 2002; Nestel et al., 1997; Hodgson et al., 1998). In the present study, β -conglycinin containing low or high levels of isoflavones and saponins compared with casein at concentrations any time point (data not shown) did not significantly alter plasma lipids in subjects with LDL cholesterol above the median (> 4.02 mmol/L) of the population studied.

A meta-analysis of data from 38 clinical studies indicated that the changes in plasma total and LDL cholesterol concentrations were directly related to the initial plasma cholesterol concentrations (Anderson et al., 1995). Subjects with normal cholesterol levels (5.2 mmol/L) or mild hypercholesterolemia (initial values of 5.2 to 6.6 mmol/L) had nonsignificant reductions of 3.3% and 4.4%, respectively, while receiving the soy protein diet. Subjects with moderate hypercholesterolemia (initial values of 6.7 to 8.6 mmol/L) or with severe hypercholesterolemia (initial values above 8.66 mmol/L) had significant reductions of 7.4% and 19.6%, respectively. The pattern of changes in plasma LDL cholesterol was similar to the pattern for plasma total cholesterol concentrations. Potter et al. (1993) showed that 50 g of isolated soy protein/d significantly lowered total and LDL cholesterol levels by 12% and 11.5%, respectively, compared with nonfat dry milk in mildly hypercholesterolemic men with an average baseline cholesterol concentration of 5.9 mmol/L. Teixeira et al. (2000) reported that 20 g of isolated soy protein did not significantly change the concentrations of total and non-HDL cholesterol at week 3 in moderately hypercholesterolemic men aged 23-74 y (total cholesterol between 5.70 mmol/L and 7.70 mmol/L). At week 6, 20 g of isolated soy protein significantly decreased total and non-HDL cholesterol concentrations by 6.8% and 7.9%, respectively, compared with casein. Either consumption of greater amounts of soy protein/d for shorter periods of time or 28 g of soy protein/d fed for longer periods of time may be needed to achieve significant lowering of plasma lipids concentrations compared with casein.

The dietary P:S ratio may have masked the effectiveness of soy protein in this study. Widhalm (1986) reported that a linoleic acid-rich soy protein diet resulted in marked reductions in plasma cholesterol (30-32%), LDL cholesterol (36-38%), HDL cholesterol (26-

28%), and apo B (30-33%) compared with cow-milk protein diet in children heterozygous for familial hypercholesterolemia. However, Laurin et al. (1991) found that children with familial hypercholesterolemia fed soy protein had no changes in plasma cholesterol, LDL cholesterol, or apolipoprotein concentrations compared with those fed cow-milk protein. It seems likely that the lower P:S in Laurin's study (P:S=0.3) compared with Widhalm's (P:S=1.5) may partly explain the different effects of soy protein. Sirtori et al. (1979) showed that a soy protein diet containing a very low P:S (0.1) is less effective for inducing significant responses in plasma total and LDL cholesterol. The P:S ratio of 0.7, as determined by food records during the treatment period in this study, at day 14 and 28 may have masked a small effect of β -conglycinin containing high levels of isoflavones and saponins to lower plasma total and LDL cholesterol concentrations. Increased polyunsaturated fatty acids over time in all treatments may be due in part to consumption of muffin containing vegetable oil.

We observed that subjects fed β -conglycinin containing high levels of isoflavones and saponins had significantly decreased plasma total and LDL cholesterol concentrations at days 14 and 28 compared with day 0. There were no significant differences in plasma total and LDL cholesterol between casein and β -conglycinin containing high levels of isoflavones and saponins at any time point. Crouse et al. (1999) reported that consuming 25 g isolated soy protein with 62 mg of isoflavones for 9 weeks significantly lowered plasma total and LDL cholesterol by 4% and 6%, respectively, compared with casein in moderately hypercholesterolemic subjects (initial LDL cholesterol levels between 3.62 and 5.17 mmol/L). In the present study, 28 g of β -conglycinin containing approximately 55 mg of isoflavones was consumed for 4 week and the initial LDL cholesterol of subjects was between 2.58 and 5.17 mmol/L. If a higher intake of isoflavones had been consumed for a

longer period, or subjects had high initial cholesterol concentrations, perhaps a significant improvement in plasma total and LDL cholesterol would have been seen in these subjects fed β -conglycinin containing high levels of isoflavones and saponins. β -conglycinin containing low levels of isoflavones and saponins did not lower plasma cholesterol levels over time in this study. It may have been possible that the protein structure was damaged during the ethanol-washing of β -conglycinin, thus limiting the β -conglycinin efficacy in lowering plasma lipid concentrations. However, other studies showed that alcohol-washed soy protein was effective in lowering plasma lipid concentrations (Song et al., 2003; Blair et al., 2002; Balmir et al., 1996). Compared with casein, ethanol-washed isolated soy protein lowered plasma total and non-HDL cholesterol levels in male and female hamsters (Song et al., 2003). Ethanol-acetone extracted soy protein significantly lowered plasma total and non-HDL cholesterol in rats compared with casein (Balmir et al., 1996). Further studies are needed to investigate the impact of alcohol extraction on the bioactivity of soy protein.

An unexpected finding in this study was that LDL cholesterol was significantly decreased at day 28 compared with day 0 in women fed casein. Gardner et al. (2001) observed that a group fed milk protein had nonsignificant effects in reducing plasma total and LDL cholesterol over time. The changes in plasma total and LDL cholesterol were not significantly different between the milk protein and the isolated soy protein containing either trace amounts of isoflavones or 80 mg aglycone isoflavones. It seems that unidentified components associated with the casein may have caused this effect. Van der Meer et al. (1985) showed that at the normal calcium level (0.84%, w/w), casein, as compared with soy protein, increased the concentration in serum of total and free cholesterol and the ratio of free cholesterol to phospholipid in young male rabbits. An increased amount of dietary calcium

(1.44%, w/w) inhibited the effects of casein to increase intestinal phosphate absorption and the serum lipid parameters. In contrast, calcium levels did not change these parameters in rabbits fed a soy protein diet. In the present study, there were no apparent differences in calcium intake among treatment groups, which ranged from 900 to 1100 mg per day. More research is needed to explain the cholesterol-lowering effect of casein. HDL cholesterol and triglycerides were not significantly affected by dietary treatment (Table 3), consistent with other studies (Teixeira et al., 2000; Potter et al., 1993; Crouse et al., 1999).

Our data showed that subjects fed β -conglycinin containing high levels of isoflavones and saponins had significantly decreased the ratio of total to HDL cholesterol at days 14 and 28 compared with day 0. Because subjects fed β -conglycinin containing low levels of isoflavones and saponins had lower baseline ratio of total to HDL cholesterol concentrations than other groups (Table 3), albeit not statistically significant, it might have been less likely to see a significant difference in this ratio with this treatment.

It is not well known how dietary soy protein induces changes in blood lipid concentrations. Some investigators have reported that dietary soy protein increased excretion of fecal bile acids and neutral steroids (Beynen 1990; Schwerin et al., 2002 Wright and Salter 1998; Nagaoka et al., 1999). Wright and Salter (1998) showed that fecal bile acids were 2-fold greater in soy protein-fed hamsters than in hamsters fed a casein diet. Hepatic cholesterol decreased by 32%, as the amount of soy protein consumed increased from 16.3% to 36.3%, suggesting that it is this pool of cholesterol that is used to replace the excreted bile acids. Nagaoka et al. (1999) reported that serum total cholesterol was significantly lower (46%) in rats fed soy protein peptic hydrolysate with bound phospholipids (SPHP) than in those fed casein. Fecal excretion of total steroids (acidic steroids plus neutral steroids) was 2-

fold higher in rats fed SPHP than in those fed casein. Our results showed no significant treatment effects on bile acid and neutral sterol excretion. However, the mean values of both bile acid and neutral sterol outputs tended to be higher in women fed β -conglycinin containing low or high levels of isoflavones and saponins compared with women fed casein. The lack of an effect of soy protein on bile acid and neutral sterol excretions is consistent with the fact that feeding β -conglycinin did not significantly alter plasma lipids compared with subjects fed casein in the present study.

In summary, our data in mildly hypercholesterolemic, premenopausal women demonstrated a significant reduction of plasma total and LDL-cholesterol over time during ingestion of β -conglycinin only was at contained high levels of isoflavones and saponins. A slight but nonsignificant increase in bile acid and neutral sterol outputs may have contributed to these changes, but this possibility will require further study. The respective roles in cholesterol metabolism of isoflavones, saponins, and other non-protein components associated with soy protein deserve more investigation.

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Table 1. The means of body mass index (kg/m^2), body fat (% of weight), and physical activity ($\text{kcal}/\text{kg}/\text{d}$) among treatments after 28 d treatment period¹

Groups	N	Body mass index (kg/m^2)	Body fat (%)	Physical activity ($\text{kcal}/\text{kg}/\text{d}$)
Casein	18	26.7 ± 7.4	31.6 ± 5.8	35.1 ± 1.9
β-conglycinin (+)	16	24.6 ± 3.1	31.1 ± 4.4	35.9 ± 4.3
β-conglycinin (-)	18	25.6 ± 4.8	30.9 ± 5.2	34.5 ± 1.6

¹ Values represent means ± SD.

β-conglycinin (+): β-conglycinin containing isoflavones and saponins

β-conglycinin (-): β-conglycinin deficient in isoflavones and saponins

Table 2. The means of total fatty acids, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids (all as mg/day) from 24-hour food record data compared across treatments¹

	Casein (n=18)	β-conglycinin (+) (n=16)	β-conglycinin (-) (n=18)
Total fatty acids²			
Baseline	50 ± 28	62 ± 20	64 ± 30
Day 14	61 ± 21	65 ± 22	71 ± 25
Day 28	55 ± 32	64 ± 24	65 ± 22
Saturated fatty acids³			
Baseline	17 ± 11	22 ± 11	22 ± 13
Day 14	19 ± 9	20 ± 10	20 ± 7
Day 28	19 ± 12	18 ± 10	21 ± 11
Monounsaturated fatty acids³			
Baseline	14 ± 9	19 ± 7	16 ± 12
Day 14	16 ± 7	18 ± 8	18 ± 9
Day 28	13 ± 9	19 ± 12	18 ± 7
Polyunsaturated fatty acids³			
Baseline	9 ± 4	9 ± 4	9 ± 6
Day 14	15 ± 4 ^a	15 ± 5 ^a	19 ± 10 ^a
Day 28	13 ± 9 ^a	14 ± 5 ^a	15 ± 6 ^a

¹ Values represent means ± SD.

^{2,3} For females 19-40 years, recommended total fatty acid intake is 73 gm/d, saturated fatty acid or monounsaturated fatty acid or polyunsaturated fatty acid is 24 gm/d.

^a Significantly different from baseline at $p < 0.05$

β-conglycinin (+): β-conglycinin containing isoflavones and saponins

β-conglycinin (-): β-conglycinin deficient in isoflavones and saponins

Table 3. Lipid and lipoprotein concentrations at day 0, 14, and 28 in participants consuming 28 g of β -conglycinin containing variable amounts of isoflavones and saponins or 28 g of casein¹

	Casein (n=18)	β-conglycinin (+) (n=16)	β-conglycinin (-) (n=18)
Cholesterol (mmol/L)			
Baseline	5.60 \pm 0.87	5.48 \pm 0.68	5.60 \pm 1.03
Day 14	5.44 \pm 0.91	5.12 \pm 0.69 ^a	5.43 \pm 0.80
Day 28	5.44 \pm 0.83	5.18 \pm 0.59 ^a	5.42 \pm 0.86
LDL (mmol/L)			
Baseline	3.62 \pm 0.65	3.62 \pm 0.65	3.56 \pm 0.76
Day 14	3.42 \pm 0.67	3.28 \pm 0.68 ^a	3.43 \pm 0.68
Day 28	3.34 \pm 0.60 ^a	3.32 \pm 0.58 ^a	3.40 \pm 0.59
HDL (mmol/L)			
Baseline	1.36 \pm 0.53	1.28 \pm 0.18	1.38 \pm 0.34
Day 14	1.40 \pm 0.52	1.31 \pm 0.18	1.40 \pm 0.35
Day 28	1.36 \pm 0.50	1.32 \pm 0.19	1.42 \pm 0.34
Triglyceride (mmol/L)			
Baseline	1.38 \pm 0.50	1.27 \pm 0.38	1.46 \pm 0.70
Day 14	1.36 \pm 0.54	1.18 \pm 0.41	1.31 \pm 0.61
Day 28	1.61 \pm 0.63	1.19 \pm 0.40	1.31 \pm 0.82
Total/HDL cholesterol			
Baseline	4.54 \pm 1.29	4.34 \pm 0.79	4.24 \pm 1.02
Day 14	4.18 \pm 0.98 ^a	4.00 \pm 0.89 ^a	4.06 \pm 0.98
Day 28	4.39 \pm 1.24	4.02 \pm 0.87 ^a	3.99 \pm 0.98

¹ Values represent means \pm SD.

^a Significantly different from baseline at $p < 0.05$

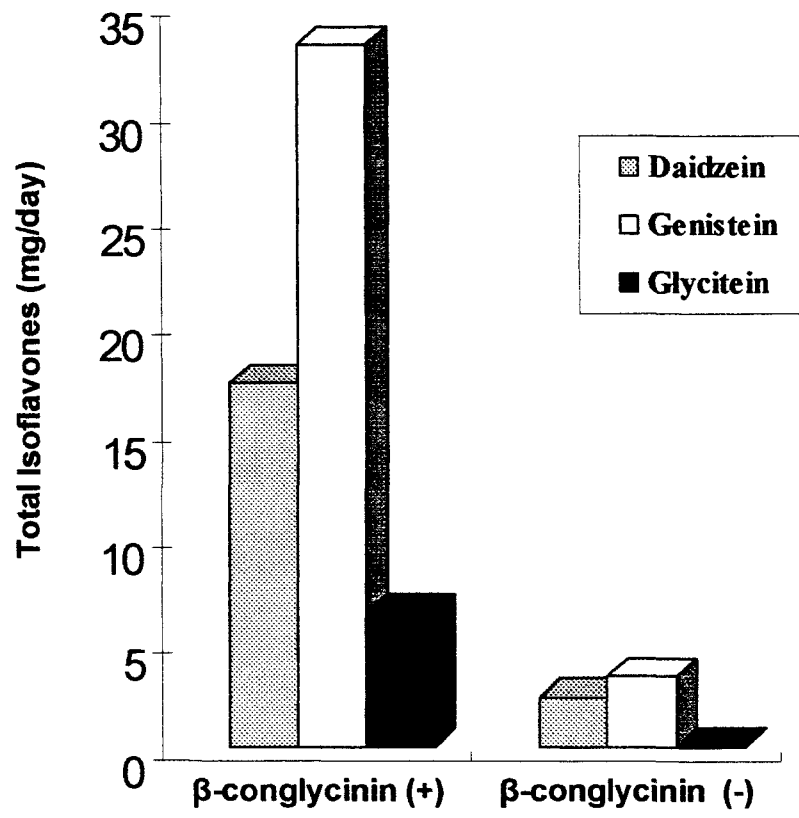
β -conglycinin (+): β -conglycinin containing isoflavones and saponins

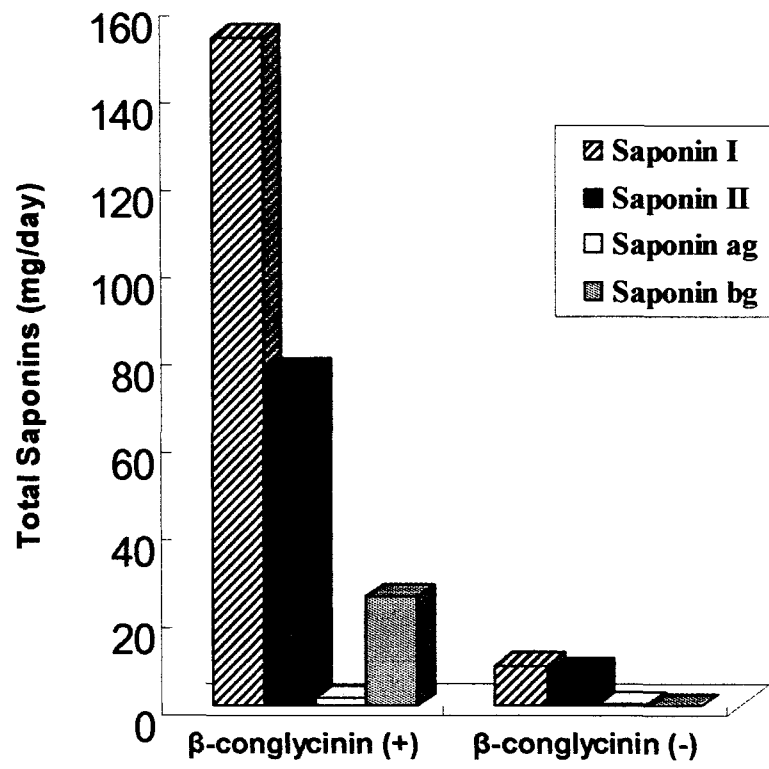
β -conglycinin (-): β -conglycinin deficient in isoflavones and saponins

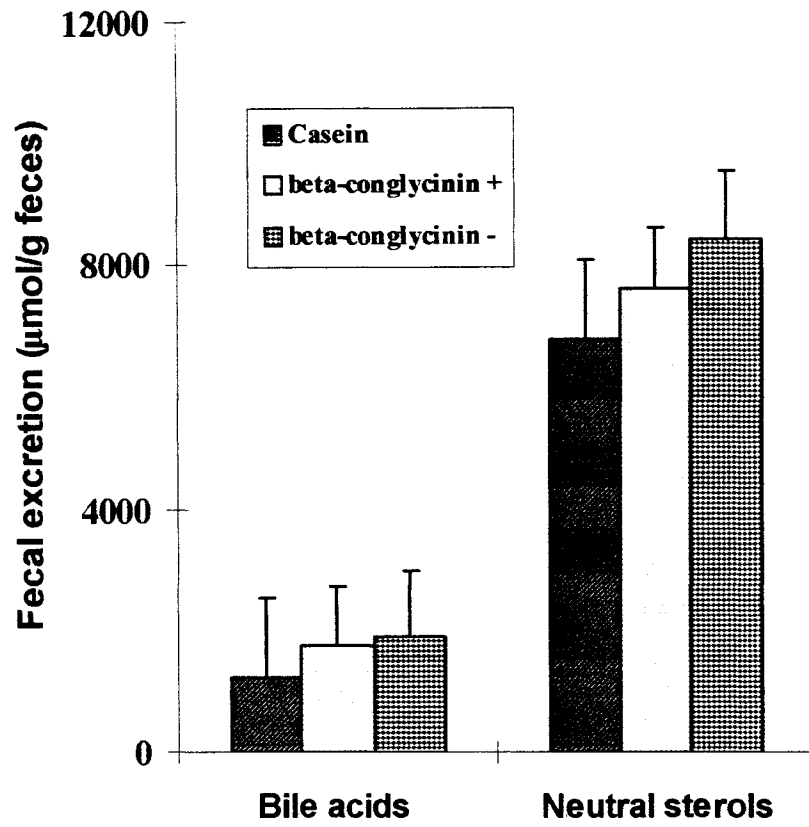
Fig 1. Isoflavone contents in a diet containing intact β -conglycinin (β -conglycinin+) or ethanol-washed β -conglycinin (β -conglycinin-).

Fig 2. Saponin contents in a diet containing intact β -conglycinin (β -conglycinin+) or ethanol-washed β -conglycinin (β -conglycinin-).

Fig. 3 Mean values for fecal output of bile acids and neutral sterols in subjects who consumed β -conglycinin with isoflavones and saponins (β -conglycinin+) or β -conglycinin deficient in soy isoflavones and saponins (β -conglycinin-). Bile acid and steroid excretion is expressed per gram feces (dry weight). Values are represented as mean \pm SD.







THE ISOFLAVONE GLYCITEIN LOWERED PLASMA CHOLESTEROL IN FEMALE GOLDEN SYRIAN HAMSTERS

A paper to be submitted to Experimental Biology and Medicine

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ABSTRACT

The soybean isoflavones, daidzein, genistein, and glycitein, were hypothesized to act as cholesterol-lowering components, separate from soy protein. Pure synthetic daidzein, genistein, or glycitein was fed to female Golden Syrian hamsters (11-12 weeks of age, 10 hamsters/treatment) for 4 weeks in a diet containing ~37% of energy as fat (62% from coconut oil), 25% casein and 0.1% cholesterol. The content of each isoflavone was ~30% less than the total amount of isoflavones in a diet based on 25% isolated soy protein, 0.9 mmol/kg diet. Hamsters fed glycitein had significantly lower plasma total cholesterol by 15% and lower non-HDL cholesterol by 24% compared with those fed casein ($P < 0.05$). Daidzein and genistein's effects on these lipids did not differ from the effects of either casein or glycitein. Plasma HDL cholesterol and triglyceride concentrations were not significantly affected by dietary treatments. The percentage of ingested dose recovered in urine for each isoflavone was glycitein > daidzein > genistein (32.2% > 4.6% > 2.2%). These data suggest that glycitein's greater cholesterol-lowering effect was due to its greater bioavailability, as reflected in urinary recovery of glycitein compared with the other purified isoflavones.

Keywords: daidzein, genistein, glycitein

INTRODUCTION

Soy isoflavones are major phytoestrogens in soybeans and soy foods. There is a great interest in soy isoflavones, daidzein, genistein, and glycitein, regarding their possible effects on plasma lipid levels. The role of soy isoflavones in lowering plasma concentrations of lipoproteins and cholesterol has been studied in animals (Balmir, et al., 1996; Tovar-Palacio et al., 1998; Anthony et al., 1996 and 1997; Kirk et al., 1998; Ni et al., 1999; Clarkson et al., 2001; Song et al., 2003) and humans (Crouse et al., 1999; Merz-Demlow et al., 2000; Gardner et al., 2001; Wangen et al., 2001).

Animal studies showed that isolated soy protein containing isoflavones or alcohol extracts of soy protein rich in isoflavones have a greater hypocholesterolemic effect on plasma total and non-HDL cholesterol concentrations. Soy protein isolate (ISP) containing isoflavones (1.0 mmol/kg diet) significantly lowered plasma total and LDL+VLDL cholesterol concentrations in ovariectomized monkeys compared with isoflavones-depleted ISP (0.005 mmol/kg diet) (Clarkson et al., 2001). Balmir et al. (1996) reported a significant reduction in plasma LDL cholesterol in male hamsters fed diets containing intact ISP, ISP with added ethanol extract (providing 0.6 mmol total isoflavones/kg diet), or casein with soy ethanol extract (providing 0.3 mmol total isoflavones/kg diet) compared with those fed casein. Several human studies also reported that consumption of a high level of isoflavones in the diet have more effective in improving plasma lipid profiles. Crouse et al. (1999) found that consumption of 25 g isolated soy protein with 62 mg isoflavones (approximately 0.5 mmol/kg diet) significantly reduced plasma total and LDL cholesterol concentrations compared with casein. Plasma LDL cholesterol concentration was significantly lowered in premenopausal women consuming 53 g isolated soy protein with 129 mg isoflavones

(approximately 1.0 mmol/kg diet) compared with women consuming isolated soy protein contained 10 mg isoflavones (approximately 0.08 mmol/kg diet) (Merz-Demlow et al., 2000).

Recent studies investigated the cholesterol-lowering effects of pure synthetic isoflavones or isolated pure isoflavones from soybean fraction (Uesugi et al., 2001; Demonty et al., 2002; Nogowski et al., 1998; Song et al., 2003). Ovariectomized rats fed isolated pure isoflavone glycosides daidzin (25 or 50 mg/kg body weight/d) or genistin (50 mg/kg body weight/d) orally using a stomach tube showed significantly lower plasma total cholesterol and triglyceride concentrations compared with casein-fed rats (Uesugi et al., 2001). Plasma triglyceride was significantly lowered in Sprague-Dawley rats fed a mixture of synthetic daidzein and genistein (1.4 mmol/kg diet or ~23.6 mg/kg body weight/d) compared with those fed casein (Demonty et al., 2002). Song et al. (2003) reported that hamsters fed pure synthetic daidzein (1.3 mmol/kg diet or ~16 mg/kg body weight/d) had significantly lower plasma total and non-HDL cholesterol compared with those fed casein.

Isoflavones contents in urine or plasma may be considered as a biological marker of soy consumption and it is relatively easy to quantify the amount of isoflavones. Xu et al. (1994) found that daidzein was more bioavailable than genistein, as reflected in urinary recovery of daidzein (21%) compared with that of genistein (9%). Zhang et al. (1999) reported that the average 48 h urinary recoveries of daidzein, glycitein, and genistein were approximately 52%, 47%, and 37%, respectively, suggesting that bioavailability of daidzein and glycitein were similar, both being greater than genistein.

We hypothesized that soy isoflavones are responsible in large part for the beneficial effects of soy on plasma lipid concentrations and that glycitein may have more cholesterol-

lowering effects because glycitein is more absorbable compared with the other two purified isoflavones in hamsters, based on previous unreported studies in our laboratory. To test these hypotheses, we fed pure synthetic daidzein, genistein, or glycitein to hamsters and then measured plasma lipid concentrations and determined the association between urinary recovery of each isoflavone and plasma lipid concentrations. Hamsters were selected as an animal model because there are several similarities in cholesterol metabolism between hamsters and humans. Hamsters fed a diet enriched with saturated fat show a substantial increase in plasma total and LDL cholesterol (Terpstra et al., 1991). There is a comparable bile acid pool composition between hamsters and humans (Bravo et al., 1994).

MATERIALS AND METHODS

Chemicals and Diets

The method of Chang et al. (1994) was modified to synthesize daidzein (Song et al., 1998). Genistein was synthesized by a method of Chang et al. (1994). Glycitein was synthesized according to Lang'at-Thoruwa et al. (2003). The purity of isoflavones, determined by HPLC chromatogram peak area and Beckman Gold HPLC system peak purity software, was > 97%. All chemicals for isoflavone synthesis were purchased from Sigma-Aldrich (St. Louis, MO).

All dietary ingredients except rice flour were obtained from Harlan Teklad (Madison, WI). Rice flour was purchased from Bioserve (Frenchtown, NJ). Four treatments were fed: casein as control, daidzein, genistein, or glycitein (Table 1). The content of each isoflavones was ~30% less than the total molar isoflavones content of isolated soy protein, 0.9 mmol/kg diet. The dose of each isoflavones was approximately 12.4 to 13.5 mg/kg body weight/day.

The casein diet did not contain isoflavones. The experimental diets contained ~37% of energy as fat, 25% casein, and 0.1% cholesterol. Rice flour was used as a carbohydrate source because rice flour prevents “wet tail” disease, a form of chronic diarrhea with a high rate of mortality for hamsters (Terpstra et al. 1991).

Animals

The use of animals and the experimental protocol were approved by Iowa State University Animal Care Center Committee. Forty female Golden Syrian hamsters, 11-12 weeks old, were purchased from Harlan Teklad (Madison, WI) and housed individually in temperature-controlled room (23°C) with a 12-h light : dark cycle. Hamsters were randomly assigned to four treatments with the same average body weight in each group. Hamsters had free access to food and water during the 4-week experimental period. Body weights were measured weekly and food intakes were measured daily. At the end of the feeding period, diets were withdrawn from hamsters 16-18 hours before they were sacrificed under CO₂. Blood was collected by cardiac puncture and centrifuged at 5000 x g for 15 min at 4°C to prepare plasma that was stored at -20°C until analysis.

Plasma lipid analysis

Plasma total cholesterol and HDL cholesterol concentration were measured with Sigma diagnostics kits (St. Louis, MO). Plasma triglycerides were measured with Thermo Trace kits (Louisville, CO). Non-HDL cholesterol was calculated by subtraction of HDL cholesterol from total cholesterol and represented LDL+IDL+VLDL cholesterol.

Analysis of urinary excretion of isoflavones

Hamsters were put in metabolic cages for 24 hours to collect urine during the last 2 or 3 days of the feeding period. Urine sample (5 mL) was mixed with 5 mL sodium acetate buffer (0.2 M, pH 5.5), 50 μ L of β -glucuronidase/sulfatase (H_2 type, Sigma-Aldrich, St. Louis, MO), and 50 μ L of 2,4,4-trihydroxydeoxybenzoin (THB as an internal standard, 2 mg/ml) in a tube. After incubation for 20 hours at 37°C on a shaker, 9 mL of sodium phosphate buffer (10 mM, pH 7.0) was added and the solution was passed through an Extrelut QE column (EM Science, Gibbstown, NJ). The column was washed 2 times with 18 mL ethyl acetate (HPLC grade) and one time with 10 mL ethyl acetate (HPLC grade). The effluent was collected in a round-bottom flask, and dried in a rotary evaporator (Buchler Instrument, Fortlee, NJ). The dried residues were dissolved in 9.8 mL 20% ethanol and acidified by 200 μ L of 1 N HCl. Five mL of ethanol-dissolved residue was slowly applied to a pre-conditioned Sep-Pak cartridge (C18, Waters Corp. Milford, MA). The cartridge was washed 2 times with 2 mL distilled water to remove the water soluble impurities and the eluent recovered in 2 mL of 80% methanol. The extract was vortexed and filtered through a 0.45 mm PTFE filter (Alltech Associates Inc., Deerfield, IL) prior to HPLC analysis.

The HPLC analysis was carried out on a Hewlett Packard 1050 Series HPLC system. This system consisted of a photodiode array detector (PDA), a quadruplicate pumping system, an autosampler and an HP Vectra 486/66 personal computer with Chem Station 3D software (Hewlett Packard Company, Scientific Instruments Division, Palo Alto, CA). Daidzein, genistein, glycitein, and THB were detected and quantified on a YMC-pack ODS-AM C18 reverse phase column (5 μ m pore size, 25 cm x 4.6 mm, YMC, Inc., Wilmington, NC). Elution was carried out at a flow rate of 1 mL/min at ambient temperature with the

following solvent system: 0.1% acetic acid in Milli-Q H₂O (Millipore Co., Bedford, MA) (solvent A) and methanol (solvent B). After injection of 20 µL sample, solvent B was increased from 30% to 50% in 45 min, then held at 50% for 10 min, and decreased to 30% in 10 minutes. Analytes were monitored with PDA from 190 to 350 nm. Ultraviolet absorbance spectra were recorded and area responses were integrated by Chem Station 3D Software to identify isoflavones. Recovery based on the THB internal standard was 84%, and recovery-adjusted urinary excretion of each isoflavone concentration as a percentage of ingested dose was reported.

Statistical analysis

All statistical analyses were conducted with the Statistical Analysis System (SAS, Release 8.2, SAS Institute Inc., Cary, NC). Values were expressed as means \pm SD. The results were analyzed by one-way analysis of variance (ANOVA). Differences between treatments were determined by least significant difference test. An α of 0.05 was used to determine statistically significant differences.

RESULTS

Body weights and food intakes

The powdered diets were well accepted by hamsters throughout the experiment. There were no daily food intake differences among dietary treatment groups (Table 2). Hamsters in all groups had similar initial mean body weights. No significant differences were found among the different groups for the final mean body weight (Table 2).

Effects of isoflavones on plasma lipid concentrations

Hamsters fed glycitein had significantly lower total cholesterol and non-HDL cholesterol concentrations ($P < 0.05$) compared with those fed casein (Table 3). Plasma HDL cholesterol and triglyceride concentrations were not significantly affected by dietary treatments (Table 3).

Percentage of urinary recovery of each isoflavone

The percentage of urinary recovery of ingested dose of each isoflavone was glycitein ($32.2 \pm 29.7\%$) > daidzein ($4.6 \pm 1.8\%$) > genistein ($2.2 \pm 1.7\%$) (Figure 3). Hamsters fed glycitein had significantly greater urinary isoflavone recovery ($P < 0.05$) compared with those fed daidzein or genistein (Figure 3). Seemingly, glycitein was more available in the body compared with the other two dietary isoflavones.

DISCUSSION

The present study was designed to determine if daidzein, genistein, or glycitein lowered plasma cholesterol in female Golden Syrian hamsters fed these compounds. Pure synthetic isoflavones were added to a casein-based diet to avoid the possible confounding effects of other alcohol extractable components of soy protein such as soyasaponins.

Hamsters fed pure synthetic glycitein had lower plasma total and non-HDL cholesterol concentrations by 15% and 24%, respectively, compared with those fed casein. Daidzein or genistein showed intermediate cholesterol-lowering effects compared with either casein or glycitein (Table 3). These results confirmed our hypothesis that soy isoflavones play a role in lowering plasma cholesterol concentrations, separate from soy protein.

In a previous study (Song et al., 2003), hamsters fed pure synthetic daidzein at 1.3 mmol/kg diet (or ~16 mg/kg body weight/d) for 10 weeks had significantly lower plasma total and non-HDL cholesterol concentrations by 22-24% and 30-38% compared with those fed casein. In the present study, the content of daidzein was ~30% less than Song's study (1.3 mmol daidzein/kg diet), 0.9 mmol/kg diet (or 12 mg to 13 mg/kg body weight/d). After a 4 wk feeding period, daidzein lowered plasma total and non-HDL cholesterol by 7% and 14%, respectively, compared with casein. Perhaps if a longer experimental period was conducted, a significant reduction of plasma total and non-HDL cholesterol might be observed with daidzein as well. Demonty et al. (2002) reported that a mixture of pure synthetic daidzein and genistein at 1.4 mmol/kg diet (or ~23 mg/kg body weight/d) significantly lowered plasma triglycerides by 26% compared with casein in Sprague-Dawley rats. Ovariectomized rats fed isolated pure isoflavone glycosides daidzin (25 or 50 mg/kg body weight/d) or genistin (50 mg/kg body weight/d) orally using a stomach tube resulted in significantly lower plasma total cholesterol (by 23-44%) and triglyceride (by 24-46%) concentrations compared with casein (Uesugi et al., 2001). Based on the results from these studies, soy isoflavones have the ability to lower plasma lipid concentrations, separate from soy protein in rodents.

Potential mechanisms of soy isoflavones in lowering plasma cholesterol levels are not clear. Soy isoflavones have similar structure to natural estrogens and can bind estrogen receptors (ER- α and - β), especially ER- β . The loss of estrogen increased plasma concentrations of total cholesterol, LDL cholesterol, and triglycerides and decreased HDL cholesterol in women (Jensen, 1992; Gaspard et al., 1995). Jensen (1992) investigated the changes of plasma lipid concentrations after natural menopause in 170 premenopausal women. After menopause, women significantly increased plasma total (by 6%) and LDL

cholesterol (by 8%) and decreased HDL cholesterol (by 7%) level. It may be possible that soy isoflavones may affect plasma lipid concentrations through their estrogenic activity. Angelin et al. (1992) obtained liver biopsies from male patients with either estrogen or non-estrogen treatment (control) and examined hepatic cholesterol metabolism. Hepatic ^{125}I -LDL binding activity (reflecting the expression of the LDL receptor) was increased in the estrogen-treated men compared with control. Kirk et al. (1998) reported that soy protein isolate containing isoflavones at 1.33 mmol/kg diet lowered plasma LDL cholesterol by 30% in C57BL/6 mice compared with soy protein lacking isoflavones. However, no cholesterol-lowering effect by soy protein was observed in LDL receptor-deficient mice. These findings suggest that soy isoflavones may lower LDL cholesterol concentration through increased gene expression and activity of hepatic LDL receptor. Decreased non-HDL cholesterol levels observed in the present study may be explained by this possible mechanism. The potential mechanisms of isoflavones to reduce plasma lipid concentrations should be further investigated.

In this study, there were no significant differences in plasma HDL cholesterol and triglyceride concentrations among different dietary treatments. These results were consistent with other studies (Balmir, et al., 1996; Ni et al., 1999). Balmir et al. (1996) reported that plasma HDL cholesterol and triglyceride concentrations were not significantly affected by dietary treatments (casein, casein with added alcohol extract containing approximately 0.3 mmol total isoflavones/kg diet, casein with twice the level of extract providing approximately 0.6 mmol total isoflavones/kg diet, isolated soy protein (ISP), and ISP with alcohol extract) in hamsters. Female hamsters fed daidzein had no difference in plasma HDL cholesterol compared with casein (Song et al., 2003).

The percentage of urinary recovery of ingested doses of each isoflavone was greater in hamsters fed glycitein than in those fed the other two isoflavones (Figure 1). The percentage of urinary recovery of each isoflavone was glycitein > daidzein > genistein in this study. These results were similar to that of a previous study (unpublished data) in our laboratory that urinary isoflavone recovery was glycitein > daidzein > genistein in hamsters fed Novasoy containing 1.2 mmol or 1.8 mmol total isoflavones/kg diet. A different pattern of urinary recovery in human was reported by Zhang et al. (1999). The urinary recoveries in humans of daidzein, glycitein, and genistein were approximately 52%, 47%, 37%, respectively. The results suggest some differences between humans and hamsters probably due to differences between the species in metabolism and degradation of each isoflavone by intestinal microbes. In a previous study by Zheng et al. (2003), women who showed low fecal genistein disappearance rate and faster gut transit time had greater genistein bioavailability, as reflected in urinary recovery of genistein compared with women who had high genistein disappearance rate and long gut transit time. When seven subjects consumed isoflavones from soymilk, two of the subjects showed greater urinary recovery of ingested doses of isoflavones compared with other subjects (Xu et al., 1995). Hendrich et al. (1998) showed three different isoflavone disappearance phenotypes in humans and a negative correlation between plasma isoflavone concentrations and fecal isoflavone disappearance rate constant in 8 young adult men. These findings suggested that individual variability in metabolic response to isoflavones may be due to differences in the relative ability of gut microorganisms to degrade isoflavones, perhaps accounting for differences among species and isoflavones in apparent isoflavone absorption.

In conclusion, this study demonstrated glycitein's ability to lower plasma lipid concentrations. In female hamsters, glycitein's greater cholesterol-lowering effect is probably due to greater bioavailability, as reflected in urinary recovery of glycitein compared with the other purified isoflavones.

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Table 1. Composition of experimental diets.

Ingredient	Casein	Daidzein g/kg	Genistein	Glycitein
Casein ¹	250	0	0	250
Isoflavones	0	0.23	0.24	0.25
Coconut oil	100	100	100	100
Safflower oil	20	20	20	20
Soybean oil	40	40	40	40
Cellulose	75	75	75	75
Wheat bran	75	75	75	75
Choline chloride	3	3	3	3
Vitamin mix ²	10	10	10	10
Mineral mix ³	35	35	35	35
Potassium bicarbonate	20	20	20	20
Cholesterol	1	1	1	1
Rice flour ⁴	371	370.77	370.76	370.75

¹Vitamin-free casein (Harlan/Teklad, Madison, WI)

²Vitamin mixture #400160 (Harlan/Teklad, Madison, WI)

³Mineral mixture #170910 (Harlan/Teklad, Madison, WI)

⁴Rice flour (Bioserve, Frenchtown, NJ).

Table 2. Body weight change and food intake of hamsters fed casein or casein-based diets with each soy isoflavone¹

Treatment	Food Intake g/d	Initial body weight g	Final body weight g
Casein	7.5 ± 1.2	111 ± 10	126 ± 9
Daidzein	7.7 ± 1.8	111 ± 10	126 ± 11
Genistein	7.4 ± 1.6	112 ± 11	128 ± 13
Glycitein	7.3 ± 1.5	111 ± 11	127 ± 13

¹Values represent means ± SD, n=10.

Table 3. Plasma cholesterol levels in hamsters fed casein diets containing daidzein, genistein, or glycitein^{1,2}

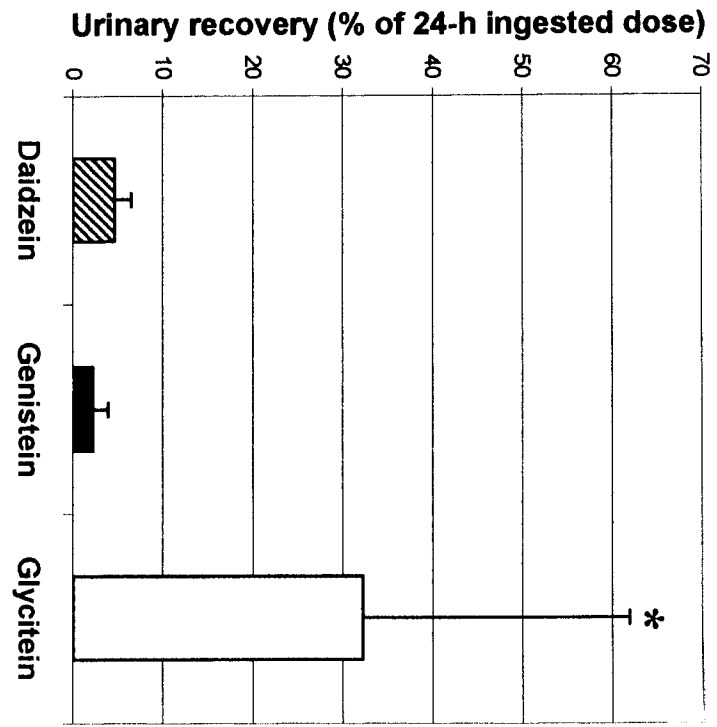
Treatment	Total cholesterol mmol/L	HDL cholesterol mmol/L	Non-HDL ³ cholesterol mmol/L	Total cholesterol /HDL cholesterol	Triglyceride mmol/L
Casein	6.33±1.32 ^a	3.75±0.53	2.58±0.81 ^a	1.69±0.24 ^a	2.67±0.24
Daidzein	5.87±0.99 ^{ab}	3.66±0.58	2.22±0.75 ^{ab}	1.61±0.26 ^{ab}	2.22±0.57
Genistein	5.61±0.87 ^{ab}	3.43±0.64	2.19±0.53 ^{ab}	1.64±0.14 ^{ab}	2.40±0.68
Glycitein	5.34±0.61 ^b	3.38±0.86	1.95±0.37 ^b	1.58±0.19 ^b	2.29±0.75

¹Values represent means ± SD, n=10;

²Within a column, means with different superscripts are different (P < 0.05);

³Represents the VLDL+IDL+LDL fractions (by difference: Total - HDL).

Figure 1. Percentage of urinary isoflavone recovery is a percentage of daily ingested dose in hamsters fed daidzein, genistein, or glycitein diets ($P < 0.05$). Data are reported as mean \pm SD, significant difference is noted by an asterisk.



**GROUP B SOYASAPONINS LOWER PLASMA CHOLESTEROL AND
INCREASE FECAL BILE ACIDS IN FEMALE GOLDEN SYRIAN
HAMSTERS**

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ABSTRACT

A study was conducted in hamsters to determine if group B soyasaponins affect plasma lipid concentrations by increasing excretion of fecal bile acids and neutral sterols, to investigate the relationship between group B soyasaponin metabolite and plasma lipid levels, and to identify group B soyasaponin metabolites. Twenty female Golden Syrian hamsters, 11-12 week old, 85-125 g, were randomly assigned to one of two dietary treatments fed for 4 weeks. The content of group B soyasaponins was 2.2 mmol/kg diet and no isoflavones were detected in the group B soyasaponin diet. Hamsters fed group B soyasaponins had significantly lower plasma total cholesterol (by 20%), non-HDL cholesterol (by 33%), and triglyceride (by 18%) concentrations compared with those fed casein ($P < 0.05$). The ratio of total to HDL cholesterol was significantly lower in group B soyasaponins than casein ($P < 0.05$). The excretion of fecal bile acids and neutral steroids were significantly greater in the soyasaponin group compared with the casein group ($P < 0.05$). Thus, group B soyasaponins lowered plasma total and non-HDL cholesterol levels by a mechanism involving greater excretion of fecal bile acids and neutral steroids compared with casein. Hamsters fed group B soyasaponins sorted into two fecal soyasaponins metabolite excretion phenotypes: high ($n=3$)

and low excretors (n=7). When low and high producers of a putative soyasaponin metabolite were compared for plasma cholesterol status, the high producers showed significantly lower total cholesterol/HDL cholesterol ratio compared with the low producers (1.38 ± 0.7 v. 1.59 ± 0.13), $P < 0.03$. Greater production of group B soyasaponin metabolite in hamsters was associated with better plasma cholesterol status, suggesting that gut microbial variation in soyasaponin metabolism may influence the health effects of group B soyasaponins.

Keywords: group B soyasaponin, soyasaponin metabolite

INTRODUCTION

Soyasaponins are one of the major class of phytochemicals associated with soy protein. The primary saponins in soybeans are group A and B soyasaponins on the basis of their aglycone parts, soyasapogenols A and B (Gurfinkel et al., 2002; Rupasinghe et al., 2003). The content of group B soyasaponins in whole soybean seeds is about 60-75% by weight of total soyasaponins (Gu et al., 2002; Ireland et al., 1986). Soybeans, isolated soy protein, and soy products provide soyasaponins approximately 1.4 to 5.9 $\mu\text{mol/g}$, 9.5 to 10.6 $\mu\text{mol/g}$, and 1.5 to 4.5 $\mu\text{mol/g}$ dry weight basis, respectively (Gu et al., 2002; Hu et al., 2002; Tsukamoto et al., 1995).

Soyasaponins have been suggested as a factor capable of lowering plasma cholesterol concentrations (Oakenfull et al., 1984; Potter et al., 1995; Ueda et al., 1996; Fukui et al., 2002). Plasma total cholesterol was significantly decreased in male Sprague-Dawley rats fed soy protein isolate, either intact or depleted of isoflavones but containing 49% of the amount of saponins present in the soy protein isolated-based diet compared with those fed casein (Fukui et al., 2002). Rats fed a 1% cholesterol diet with 1% purified soyasaponins had

significantly lowered plasma and hepatic total cholesterol levels compared with those fed the 1% cholesterol diet (Oakenfull et al., 1984).

The potential mechanisms responsible for the hypocholesterolemic effects of soyasaponins or synthetic saponins have been investigated (Oakenfull et al., 1984; Oakenfull and Sidhu, 1990; Morehouse et al., 1999; Oakenfull, 2001). Synthetic saponins, pamaqueside and tiqueside, significantly decreased intestinal absorption of cholesterol compared with a control diet (Morehouse et al., 1999). Oakenfull et al. (1984) found that 1% soyasaponins significantly increased fecal bile acid and neutral sterol excretion compared with a control diet without soyasaponin. Studies have yielded evidence that cholesterol-lowering effects of saponins may be mediated by decreased intestinal absorption of cholesterol or enhanced fecal excretion of bile acids or neutral sterols.

Little is known about the metabolism and bioavailability of soyasaponins (Gestetner et al., 1968; Gurfinkel and Rao, 2003; Hu et al., in press, a and b). In an *in vitro* fermentation system, soybean saponins were hydrolyzed to soyasapogenols (aglycone saponins), partially hydrolyzed forms, and sugars by colonic microflora (Gurfinkel and Rao, 2003; Hu et al., in press, a). *In vivo*, neither soyasaponins nor soyasapogenols were detected in the blood of rats, mice, and chicks (Gestetner et al., 1968) and in human urine (Hu et al., in press, b).

The present study was designed to investigate whether group B soyasaponins affect plasma lipid concentrations by increasing excretion of fecal bile acids and neutral sterols, to investigate the relationship between group B soyasaponin metabolite and plasma lipid levels, and to identify group B soyasaponin metabolites in female hamsters. To avoid the confounding effects of other alcohol-extractable components in soy, crude group B soyasaponins were fed in this study.

MATERIALS AND METHODS

Diets

Crude group B soyasaponins were generously donated by Dr. Mark Berhow (United States Department of Agriculture, National Center for Utilization of Agricultural Products, Peoria, IL). The soyasaponin fractions of crude group B soyasaponins were analyzed by HPLC (Hu et al., 2002).

All dietary ingredients except rice flour were obtained from Harlan Teklad (Madison, WI). Rice flour was purchased from Bioserve (Frenchtown, NJ). Two treatments were fed: casein as control or group B soyasaponins (Table 1). The amount of group B soyasaponins fed in this study (Table 2) was similar to the amount of soyasaponins in a diet based on 25% isolated soy protein, 2.2 mmol/kg diet. The dose of group B soyasaponins was approximately 128 mg/kg body weight/d. No isoflavones were detected in group B soyasaponin diet. The experimental diets contained ~37% of energy as fat, 25% casein and 0.1% cholesterol. Rice flour was used as a carbohydrate source to replace cornstarch in the standard rodent diet because rice flour prevents a chronic diarrhea that cause a high rate of mortality for hamsters (Terpstra et al. 1991).

Animals

The use of animals and the experimental protocol were approved by Iowa State University Animal Care Center Committee. Twenty female golden Syrian hamsters, 11-12 week old, 85-125 g, were purchased from Harlan Teklad (Madison, WI) and housed individually in temperature-controlled room (23°C) with a 12 hours light : dark cycle.

Hamsters were randomly assigned to two treatments with the same average body weight in each group. Hamsters had free access to food and water during the 4-week experimental period. Body weights were measured weekly and food intakes were measured daily. At the end of the feeding period, diets were withdrawn from hamsters 16-18 hours before they were sacrificed under CO₂. Blood was collected by cardiac puncture and centrifuged at 5000 x g for 15 min at 4°C to prepare plasma that was stored at -20°C until analysis.

Plasma lipid analysis

Plasma total cholesterol and HDL cholesterol concentration were measured with Sigma diagnostics kits (St. Louis, MO). Plasma triglycerides were measured with Thermo Trace kits (Louisville, CO). Non-HDL cholesterol was calculated by subtraction of HDL cholesterol from total cholesterol and represented LDL+IDL+VLDL cholesterol.

Analysis of fecal bile acids and neutral sterols

Fecal samples were collected over 24-hour intervals for the last 2 or 3 days of the feeding period. Quantitation of bile acids and neutral sterols in feces was measured by gas-liquid chromatography according to the method of Batta et al. (2002). Two hundred microliters of n-butanol which contained cholic acid and 5 α -cholestane (internal standards) and 50 μ L of concentrated hydrochloric acid were added to 15 mg of freeze-dried fecal samples and to the standards (10 and 20 μ g of each bile acid and neutral sterol). The mixture was heated at 60°C for 4 hours and then solvents evaporated at 60°C under N₂. The butyl esterified bile acids, the neutral sterol standards, and fecal samples were reacted with 100 μ L of Sil-prep (trimethyl silylation reagent, Alltech Associates Inc., Deerfield, IL) for 30

minutes at 55°C. Solvents were evaporated at 55°C under N₂. Trimethyl silyl ether derivative of fecal samples and standards were resuspended in 200 µL hexane, centrifuged to separate the fecal debris, and injected 2 µL onto the gas-chromatography column.

Analysis was performed using a Varian Chrompak CP-Sil 5 CB Low Bleed/MS fused silica capillary column, 25 M x 0.25 mm ID x 0.25 mm film thickness (Supelco Park, Bellefonte, PA) installed in a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and autosampler. Carrier gas was helium at a flow rate of 1.5 mL/min. Injector temperature was 260°C and detector temperature was 290°C. Peaks were identified by comparing retention times with those of authentic bile acids (hyodeoxycholic acid, deoxycholic acid, hyocholic acid, cholic acid, chenodeoxycholic acid, ursodeoxycholic acid, ursocholic acid, and lithocholic acid) or neutral sterol standards (coprostanol, campesterol, stigmasterol, stigmastanol, cholestanone, cholesterol, cholestenone, cholestane, β-sitosterol, lanosterol, and lathosterol) (Steraloids Inc., Newport, RI). The weight percentage of each bile acid and neutral sterol was determined by integration of the peak areas.

Extraction of fecal samples

A method of Hu et al. (2004a) was used to extract fecal samples. Hamsters were put in metabolic cages for 24 hours to collect fecal samples. One gram of ground fecal sample was weighed and extracted with 50 mL of 70 % ethanol at room temperature for 2 hours. The extract was filtered through Whatman filter paper. Then the filtrate was collected in a round-bottom flask, and dried in a rotary evaporator (Buchler Instrument, Fort Lee, NJ). The dried residues were dissolved in 5 mL of 20% methanol and slowly applied onto a pre-conditioned

Sep-Pak cartridge (C18, Waters Corp. Milford, MA). The cartridge was washed with 5 mL of distilled water to remove the water soluble impurities followed by 5 mL of 30% methanol. The eluent was recovered in 1 mL of HPLC grade methanol. The extract was vortexed and filtered through a 0.45 mm PTFE filter (Alltech Associates Inc., Deerfield, IL) prior to HPLC analysis.

Group B soyasaponin metabolite was determined by HPLC system as previously reported (Hu et al., 2002). Elution was carried out at a flow rate of 1 mL/min with the following solvent system: 0.05% trifluoroacetic acid in Milli-Q water (Millipore Co., Bedford, MA) (solvent A) and acetonitrile (solvent B). After injection of 50 μ L sample, solvent B was increased from 73% to 100% linearly in 35 min; then, solvent was recycled back to 73% in 4 minutes. The column temperature was 30°C. Ultraviolet absorbance monitored analytes from 190 to 350 nm using a photodiode array detector.

Preparation of soyasapogenol B and identification of soyasaponin metabolite

Soyasapogenol B was prepared according to Hu et al. (2004a) and uses as a standard to compare with soyasaponin metabolite. The chemical structure of soyasaponin metabolite was identified by ^1H , ^{13}C -NMR, electrospray ionized (ESI) mass spectroscopy, and infrared absorbance analyses. ^1H NMR and ^{13}C Attached Proton Test NMR spectra were acquired on a Varian VXR-300 spectrometer (Varian Inc., Palo Alto, CA). A Finnigan TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a Finnigan ESI interface was used in the positive Q1MS mode. The sample was dissolved in methanol- d_4 or chloroform- d_6 (Cambridge Isotope Laboratories, Inc., Andover, MA). The ^1H and ^{13}C -NMR

spectra data of soyasapogenol B were in good agreement with those reported by Kudou et al. (1993) and Hu et al. (2004a).

Statistical analysis

Statistical analyses were conducted with the Statistical Analysis System (SAS, Release 8.2, SAS Institute Inc., Cary, NC). Values were expressed as means \pm SD. A cluster analysis was performed to classify fecal soyasaponin metabolite excretion phenotypes. The results were analyzed by one-way analysis of variance (ANOVA). Differences between treatments were determined by least significant difference test. An α of 0.05 was used to determine statistically significant differences.

RESULTS

Hamster body weights and food intakes

The powdered diets were well accepted by hamsters throughout the experiment. Daily food intakes did not differ between casein and group B soyasaponin treatments (Table 3). Hamsters in both groups had similar initial mean body weight. There were no final weight differences between casein and group B soyasaponin groups (Table 3).

Effects of group B soyasaponins on plasma lipid concentrations

Total cholesterol, non-HDL cholesterol, triglyceride concentrations and total cholesterol/HDL cholesterol ratio were significantly less in hamsters fed group B soyasaponins ($P < 0.05$) compared with casein-fed hamsters (Table 4). There were no

differences in HDL cholesterol concentrations between groups fed casein or group B soyasaponin groups (Table 4).

Fecal bile acids and neutral sterols

There were significant effects of dietary treatment on both bile acid and neutral sterol outputs (Figure 1). Mean value of fecal bile acid and neutral sterol outputs was significantly greater in hamsters fed group B soyasaponins ($P < 0.05$) compared with those fed casein (Figure 1).

Fecal soyasaponin metabolite excretion

The retention time of a putative soyasaponin metabolite was ~22 min on HPLC system (Figure 2B) whereas the retention time of soyasapogenol B was ~15 min (Figure 2A). According to a cluster analysis, hamsters fed group B soyasaponins sorted into two fecal soyasaponin metabolite excretion phenotypes: high ($n=3$) and low ($n=7$) excreters (Figure 3).

Effects of group B soyasaponin metabolite on plasma lipid concentrations

There were no significant differences in plasma total, HDL cholesterol, non-HDL cholesterol, and triglyceride concentrations between high producer and low producer of soyasaponin metabolite (Table 5). The high producers of soyasaponin metabolite showed improved plasma cholesterol levels (the ratio of total/HDL cholesterol) compared with the low producers of the soyasaponin metabolite ($P < 0.03$) (Table 5).

Identification of soyasaponin metabolite

¹H-NMR (400 MHz) spectra of soyasaponin metabolite showed signals at δ 5.53 (m, 4H), 2.96 (t, $J = 6.0$ Hz, 2H), 2.46 (t, $J = 7.6$ Hz, 3H), 2.25 (d, $J = 6.4$ Hz, 4H), 1.78 (s, 3H), 1.50 (d, $J = 15.2$ Hz, 24H), and 1.06 (m, 6H). The ¹³C-NMR (100 MHz) spectrum of soyasaponin metabolite exhibited carbon signals at 177.9, 131.1, 131.0, 129.3, 129.2, 37.9, 35.9, 35.1, 32.8, 31.3, 31.0, 30.9, 30.8, 30.7, 30.5, 30.4, 28.3, 26.7, and 26.3 ppm. The ¹³C-NMR spectrum at 131.1, 130.0, 129.3, 129.2, and 177.9 ppm indicated the presence of two double bonds and one carbonyl stretching band. The positive mode ESI spectra of soyasapogenol B and soyasaponin metabolite were ion peak at m/z 332 $[M+5CH_3OH+2Na]^2$ and m/z 393 $[M-COOH]^+$, respectively. The carboxyl group of soyasaponin metabolite was confirmed with its infrared absorbance spectrum, absorbing in the region 1600-1800 cm^{-1} . The molecular formula and weight of soyasaponin metabolite were estimated by elemental analysis, $C_{30}H_{46}O_2$ and 438, respectively. The molecular weight is a value calculated based on the molecular formula. The putative structure of this soyasaponin metabolite was shown in Figure 4.

DISCUSSION

The present data demonstrated that group B soyasaponins lowered plasma total, non-HDL cholesterol, triglyceride concentrations and the ratio of total to HDL cholesterol in female hamsters (Table 4). Greater fecal excretion of bile acids and neutral sterols were observed in hamsters fed soyasaponins compared with those fed casein (Figure 1). The results were similar to that of Oakenfull et al. (1984) that 1% cholesterol diet plus 1% purified soyasaponins significantly lowered plasma (26%) and hepatic (33%) total cholesterol by increasing excretion of fecal bile acids and neutral sterols in rats compared

with 1% cholesterol diet. In the present study, significant reduction of non-HDL cholesterol and triglyceride levels in hamsters fed soyasaponins may be a consequence of decreased hepatic cholesterol reserve as a result of inhibiting intestinal absorption of cholesterol or increasing fecal excretion of bile acids and neutral sterols. Taken together, these data suggested that cholesterol-lowering effect of group B soyasaponins was mediated by increased fecal outputs of bile acids and neutral sterols and inhibition of intestinal absorption of cholesterol and bile acids.

Fewer studies have been reported about the metabolic fate of soyasaponins (Gestetner et al., 1968, Gurfinkel and Rao, 2003; Hu et al., in press, a and b). *In vitro* fermentation, Gurfinkel and Rao (2003) found that group A and B soyasaponins were hydrolyzed by colonic microflora to produce soyasapogenol A and soyasapogenol B, respectively. Hu et al. (2004a) reported that two gut microbial metabolites of soyasaponin I, soyasaponin III and soyasapogenol B, were identified when human feces were incubated in a brain-heart-infusion media with soyasaponin I (10 μmol /g feces) for 48 hours at 37°C. *In vivo* studies, Gestetner et al. (1968) found that soyasaponins or soyasapogenols were not detected in the blood of rats, mice, and chicks. Only soyasapogenol B (8.4% of ingested soyasaponin dose recovered in feces) was detected in human feces after a single dose of soy extract containing 434 μmol of group B soyasapogenins. Neither soyasapogenol B nor other soyasaponins were found in human urine samples (Hu et al., in press, b). These findings suggested that soyasaponins might not be absorbed from the digestive tract, but all rather metabolized by intestinal microorganisms, and excreted in the feces.

In the present study, a new metabolite of group B soyasaponins was detected in hamster feces after a 4-week feeding period, but neither soyasaponins nor soyasapogenol B

were found (Figure 2). These results suggested that soyasaponins were further metabolized or degraded by intestinal microflora. All hamsters fed group B soyasaponins produced soyasaponin metabolite. By cluster analysis, we found that there were two fecal soyasaponin metabolite excretion phenotypes: high and low. When low and high producers of a putative soyasaponin metabolite were compared for plasma cholesterol status, the high producers of soyasaponin metabolite showed significantly lower total/HDL cholesterol ratio ($p < 0.03$) (Table 5). Nonsignificant reduction in plasma non-HDL cholesterol ($p < 0.07$) was observed in the high producers of soyasaponin metabolite. Perhaps if a larger number of animals were analyzed, the presence of this saponins metabolite might be associated with a significant improvement in plasma non-HDL cholesterol concentration.

In conclusion, group B soyasaponins lowered plasma cholesterol levels by a mechanism involving greater excretion of fecal bile acids and neutral steroids compared with casein. Greater production of soyasaponin metabolite in hamsters was associated with better plasma cholesterol status. It is difficult to understand or assess the biological activity and health benefits of soyasaponins because of the lack of information about the metabolic fate of soyasaponins in animals and humans. Therefore, further study will be needed to investigate the role of group B soyasaponin metabolite on plasma cholesterol concentrations.

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Table 1. Composition of experimental diets.

Ingredient	Casein	Soyasaponins
	g/kg	
Casein ¹	250	250
Soyasaponins	0	2.4
Coconut oil	100	100
Safflower oil	20	20
Soybean oil	40	40
Cellulose	75	75
Wheat bran	75	75
Choline chloride	3	3
Vitamin mix ²	10	10
Mineral mix ³	35	35
Potassium bicarbonate	20	20
Cholesterol	1	1
Rice flour ⁴	371	368.5

¹Vitamin-free casein (Harlan/Teklad, Madison, WI)

²Vitamin mixture #400160 (Harlan/Teklad, Madison, WI)

³Mineral mixture #170910 (Harlan/Teklad, Madison, WI)

⁴Rice flour (Bioserve, Frenchtown, NJ).

Table 2. Dietary soyasaponin contents (mg/g diet)

Soyasaponin	Soyasaponin	Soyasaponin	Soyasaponin	Soyasaponin	Soyasaponin
V	I	II	α g	β g	β a
--	1.36	1.04	0	0	0

No isoflavones were detected.

Table 3. Body weight change and food intake of hamsters fed casein or casein-based diets with each soy isoflavone¹

Treatment	Food Intake g/d	Initial body weight g	Final body weight g
Casein	7.5 ± 1.2	111 ± 10	126 ± 9
Soyasaponin	7.2 ± 1.5	112 ± 11	128 ± 14

¹Values represent means ± SD, n=10.

Table 4. Plasma cholesterol levels in hamsters fed casein or group B soyasaponin diets^{1,2}

Treatment	Total cholesterol mmol/L	HDL cholesterol mmol/L	Non-HDL ³ cholesterol mmol/L	Total cholesterol /HDL cholesterol	Triglyceride mmol/L
Casein	6.33±1.32 ^a	3.75±0.53	2.58±0.81 ^a	1.69±0.24 ^a	2.67±0.24 ^a
Soyasaponin	5.14±0.79 ^b	3.43±0.69	1.71±0.49 ^b	1.50±0.14 ^b	2.18±0.57 ^b

¹Values represent means ± SD, n=10;

²Within a column, means with different superscripts are different (P < 0.05);

³Represents the VLDL+IDL+LDL fractions (by difference: Total - HDL)

Table 5. Plasma lipid status of high or low producers of soyasaponin metabolite^{1,2}

	Total cholesterol mmol/L	HDL cholesterol mmol/L	Non-HDL ³ cholesterol mmol/L	Total cholesterol /HDL cholesterol	Triglyceride mmol/L
High producers (n=3)	4.68±0.18	3.39±0.18	1.28±0.18	1.38±0.07 ^b	1.73±0.49
Low producers (n=7)	5.35±0.88	3.37±0.47	1.98±0.55	1.59±0.13 ^a	2.38±0.71

¹Values represent means ± SD;

²Within a column, means with different superscripts are different (P < 0.03);

³Represents the VLDL+IDL+LDL fractions (by difference: Total - HDL)

Figure 1. Fecal excretion of bile acids and neutral steroids per day. Data are reported as mean \pm SD. An asterisk indicates $P < 0.05$ vs casein.

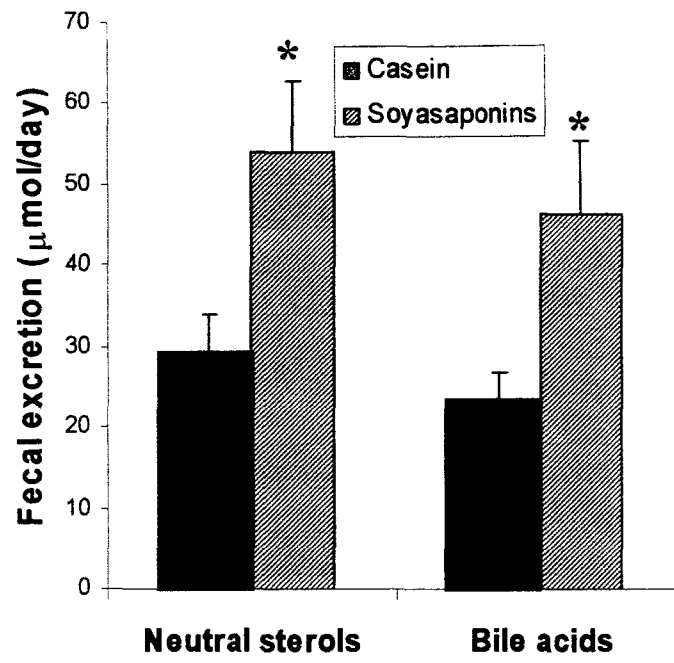
Figure 2. HPLC chromatograms.

A: Chromatogram of soyasapogenol B

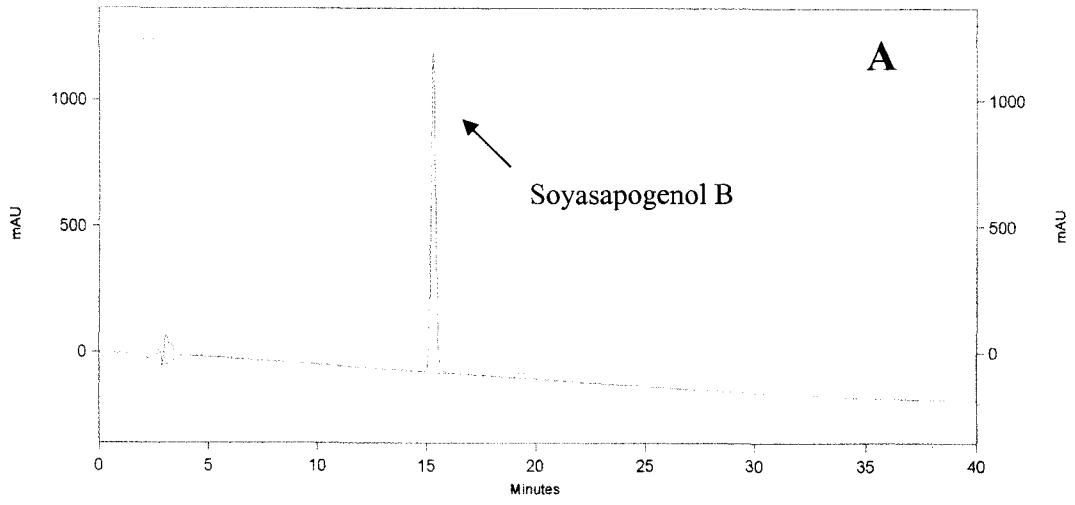
B: Chromatogram of putative fecal group B soyasaponin metabolite

Figure 3. Fecal soyasaponin metabolite excretion phenotypes.

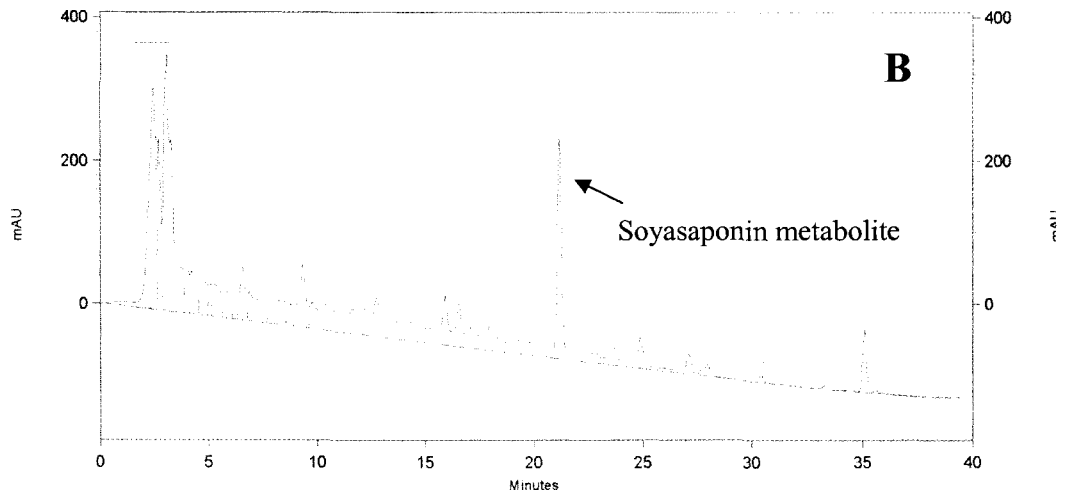
Figure 4. Putative structure of soyasaponin metabolite.

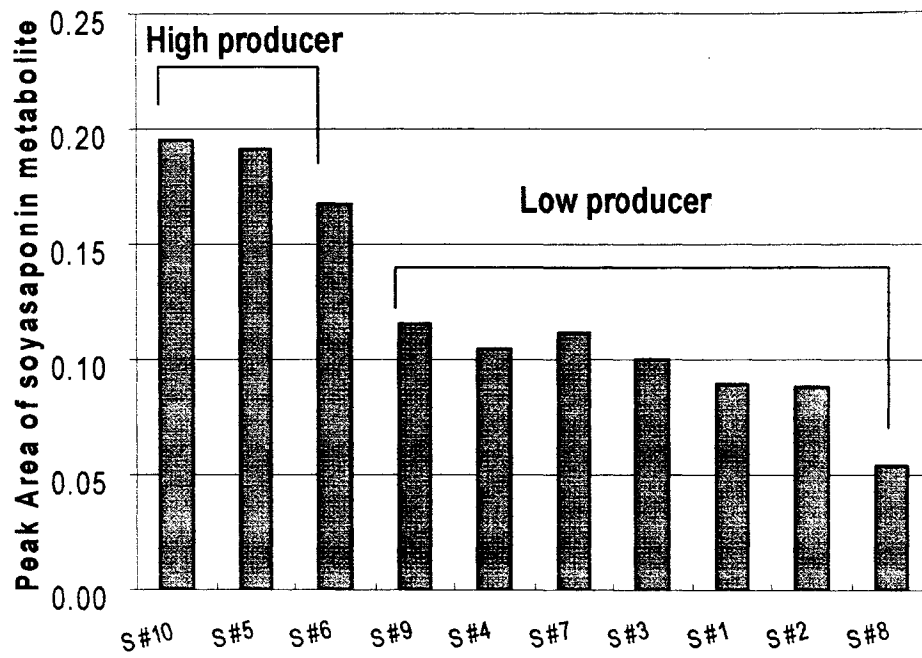


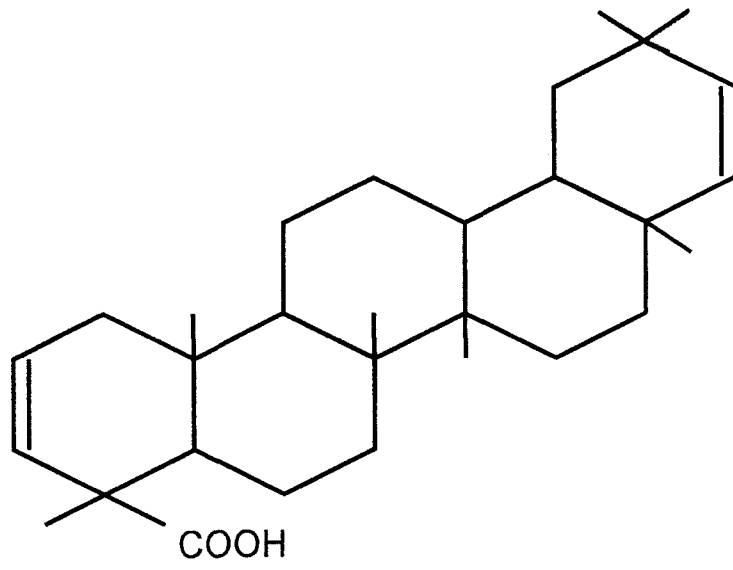
Saponin analysis 205nm



Saponin analysis 205nm







GENERAL CONCLUSIONS

Soy protein and soy protein-associated components including isoflavones and soyasaponins have received attention regarding their possible role in lipid metabolism. Not many studies have investigated the hypocholesterolemic effect of the soy protein fraction, specifically β -conglycinin, in animals and humans. Most of the studies used an isolated soy protein extract rich in isoflavones and soyasaponins or an alcohol-washed isolated soy protein deficient in isoflavones and soyasaponins for investigating the cholesterol-lowering effects of isoflavones. Also, the content of soyasaponins was not well determined in other studies. Therefore, it is not clear which soy components are responsible for the cholesterol-lowering effects. In order to clarify which components of soy protein reduce plasma lipid concentrations, it is necessary to exam the isoflavones or soyasaponins, separate from soy protein.

In our first study, we found that β -conglycinin with low levels of isoflavones and soyasaponins was ineffective at lowering plasma cholesterol over time in mildly hypercholesterolemic women. This study suggested that isoflavones and soyasaponins seem to be needed in companions for β -conglycinin to exert a cholesterol lowering effect. To avoid the possible confounding effects of other alcohol extractable components of soy protein, such as soyasaponins, pure synthetic isoflavones were added to a casein-based diet. Glycitein's greater hypocholesterolemic effect due to greater bioavailability, as reflected in urinary recovery of glycitein compared with the other purified isoflavones. Further studies can be in the directions of investigating the potential mechanisms of each purified isoflavones to lower

plasma lipid concentrations, and examining the effects of different doses of each isoflavones in reducing plasma lipid levels (dose-response study).

Not many of the biological activities associated with soyasaponins have been investigated because of the lack of availability of large quantities of purified soyasaponins and efficient methods for the detection and identification of soyasaponins and their metabolites. Our study used crude group B soyasaponins not containing isoflavones to avoid the possible confounding effects of other alcohol extractable components of soy protein. Group B soyasaponins lowered plasma lipid concentrations by increasing excretion of fecal bile acids and neutral sterols. Further studies will be needed to determine which soyasaponins fractions of soy, including soyasaponin α g, β g, β g, V, I, or II, have more cholesterol-lowering effect. A new soyasaponin metabolite was detected in hamster's fecal sample after a 4 week feeding period. The high producers of this metabolite showed improved plasma lipid status (the ratio of total/HDL cholesterol). It is difficult to understand or assess the biological activity and health benefits of soyasaponins because of the lack of information about the metabolic fate of soyasaponins in animals and humans. Therefore, further study will be needed to investigate the role of soyasaponin metabolite on plasma lipid concentrations.

In summary, both soy isoflavones and soyasaponins in nutritionally relevant concentrations contribute to the cholesterol-lowering effects of soy foods and soy protein ingredients.

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