# Protein–Saponin Interaction and Its Influence on Blood Lipids

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Protein source and saponins have been shown to influence lipid metabolism; however, little is known regarding the chemical interactions between proteins and saponins or the impact of addition of dietary saponins to different proteins on cholesterolemia. In the present study, quillaja saponin was added to casein and to isolated soy protein (ISP) and the saponin–protein interaction was investigated by gel electrophoresis and fluoroscopy. The impact on cholesterol metabolism also was investigated in gerbils. Results from the interaction studies showed that high molecular weight complexes were formed gradually between saponins and caseins, with β-casein being most susceptible. The resulting complexes differed drastically in charge and molecular weight. In contrast, soy proteins formed insoluble aggregates during heating independently of the presence of added quillaja saponin. Data from the animal study showed that addition of saponin to ISP did not affect serum lipids, while addition of saponin to casein resulted in significant decreases of LDL cholesterol and LDL/HDL ratios, resulting in values similar to those of ISP-fed animals. These results indicate that the effect of saponins on serum lipid profiles is dependent on the source of dietary protein. This could be explained by the finding that quillaja saponin reacted differently with caseins as compared to soy protein isolate.

## INTRODUCTION

The cholesterolemic effects of dietary proteins have been demonstrated in humans (Erdman and Fordyce, 1989; Carroll, 1991) and various animal models including rabbits (Hamilton and Carroll, 1976), rats (Nagata et al., 1981), swine (Kim et al., 1978), mice (Roy and Schneeman, 1981), and gerbils (Forsythe, 1986; DiFrancesco et al., 1990a,b). In general, vegetable proteins have hypocholesterolemic effects, while animal proteins have hypercholesterolemic effects. The mechanisms for protein-induced changes in blood lipids have not been elucidated and appear to be complex (Pfeuffer, 1988; Hagemeister et al., 1990; Scholz-Ahrens et al., 1990). Some have suggested that proteins from plant vs animal origin exert their lipemic effects at the intestinal level, affecting lipid absorption and enterohepatic circulation of cholesterol, thereby altering lipid metabolism and lipoprotein receptor activity (Samman et al., 1989; Ho et al., 1989). Others have suggested that absorbed amino acids directly affect lipid metabolism and receptor activity (Gaddi et al., 1991). It has also recently been postulated that the cholesterolemic effects may be due to nonprotein compounds such as one of the wide range of phytochemicals known to be present in vegetable proteins but absent in animal proteins.

One type of compound found commonly in proteins of plant origin but not in those of animal origin are the saponins. Saponins added to the diet have been reported to decrease plasma lipids in experimental animals (Oakenfull, 1981). These compounds can be obtained from several different plant species, and their ability to decrease plasma cholesterol level is dependent on their chemical structure, which is specific to each species of plant from which it is obtained (Manilow, 1984). Because of this, saponins of different origin have been examined and those from soybeans, quillaja, alfalfa, and soapwort have proven to decrease plasma cholesterol when added to the diets of rats (Oakenfull, 1981; Sidhu and Oakenfull, 1986).

Investigations on the impact of soy protein on cholesterolemia in humans have reported a wide variation in responsiveness among studies (Carroll, 1991; Erdman and Fordyce, 1989). One possible reason for the inconsistency is the use of a variety of differently processed soy protein products, containing different amounts of saponins. The interaction between proteins and saponins is poorly understood, and it is not known how the addition of saponins to diets containing protein from plant or animal origins affects lipemia. Thus, the following experiment was designed to begin to define the interactions of saponins with proteins from animal and plant origin and to determine if the lipemic responses to dietary proteins are altered by the addition of saponins.

## MATERIALS AND METHODS

Quillaja saponin was provided from Calbiochem (La Jolla, CA). β-Lactoglobulin and α-, β-, and κ-caseins were from Sigma Chemical Co. (St. Louis, MO), cholesterol and triglyceride reagents and standards from Sigma Diagnostics (St. Louis, MO), and heparin–agarose chromatographic column beds from Isolab Inc. (Akron, OH). Diet ingredient information is given in Table I.

**Animals and Diets.** Male outbred gerbils (*Meriones unguiculates*, Tumblebrook Farms, Brent Lake, NY) weighing an average of 67 ± 6 g were individually housed in a temperature-controlled room (25 °C) with an alternating 12-h light/dark cycle. Upon arrival, animals were fed standard rodent chow for adaptation. After 3 days, gerbils were randomly assigned to one of four dietary treatment groups of 15 animals/group. Diets were similar in all respects with the exception of dietary protein source coming from either isolated soy protein (ISP) or casein and the addition or omission of quillaja saponin (Table I). Cellulose was eliminated in the saponin-containing diets to equalize the fiber content of each diet. Feed and water were provided *ad libitum*, and weight gain was monitored throughout the experimental period. After 28 days, gerbils were fasted for 12 h and sacrificed by decapitation following $CO_2$ sedation. Blood was collected and serum obtained by centrifugation at 35000*g*, 4 °C, for 20 min.

**Analytical Techniques.** LDL + VLDL (LDL) and HDL lipoprotein fractions were separated immediately using heparin–agarose chromatographic column beds (Bentzen et al., 1982).

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Table I. Diet Composition (Percent by Weight)

| component | dietary treatment group: ISP | ISP + saponin | casein | casein + saponin |
|---|---|---|---|---|
| casein[a] | 0 | 0 | 18.00 | 18.00 |
| ISP[b] | 18.00 | 18.00 | 0 | 0 |
| sucrose | 16.00 | 16.00 | 16.00 | 16.00 |
| lard | 16.00 | 16.00 | 16.00 | 16.00 |
| corn oil | 1.00 | 1.00 | 1.00 | 1.00 |
| vitamin mix[c] | 1.00 | 1.00 | 1.00 | 1.00 |
| mineral mix[d] | 5.00 | 5.00 | 5.00 | 5.00 |
| cholesterol[e] | 0.10 | 0.10 | 0.10 | 0.10 |
| dextrose | 33.90 | 32.90 | 33.90 | 32.90 |
| cellulose[f] | 9.00 | 0 | 9.00 | 0 |
| saponin[g] | 0 | 10.00 | 0 | 10.00 |

[a] Lactic casein, New Zealand Dairy Board, Wellington, New Zealand. [b] Soy protein isolate, Supro 610, Protein Technologies International, St Louis, MO. [c] Teklad vitamin mix, Teklad, Madison, WI. Vitamin mix supplied the following vitamins (in mg/kg of diet): vitamin $B_{12}$ (0.1% trituration in mannitol), 30; calcium pantothenate, 66; dihydrogen citrate, 3500; folic acid, 2; inositol, 110; menadione (vitamin K), 50; niacin, 99; pyridoxine hydrochloride, 22; riboflavin 22; thiamin hydrochloride, 22; dry retinyl palmitate (500 000 IU/g), 40; dry ergocalciferol (500 000 IU/g), 4.4; dry tocopheryl acetate (500 IU/g), 2423. [d] Mineral mix, Hegsted IV, Teklad, Madison, WI. Mineral mix supplied the following minerals (in g/kg of diet): calcium carbonate, 15.0; potassium phosphate, dibasic, 16.1; calcium phosphate, dibasic, 3.7; magnesium sulfate, 5.1; sodium chloride, 8.4; ferric citrate (USP 16.7% Fe), 1.4; potassium iodide, 0.034; manganese sulfate, 0.19; zinc chloride, 0.012; cupric sulfate, 0.015; nonnutritive fiber, 0.061. [e] Cholesterol, Sigma Chemical, St. Louis, MO. [f] Cellulose, Solka Floc, James River Corp., Berlin, NH. [g] Quillaja saponin, approximately 10% saponin/90% cellulose.

Serum total, LDL and HDL cholesterol, and total triglyceride concentrations were quantified using commercially available kits (Allain et al., 1974; McGowen et al., 1983) (Sigma).

**Saponin–Protein Interactions.** Fifteen milligram per milliliter solutions of each $\alpha$-, $\beta$-, $\kappa$-, and whole casein and isolated soy protein (ISP) were prepared in a PBS buffer (1.09 g of $Na_2PO_4$, 0.31 g of $KH_2PO_4$, 5.85 g of NaCl/L). To each vial was added 40 $\mu$L of 0.1 M NaOH to 1 mL of solution. Three hundred microliters of the solutions was taken and used as standards, and 20 mg of quillaja saponin was added to the 700 $\mu$L left. The reaction mixtures were heated at 78 °C in a water bath. At time intervals of 0, 1, 3, 5, 8, 11, and 26 min, the 40-$\mu$L sample was removed. Solutions were mixed by vortexing intermittently between times of sample removal.

**Gel Electrophoresis.** *Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis* was performed according to the method of Laemmli (1970). Fifteen microliters of protein sample was added to 15 mL of SDS sample buffer containing 5.0% $\beta$-mercaptoethanol. Samples containing 70 $\mu$g of protein were loaded into gels of 12% acrylamide. *For urea–polyacrylamide gel electrophoresis*, sample preparation was done by mixing 40 $\mu$L of each protein sample with 40 $\mu$L of 9 M urea and holding for 60 min at room temperature. Samples were then mixed with 80 $\mu$L of sample buffer containing 18% $\beta$-mercaptoethanol and 2.0% bromophenol blue in 6.0 M urea. Urea–polyacrylamide gel electrophoresis (PAGE) was done according to the method of Medrano and Sharrow (1989), with running gel at pH 8.9 consisting of 0.375 M Tris, 4 M urea, and 9.5% acrylamide. The stacking gel consisted of 0.375 M Tris and 4.6% acrylamide at pH 6.7. A sample of 37.5 $\mu$g of protein was loaded into each well. Running conditions and staining of the gels were carried out conventionally.

**Lactose–Protein Interactions.** A mixture of $\beta$-lactoglobulin (2 mg/mL) and lactose (2 mg/mL) was prepared in water and freeze-dried. The samples were heated in a water bath at 64 °C for 0, 10, 30, 60, 120, 240, 360, and 900 min. Control samples containing protein only were also heated. Samples were suspended in a 200 $\mu$L of PBS buffer with 20 $\mu$L of 0.1 M NaOH.

**Fluorescence Spectroscopy.** The fluorometric method of Böhlen et al. (1973) was used by pouring 50 $\mu$L each protein solution (1.05 mg/mL) into test tubes and then adding 3.0 mL of 50 mM sodium phosphate buffer (pH 8.0). While each test tube was vortexed, 0.5 mL of fluorescamine in dioxane (30 mg/mL) was rapidly added using a syringe. The relative fluorescence was measured using an Aminco-Bowman spectrofluorometer with excitation wavelength of 390 nm and emission wavelength of 475 nm.

Table II. Weight Gain and Feed Intake (Grams)[a,b]

| | dietary treatment group: ISP | ISP + saponin | casein | casein + saponin |
|---|---|---|---|---|
| weight gain | 5.8 ± 3.8[a] | 0.1 ± 3.5[b] | 7.1 ± 5.5[a] | 1.5 ± 7.5[b] |
| feed intake | 148 ± 17.6[a] | 139 ± 20.1[a] | 149 ± 18.5[a] | 120 ± 17.7[b] |

[a] Means ± SD. [b] Means with different superscripts in the same row are significantly different ($p < 0.05$).

Table III. Serum Lipid Concentrations (Millimoles per Liter)[a,b]

| | dietary treatment group: ISP | ISP + saponin | casein | casein + saponin |
|---|---|---|---|---|
| total | 3.4 ± 0.6[b] | 4.2 ± 1.0[b] | 6.5 ± 4.2[a] | 4.4 ± 1.0[ab] |
| HDL | 2.2 ± 0.6 | 2.5 ± 0.9 | 2.8 ± 1.1 | 2.5 ± 0.6 |
| LDL | 1.4 ± 0.3[b] | 1.7 ± 0.6[b] | 3.4 ± 2.6[a] | 1.9 ± 0.6[b] |
| LDL/HDL | 0.6 ± 0.1[b] | 0.8 ± 0.4[b] | 1.2 ± 0.4[a] | 0.8 ± 0.3[b] |
| triglycerides | 2.6 ± 0.7 | 4.0 ± 2.7 | 3.9 ± 2.1 | 4.2 ± 1.6 |

[a] Means ± SD. [b] Means with different superscripts in the same row are significantly different ($p < 0.05$).

**Statistical Analysis.** Data from the nutrition study were analyzed using the Statistical Analysis System (SAS) by analysis of variance, and mean comparisons were performed using Tukey's HSD mean comparison test (SAS, 1985).

## RESULTS

**Growth and Feed Consumption.** Total weight gain and feed intake are summarized in Table II. Addition of saponin to either protein source resulted in decreased growth ($p < 0.05$). Feed intake was similar among animals fed either casein- or ISP-based diets; however, when saponin was added to the casein-based diet, intake was depressed ($p < 0.05$).

**Lipid Analyses.** Mean serum lipid concentrations are summarized in Table III. In gerbils fed ISP alone, total and LDL cholesterol concentrations and LDL/HDL ratios were lower than in those fed casein alone ($p < 0.05$). When saponin was added to ISP, no additional lipid-lowering effect was noted above that observed in aninals fed ISP-based diets. However, in the group in which saponins were added to the casein-diet, LDL cholesterol and LDL/HDL cholesterol ratios were decreased compared to those of the casein-fed group ($p < 0.05$), resulting in values similar to both ISP-fed groups. HDL cholesterol and total triglyceride concentrations were unaffected by dietary treatment.

**Saponin–Protein Interactions (Electrophoresis).** The reaction between caseins and quillaja saponin was analyzed by SDS–PAGE and urea–PAGE. Figure 1 represents the SDS electropherogram of the reaction as it progressed during the first 30 min. It is evident that high molecular weight complexes were gradually formed (lanes 4–8) during the reaction. Soybean proteins did not result in the same type of gradual high molecular weight increase as did the caseins. Figure 2 represents the SDS electropherogram of the reaction time course of quillaja saponin with ISP under the same conditions as those used with caseins. This electropherogram shows a decrease in the intensity of all bands, and no gradual increase in the molecular weight of any of the soy proteins is apparent. Insoluble aggregates, which were retained in the stacking gel used for the analysis of the samples, were observed as a result of the reaction.

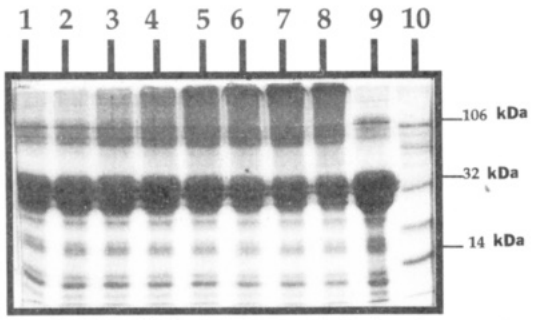


**Figure 1.** SDS–PAGE of whole casein during the reaction with quillaja saponin. Lane 1 is control casein without treatment; lanes 2–8 are samples of the reaction taken at 0, 1, 3, 5, 8, 11, and 26 min; lane 9 is casein without saponin heated for 26 min; lane 10 is the commercial molecular mass marker, the main bands, and their molecular masses are indicated at the right side of the figure.

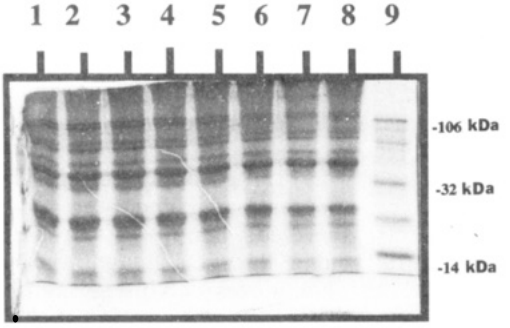


**Figure 2.** SDS–PAGE of soybean protein isolate and quillaja saponin. Lane 1 is control soy protein isolate without treatment; lanes 2–8 are samples of the reaction taken at 0, 1, 3, 5, 8, 11, and 26 min; lane 9 is the commercial molecular mass marker.

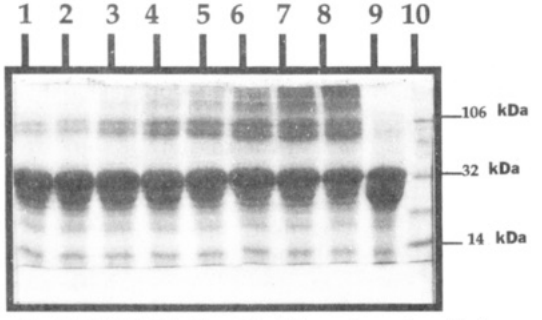


**Figure 3.** SDS–PAGE of purified $\beta$-casein and quillaja saponin. Lane 1 is control $\beta$-casein without treatment; lanes 2–8 are samples of the reaction taken at 0, 1, 3, 5, 8, 11, and 26 min; lane 9 is $\beta$-casein heated without saponin for 26 min; lane 10 is the commercial molecular mass marker, the main bands, and their molecular masses are indicated at the right side of the figure.

The gradual increase in molecular weight of the caseins was studied in each individual casein. Purified fractions of $\alpha_{s1}$-, $\beta$-, and $\kappa$-caseins were allowed to react under the same conditions, and all were analyzed by SDS and urea-PAGE. Figure 3, purified $\beta$-casein with quillaja saponin, demonstrates that $\beta$-casein was, for the most part, responsible for the gradual increase in molecular weight bands. Lane 9 in Figure 3 is $\beta$-casein heated for 30 min without the presence of saponins and indicates that the presence of high molecular weight bands is a result of the interaction with saponins. Figure 4, a urea electropherogram which separates proteins by molecular weight as well as by charge difference, demonstrates a gradual increase in number and a decrease in mobility of many protein bands. These results appear to indicate that the changes

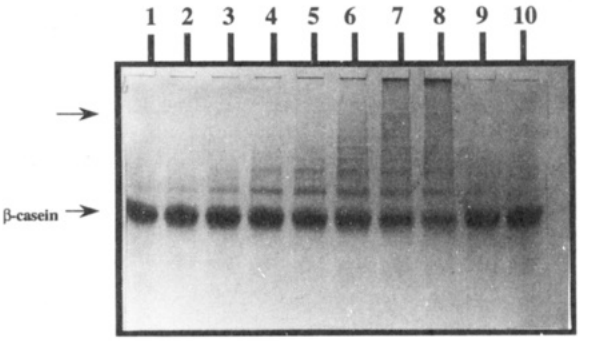


**Figure 4.** Urea–PAGE of $\beta$-casein with quillaja saponin; lanes correspond to the following times: 0, 1, 3, 5, 8, 11, and 26 min and $\beta$-casein heated for 26 min. Lanes 1 and 10 are control $\beta$-casein without treatment; lanes 2–8 are samples of the reaction taken at 0, 1, 3, 5, 8, 11, and 26 min; lane 9 is $\beta$-casein heated without saponin for 26 min. The arrows show the original mobility of $\beta$-casein and the region of lower mobility where the bands appear as the reaction time progresses.

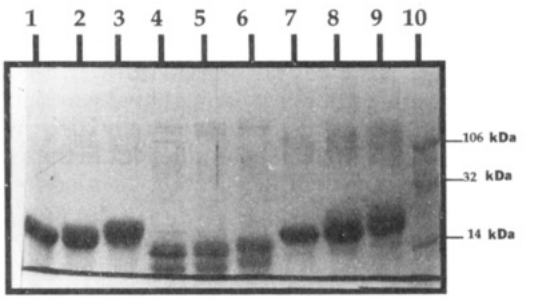


**Figure 5.** SDS–PAGE of different purified fractions of caseins with lactose. $\beta$-Casein (lanes 1–3), $\kappa$-casein (lanes 4–6), and $\alpha$-casein (lanes 7–9) are samples for each casein in increasing reaction times, corresponding to 0, 120, and 240 min.

in milk proteins due to their interaction with quillaja alter drastically the protein charge as well as their molecular weight.

The possibility that proteins and saponins, which are glycosides, were interacting via Maillard browning was explored. Analogous conditions of the reaction with saponins were tested with lactose. Figure 5 indicates the expected molecular weight changes when three purified caseins react with lactose. Lanes 1–3 represent $\beta$-casein, lanes 4–6 $\kappa$-casein, and lanes 7–9 $\alpha_{s1}$-casein. None of these proteins show any polymerization due to their interaction with lactose. The molecular weight difference is small and may correspond to the amount of lactose added onto the proteins.

**Fluorometric Measurement of Free Amino Groups.** Figure 6 shows the relative decrease of free amino groups from $\alpha$- and $\beta$-casein, respectively. This reduction of free amino groups is measured as a decrease in fluorescence. The reaction is fast and almost completed in the first 10 min. This reduction in fluorescence indicates a reduction of the free amino groups in the proteins from the lysine, arginine, asparagine, and glutamine residues. In general, $\alpha$-caseins are more acidic and have less surface hydrophobicity than $\beta$-casein. These are important differences that may indicate the type and rate of reaction of saponins with these proteins.

## DISCUSSION

Saponins present a complex chemical structure consisting of various carbohydrate moieties attached to a triterpenoid or steroidal molecule commonly referred to as the aglycon. The aglycon is very hydrophobic and the sugar very hydrophilic, which gives this molecule its

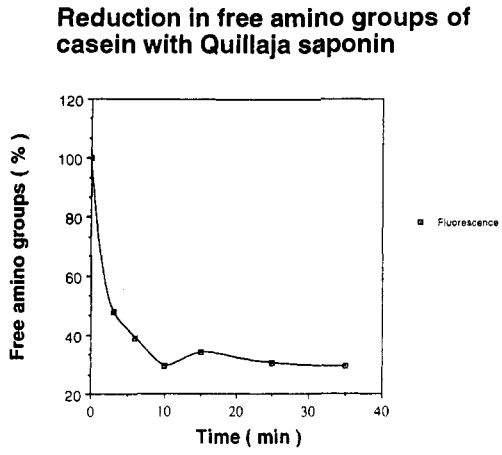


**Figure 6.** Free amine determination of caseins using the fluorescence method.

excellent emulsifying properties. In foods, these molecules are used as emulsifiers and foaming agents. The quillaja saponins are a complex mixture with a triterpenoid aglycon and a complex array of oligosaccharides and acylated residues. Several different saponins have been identified, but only one has been fully characterized (Higuchi et al., 1988). The structure includes an aldehyde in the aglycon, a glucuronopyranosyl carbohydrate, esterified material, and several hydroxyl groups. Any of these groups can react with the proteins. The conditions to study interactions between proteins and saponins in this work were designed to simulate possible processing conditions. After heating, different proteins yielded different results with relation to their interaction with quillaja saponins.

The resulting bands from the casein–saponin interaction are of higher molecular weight, as can be determined from their lower mobility on SDS–PAGE. When the different purified fractions of casein were allowed to react with quillaja saponin, it was found that β-casein is the most susceptible to the formation of higher molecular weight bands on the urea electropherograms. Urea–PAGE is sensitive to changes in the charge of the proteins as well as to their molecular weight. Figures 3 and 4 indicate that there are multiple bands generated from a purified fraction of β-casein.

It is important to determine if the interaction between the saponins and the proteins takes place via the sugar moieties of the saponin with the free amino groups of the proteins (e.g., Maillard reaction). Comparison of the resulting bands in Figures 1 and 5 shows that the interaction of proteins with saponin is faster than with lactose, and the molecular weight increase is also larger in the case of saponins. This marked difference indicates that the interaction observed between the caseins and the saponins is more complex than that of a sugar and a protein. Additional information can also be obtained by comparing the reactivity of the different caseins with saponin. The three caseins are very similar in composition and molecular weight. Subtle differences such as surface hydrophobicity, isoelectric point (acidity of the protein), and degree of phosphorylation may explain the differences in the observed reactions.

In contrast, ISP does not form any detectable bands of higher molecular weight (Figure 2). Soy proteins form insoluble aggregates during heating independently of the presence of added quillaja saponin. One possible explanation is that there are indigenous saponins present in the isolated soy protein. Another is that the soy proteins by themselves are not heat resistant, and they naturally form insoluble aggregates. Condensation between the saponin and proteins could occur in different places, and determination of the specific reaction is underway. Even highly purified soy protein isolates contain saponins, and little information is available with regard to the extent of posttranslational modifications of soy proteins and to the chemical changes during processing.

Data from the nutrition study indicate that the addition or omission of saponins in different dietary proteins affects serum cholesterol concentrations differently. When saponins were added to the casein-based diet, LDL cholesterol was reduced by approximately 44% in comparison to the casein-based diet alone. Total cholesterol was reduced by approximately 32% by the addition of saponins. However, due to the wide variation in cholesterol concentrations in gerbils fed casein, this difference was a trend only ($p < 0.10$). The LDL/HDL ratios also decreased from approximately 1.2 in animals fed casein alone to 0.8 in those fed casein plus saponins. When saponins were added to the ISP-based diets, no additional lipid-lowering effects resulted. This indicates that saponins administered in the diet have a hypocholesterolemic effect; however, it is dependent on source of dietary protein.

Results presented here correspond to some extent to findings by others on the effects of saponins on blood lipids (Oakenfull et al., 1979, 1984; Topping et al., 1980). In casein-based diets, depressions in cholesterol concentrations in rats were observed when quillaja saponins were added (Oakenfull et al., 1984). It is thought that saponins form nonabsorbable complexes with bile salts and dietary cholesterol, thereby decreasing absorption of cholesterol and enhancing bile acid excretion (Sidhu and Oakenfull, 1986). However, Topping et al. (1980) found no differences in plasma lipids in rats fed intact soy flour or saponin-removed soy flour-based diets.

Our findings from the chemistry of protein–saponin interactions indicate that saponins form very tight linkages to soy protein rapidly, whereas saponins interact with caseins much more slowly. In addition, casein interacted via a specific protein (β-casein), whereas soy protein did not. This may be part of the reason for the differences in cholesterolemia between the two sources of dietary protein. Saponins may have been more available to interact with dietary cholesterol and enterohepatic circulation in animals fed the casein-based diets. Another possibility is that the reactions between quillaja saponin and both proteins regulated resultant protein-induced changes in blood lipids by altering digestion of the proteins, resulting in a slower release of protein and/or saponin as compared to ingestion of the proteins alone.

These experiments indicate the importance of considering the overall chemistry of the systems that may lead to differences in lipid composition in the blood. While the proteins themselves may play a role in the regulation of blood lipids, there are many other factors that, despite their relatively low concentrations, may have important effects. In this case, the difference that saponins make on serum lipid composition of the casein-fed gerbils indicates that modification in the chemistry and or structure of the caseins alters their influence on blood lipids.

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