

## *Effects of Group B Soyasaponins and Soyasapogenol B on Plasma Cholesterol Levels in Golden Syrian Hamsters*

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### **Abstract**

Elevated blood cholesterol levels are associated with the development of heart disease. Group B soyasaponins have been suggested as a factor capable of decreasing plasma lipid concentrations. Soyasaponins are naturally occurring triterpenoids found in soybeans, and their sugars are cleaved from group B soyasaponins in the digestive tract leaving the aglycone form - soyasapogenol B. The objectives of this study are to determine cholesterol-lowering efficacy and bioavailability of soyasapogenol B and group B soyasaponins. Thirty six hamsters were assigned to one of three dietary treatment groups and fed for 4 weeks; 1) high fat control (HFC), 2) high fat diet with soyasapogenol B (SPG, 0.5 mmol/kg diet), and 3) high fat diet with group B soyasaponins (SSP, 1.5 mmol/kg diet). Compared to the HFC, SPG treatment was shown to decrease total cholesterol, non-HDL cholesterol, and triglyceride levels ( $P < 0.05$ ). Fecal bile acid excretion of SPG and SSP groups were greater than HFC ( $P < 0.05$ ). SSP group showed significant increased fecal neutral sterol excretion compared to the HFC and SPG ( $P < 0.05$ ). Hamsters in the SPG group showed a significantly greater bioavailability than the SSP group ( $P < 0.05$ ).

**Keywords:** Group B Soyasaponins; Soyasapogenol B; Hypocholesterolemic; *Glycine max*; Bioavailability

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### **Introduction**

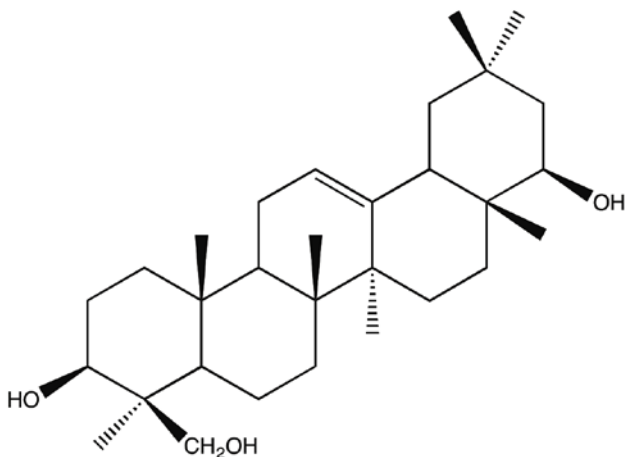
For several centuries saponin containing plants have been used as detergent (Latin word, *sapo*, which means soap), medicinal, and food sources [1]. Saponins can be defined as steroid or triterpenoid glycosides that are naturally present as secondary metabolites in plants and thought to act as a defense against external threats [2, 3]. Soy saponins, also known as soyasaponins, are triterpenoids, which are known to originate from squalene in plants [1]. Soybean (*Glycine max*) saponins are divided into three main categories: Group A, B, and E. Group B saponins are the most prevalent form of saponins in soy plants. Soyasapogenol B, the aglycone form of group B soyasaponins, is metabolized from soyasaponins by gut microflora [4, 5].

Coronary heart disease (CHD) is the most predominant form of heart disease, which is the leading cause of death in the United States [6]. Plaque build-up in the arteries is the main cause of CHD. There is a relationship between dietary component intake and atherosclerosis. Soy products have been shown to induce hypocholesterolemic effects and lead to overall better health [7, 8]. Comparisons of ethanol washed soy products to non-ethanol washed soy products have suggested that ethanol soluble components within soy products may

induce hypocholesterolemic effects [9, 10]. Group B soyasaponins, an ethanol soluble component, have significantly decreased total cholesterol and non-HDL concentrations in hamsters [11]. The observed hypocholesterolemic effects may be due to the increased fecal excretion of neutral sterols and/or bile acids [11, 12].

Soyasapogenol B (Figure 1) is thought to be the bioactive form of group B soyasaponins, but little has been investigated in an *in vivo* study. This study had two objectives: to determine the cholesterol-lowering efficacy of group B soyasaponins and soyasapogenol B by measuring the excretion of fecal bile acids and neutral sterols; and to evaluate the bioavailability of soyasapogenol B and group B soyasaponins.

**Figure 1:** Soyasapogenol B



## Materials & Methods

### Diets

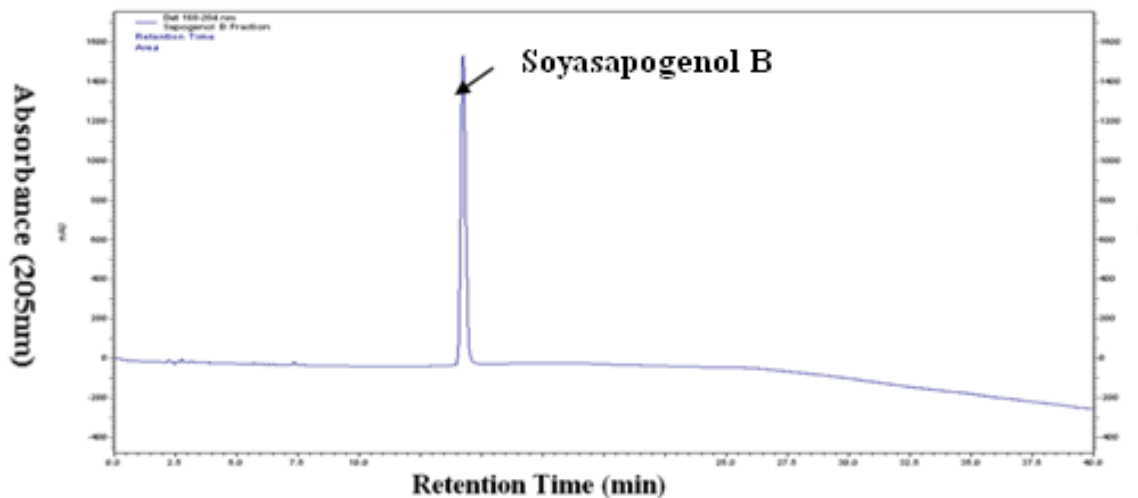
Crude group B soyasaponin powder was generously donated by Dr. Berhow from the USDA. The proportion of group B soyasaponins I, II, V in the diet are analogous to the soyasaponins that are present in commercially prepared soy protein products [13, 14]. The amount of group B soyasaponins in the diet corresponds to what would be ingested if 25% of the diet was soy protein [13]. The rice starch used in the feeding study was donated by A&B Ingredients (Fairfield, NJ).

The soyasapogenol B hydrolysis method of Hu et al. [15] was modified to increase production output. 250 mg of

soyasaponin powder was hydrolyzed then extracted on high capacity 10,000 mg C-18 solid phase extraction columns (Grace Davidson, Deerfield, IL) and corresponding amounts of all materials were increased. The final powder from the hydrolysis process contained soyasapogenol B hydrolysis artifacts. The powder was further purified on a Shimadzu semi-prep HPLC system equipped with a pump, detector, manual injector, and workstation (Shimadzu, Columbia, MD) using a reverse phase C-18 column (YMC-Pack ODS-AM S5 $\mu$ m 12 nm, 250 x 10 mm i.d., YMC, Clifton, NJ). The semi-prep method was modified from the analytical soyasapogenol B method of Lee et al. [11]. Both soyasapogenol B and group B soyasaponins were analyzed on a Beckman Coulter analytical HPLC system equipped with a pump, detector, and auto sampler (Beckman Coulter, Brea, CA) using a C-18 reverse phase column (YMC-Pack ODS-AM S5 $\mu$ m 12 nm, 250 x 4.6 mm i.d., YMC, Clifton, NJ) (Figure 2). Soyasapogenol B and group B soyasaponin compounds were tested for purity before their addition to the diets. Soyasaponin I and soyasaponin II standards were purchased from Chromadex (Irvine, CA). Soyasaponins III, V, and soyasapogenol B were collected by fractionation and used to quantify the amount of treatment compounds present in the diet by standard curve.

There were three dietary treatment groups: high fat control (HFC), high fat plus soyasaponins (SSP), and high fat plus soyasapogenol B (SPG). Feeding lasted four weeks and all dietary compounds were administered orally. The diet was based on the AIN-93M diet [16]. The high fat control and both treatment diets were high fats diets based on the diet composition used by Song et al. [17]. Casein was chosen as the protein source, in place of soy protein, to reduce the probability of bioactive soy compounds being present. Rice starch was used instead of cornstarch to prevent “wet tail” (diarrhea, which can lead to mortality in rodents) [18]. The treatment compounds in the experimental diets were substituted for rice starch. The diet was tested to ensure no isoflavones were present. All water given to the hamsters was purified by a de-ionizing system.

**Figure 2:** High-performance liquid chromatogram (HPLC) of soyasapogenol B at 205 nm



## Animals

Hamsters were chosen as an animal model because they are analogous to humans in relation to cardiovascular disease and lipid metabolism [19]. Thirty-six female Golden Syrian hamsters between the ages of 8-9 weeks were purchased from Harlan Teklad (Madison, WI). After arrival, the hamsters were allowed to acclimate for a one-week period and fed a rodent diet. The hamsters were divided into three groups (HFC, SSP and SPG) of twelve. The hamsters were placed in single occupant cages to observe exact food intake and eliminate territorial issues common to rodents. The cages were kept in a temperature controlled environment at 23°C with a 12:12 cycle of light and darkness. Hamsters had free access to food and water. Cages were cleaned once a week. Food intake was measured daily and body weight measured every three days on a GX-2000 balance (A&D, San Jose, CA). The animal setting was used when recording body weight to account for animal movement. At the end of the feeding period, all hamsters were removed from their diets sixteen to eighteen hours before sacrifice. All portions of testing involving live animals were approved by the University of Arkansas at Fayetteville IACUC committee and overseen by a Doctor of Veterinary Medicine.

## Plasma Lipid Analysis

Hamsters were sacrificed under CO<sub>2</sub> gas. The blood was collected by cardiac puncture using 22 gauge syringes (BD Franklin Lakes, NJ), which were coated in a 4.45 µmol/mL EDTA (ethylenediaminetetraacetic acid) solution (Amresco, Solon, OH). Each hamster's blood was transferred from syringe to 17.8 µmol/mL EDTA coated vacuum tubes (BD Franklin Lakes, NJ). The tubes containing blood were then transferred to ice for storage. The blood was centrifuged at 5000 x g, 48°C for 15 minutes [11]. The plasma was then pipetted into cuvettes and directly tested for total plasma cholesterol, triglyceride, and high-density lipoprotein concentrations by an Ace Alera Clinical Chemistry System (Alfa Wassermann, West Caldwell, NJ). Samples were diluted with 0.9% saline solution (Baxter, Deerfield, IL) to a 1:1 ratio before testing for high-density lipoprotein.

## Analysis of Fecal Excretion

Hamsters were placed into metabolic cages for a period of 24 hours to collect fecal samples. Feces were collected at the beginning, midpoint, and end of the study. Fecal samples were collected, weighed, and stored at -80°C. Fecal material was freeze-dried prior to analyzing. The freeze-dried feces were

tested for metabolites, bile acids, and neutral sterols. Metabolites were determined by HPLC (Beckman Coulter, Brea, CA) equipped with a reverse phase C-18 column (YMC-Pack ODS-AM S5 $\mu$ m 12 nm, 250 x 4.6 mm i.d., YMC, Clifton, NJ) using methods from Hu et al. [15]. Bile acids and neutral sterols were determined by a Hewlett Packard 6890 gas chromatograph (Palo Alto, CA) equipped with flame-ionization detector and auto-sampler. A Varian CP-Sil 5 SB (25m x 0.25 mm, Santa Clara, CA) column was used and the analytical method of Batta et al. [20] was used.

### Statistical Analysis

Data were evaluated using SAS (Release 9.4, SAS Institute Inc., Cary, NC). Results were analyzed with one-way ANOVA. Means found significant by the ANOVA analysis were separated using the least significance difference test (LSD) at P<0.05 significant level.

## Results

### Food Intake and Body weight

None of the treatment groups showed a significant difference in average daily calorie intake (Table 1). There was no significant difference in initial weight, weight gain, and final weight between the three treatment groups (Table 2).

**Table 1:** Average Food, Fat, and Calorie Intake per day of the hamster during study period of one month

Diet Group	Food Intake (g)	Fat Intake (kcal)	Calorie Intake (kcal)
HFC	8.4 $\pm$ 0.2	12.1 $\pm$ 0.4	37.9 $\pm$ 0.8
SPG	8.8 $\pm$ 0.3	12.7 $\pm$ 0.5	39.7 $\pm$ 1.1
SSP	8.6 $\pm$ 0.1	12.4 $\pm$ 0.3	38.7 $\pm$ 0.7

Values are mean  $\pm$  SEM. Values with different superscripts in a column are significantly different (P<0.05). Number of animals per diet group = 12. HFC: High Fat Control; SPG: High Fat Diet with Soyasapogenol B; SSP: High Fat Diet with Group B Soyasaponin

**Table 2:** Bodyweight (g) of the hamster during study period of one month

Diet Group	Initial Weight	Final Weight	Weight Gain
HFC	113.5 $\pm$ 2.5	131.7 $\pm$ 2.6	18.2 $\pm$ 3.3
SPG	113.5 $\pm$ 2.5	128.2 $\pm$ 2.5	14.7 $\pm$ 1.3
SSP	113.5 $\pm$ 2.5	130.7 $\pm$ 2.3	17.2 $\pm$ 1.9

Values are mean  $\pm$  SEM. Number of animals per diet group = 12. HFC: High Fat Control; SPG: High Fat Diet with Soyasapogenol B; SSP: High Fat Diet with Group B Soyasaponin

### Plasma Lipid Profile

SPG showed lower amounts of total cholesterol, triglycerides, non-HDL cholesterol and HDL cholesterol when compared to the HFC and SSP (P<0.05) (Table 3).

**Table 3:** Blood Lipid Levels (mmol/L) of the hamster at the end of the study period of one month

Diet Group	Total Cholesterol	Tri-glycerides	HDL	non-HDL
HFC	6.6 $\pm$ 0.2 <sup>a</sup>	2.2 $\pm$ 0.2 <sup>a</sup>	4.3 $\pm$ 0.1 <sup>a</sup>	2.3 $\pm$ 0.1 <sup>a</sup>
SPG	6.2 $\pm$ 0.1 <sup>c</sup>	1.9 $\pm$ 0.2 <sup>b</sup>	4.0 $\pm$ 0.1 <sup>b</sup>	2.0 $\pm$ 0.1 <sup>b</sup>
SSP	6.4 $\pm$ 0.4 <sup>b</sup>	2.2 $\pm$ 0.2 <sup>a</sup>	4.1 $\pm$ 0.3 <sup>b</sup>	2.3 $\pm$ 0.1 <sup>a</sup>

Values are mean  $\pm$  SEM. Values with different superscripts in a column are significantly different (P<0.05). Number of animals per diet group = 12. HFC: High Fat Control; SPG: High Fat Diet with Soyasapogenol B; SSP: High Fat Diet with Group B Soyasaponin

### Metabolite Excretion and Bioavailability

Fecal material from all dietary groups was tested for metabolite excretion. Only the experimental groups, SPG and SSP, showed excretion of the metabolite (soyasapogenol B). The SPG group had a significantly greater (P<0.05) amount of soyasapogenol B excretion compared to the SSP group (3.74

vs. 0.53 mol/g). Soyasapogenol B also had a significantly higher bioavailability percentage when compared to the SSP group (82.4 vs. 3.9 %).

### Bile Acids and Neutral Sterols in Feces

SPG and SSP diet fed hamsters excreted more bile acid than HFC ( $P < 0.05$ ) (Table 4). The SSP diet fed hamsters excreted significantly ( $P < 0.05$ ) more individual neutral sterols when compared to the HFC group. There are no significant differences in excretion of neutral sterol; lathosterol and campesterol between SPG and HFC diet fed hamsters.

**Table 4:** Bile Acid Excretion ( $\mu\text{mol}/\text{total feces in mg}$ ) of the hamster at the end of the study period of one month

	HFC	SPG	SSP
<b>Total Bile Acids</b>	$7.6 \pm 0.7^b$	$11.5 \pm 1.0^a$	$10.1 \pm 0.9^a$
Cholic	$1.8 \pm 0.3$	$2.6 \pm 0.4$	$2.6 \pm 0.6$
<b>Total Neutral Sterols</b>	$27.3 \pm 6.3^b$	$35.8 \pm 3.4^b$	$44.6 \pm 4.8^a$
Lathosterol	$5.7 \pm 2.0^b$	$10.0 \pm 0.9^b$	$16.9 \pm 2.5^a$
Cholesterol	$0.3 \pm 0.1$	$0.4 \pm 0.2$	$0.7 \pm 0.2$
Coprostanol	$1.7 \pm 0.2^a$	$1.1 \pm 0.1^b$	$1.7 \pm 0.1^a$
Cholestane	$1.4 \pm 0.2^b$	$2.4 \pm 0.3^a$	$2.0 \pm 0.3^{ab}$
Campesterol	$0.2 \pm 0.0^b$	$0.2 \pm 0.0^b$	$0.4 \pm 0.1^a$

Values are mean  $\pm$  SEM. Values with different superscripts in a row are significantly different ( $P < 0.05$ ). Number of animals per diet group = 12. HFC: High Fat Control; SPG: High Fat Diet with Soyasapogenol B; SSP: High Fat Diet with Group B Soyasaponin

### Discussion

The goal of this study is to determine the cholesterol-lowering efficacy of soyasapogenol B and group B soyasaponins as part of a high fat diet. This is the feeding study to use soyasapogenol B, the final metabolite of group B soyasaponins [4, 5]. This study also used lower amounts of

group B soyasaponins (1.5 mmol/kg diet) than previous research (2.2 mmol/kg diet) [11]. A lower amount of group B soyasaponins was used because of the publication of newer data reporting different levels of soyasaponins in non-ethanol washed soy protein [13]. The treatment groups, SPG and SSP, showed lower total cholesterol levels when compared to the HFC group (Table 3). Reddy et al. [21] reported that when rats were fed with high fat diet and orally administered with saponin rich aqueous leaf extract of *Gymnema sylvestre* (100 mg/kg body weight) suspended in distilled water by gastric gavage once a day for 8 weeks, the decrease the total cholesterol, triglycerides, very low density lipoproteins, LDL, and increased HDL was observed compared to high fat diet alone. However, in the present study the differences of triglycerides and non-HDL cholesterol between the SSP group and the high fat control are not significant. Extending the duration of the feeding period may cause the observed differences to become significantly larger.

Soyasaponin glucosides did not detect in the blood of rat, chicks, and mice [22]. Soyasapogenol B, the aglycone form of group B soyasaponins, is metabolized from soyasaponins by gut microflora [4, 5]. Due to these factors, bioavailability is measured by metabolite (soyasapogenol B) excretion in fecal material. Dietary analysis shows that the SPG group contained 0.5  $\mu\text{mol}$  of purified soyasapogenol B per gram of diet and the SSP group contained 1.5  $\mu\text{mol}$  of group B soyasaponins per gram of diet. No soyasapogenol B was detected in the SSP diet, nor soyasaponins detected in the SPG diet. Because SPG was fed pure metabolite, a higher bioavailability was expected when compared to SSP. A previous study determined the amount of soyasapogenol B, recovered in the fecal material of humans, and was 3.8% of the original amount of group B soyasaponins fed after a 24 hour period [15]. 3.9% of group B soyasaponins were found converted to soyasapogenol B in hamster fecal material in this study after a 24 hour collection period. 82.4% of soyasapogenol B fed to the hamsters was recovered in the fecal material. Neither this study, nor previous *in vivo* studies found group B soyasaponins present in fecal material [11, 15].

Previous *in vitro* studies have shown that soyasaponin III [5] and soyasapogenol B monoglucuronide are metabolites of soyasaponin I, but the final metabolite was suggested to be soyasapogenol B [4]. The final metabolite in this study was soyasapogenol B.

The fate of the remaining soyasaponins is unknown. None were detected in fecal material. The HPLC procedure used is sensitive enough to detect the amounts that were added to the diet per gram (0.07  $\mu\text{mol}$ ) (unpublished data). Previous research has shown that group B saponins and soyasapogenol B have low absorption rates in the intestines and are not present in urine [15] or blood [22]. It is possible that the group B soyasaponins not recovered as soyasapogenol B are metabolized differently by gut microbiota, or structurally changed in another metabolite undetected by HPLC. Research with specific gut microbiota needs to be conducted for a clearer understanding.

No significant differences of total bile acid excretions in the feces between SPG and SSP were observed in this study. Previous research has shown a significant increase in cholic and total bile acids accompany a significant ( $P < 0.05$ ) decrease in plasma lipid levels [11]. Cholic acid is a primary bile acid [23], and high levels of cholic acid excretion suggest group B soyasaponins and their metabolites can bind to it and in turn decrease the formation secondary bile acids. Both dietary treatment groups (SPG, SSP) showed a non-significant increase in cholic acid when compared to the HFC (Table 4). Lin et al. [24] used a 20  $\mu\text{mol/mL}$  solution of bile salts to evaluate the difference in bile acid binding ability between group B soyasaponins and isoflavones. Soyasaponins (3.1%) showed significantly greater binding ability than isoflavones (1.3%) ( $P < 0.05$ ) [24]. Further research should include soyasapogenol B, individual group B soyasaponins, and a wide range of bile acids.

Previous research with soyasaponins reported a significant increase in total neutral sterol, cholestane, lathosterol, and coprostanol that result from a significant decrease in plasma lipids [11]. Both dietary treatment groups,

SPG and SSP, show larger amounts of cholestane and lathosterol excretion compared to HFC (Table 4). SPG shows a significantly larger increase in cholestane and the SSP group shows a significantly larger increase in lathosterol ( $P < 0.05$ ). SPG group has lower coprostanol excretion when compared to the HFC ( $P < 0.05$ ). The SSP group has a significantly larger amount of campesterol when compared to the HFC group ( $P < 0.05$ ).

Cholesterol can be transformed into coprostanol, cholestanone, cholestenone, and cholestanol [25]. All of these sterols have been reported in hamster and human fecal material [11, 26]. Both SPG and SSP groups showed non-significant increases of cholesterol and cholestenone excretion compared to the HFC group. The decrease in coprostanol may be accounted for by the increase in cholesterol and cholestenone in this study.

The inclusion of cholestanol, a metabolite of cholesterol [27], in the neutral sterols that are analyzed in future studies may cause the SPG group to show a significantly larger total fecal excretion when compared to the HFC group.

There are several dietary components in soy foods [28]. It is possible that the observed hypocholesterolemic effects from soy derive from a combination of different components. In this study, soyasapogenol B was able to show a significant decrease in lipid plasma levels compared to the high fat control. The only previous study using isolated group B soyasaponins with a non-soy protein source found significant decreases in plasma total and non-HDL cholesterol [11]. Lower amounts of group B soyasaponins were used in this study (1.5mmol/kg diet vs. 2.2 mmol/kg diet). The quantities of soyasapogenol B needed for a feeding study are not yet available on the commercial market. Sample sizes in this study were limited by the amount of soyasapogenol B that could be produced. Hydrolysis and fractionation methods can now be used efficiently to create sizeable amounts of soyasapogenol B. This will allow for a longer duration of future feeding studies and larger sample sizes, and possibly lead to more significant changes in plasma lipid levels.

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