# Interaction between Bovine Serum Albumin and Saponin As Studied by Heat Stability and Protease Digestion

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Soyasaponins were added to bovine serum albumin (BSA), and the interaction between BSA and soyasaponin was studied by heat stability and protease digestion. Results from enzyme-linked immunosorbent assays and circular dichroism spectra showed that the heat stability of BSA was increased by soyasaponin addition. Furthermore, results from α-chymotrypsin digestion showed that BSA's susceptibility to α-chymotrypsin digestion was decreased by soyasaponin addition and that the N-terminal 26 kDa peptide fragment obtained from the hydrolysate of BSA−soyasaponin complex interacted with soyasaponin to form a protease-resistant moiety that is low in sensitivity to α-chymotrypsin.

**Keywords:** *Bovine serum albumin; soyasaponin; interaction; heat stability; immunodiffusion; ELISA; protease resistance*

## INTRODUCTION

Saponins are widely distributed in many plant species and have complex chemical structures consisting of a variety of triterpenoidal or steroidal aglycons and various carbohydrate moieties. Since the aglycon is hydrophobic and the sugar chains are hydrophilic, these molecules have some excellent foaming and emulsifying properties. Saponins are now accepted as functional components in food because of their physiological properties (Price et al., 1987).

Bovine serum albumin (BSA) is well characterized as to both its structure and its physical and physiological properties and is also known as the carrier protein of fatty acids. BSA−fatty acid complexes have reportedly been formed by electrostatic and hydrophobic interactions and showed a higher thermal stability than free BSA (Gumpen et al., 1979).

Recently, saponins added to the diet were reported to decrease plasma lipids in experimental animals. Those from soybean, quillaja, alfalfa, and soapwort have been proven to decrease plasma cholesterol in rats when added to their diets (Oakenfull, 1981; Sidhu and Oakenfull, 1986). Casein added to quillaja saponins was reported to form complexes of high molecular weight and to decrease the free amino groups of casein upon heat treatment (Potter et al., 1993). However, the interaction between protein and saponin is poorly understood.

It is very important with respect to physical and physiological functionalities to characterize the interaction between proteins and saponins in food materials such as soybeans which contain a large amount of saponins. In this study, we estimated the effect of soyasaponins on heat stability and protease digestion of BSA in light of the interaction between protein and saponin.

## MATERIALS AND METHODS

**Preparation of Soyasaponin.** Hypocotyls (100 g) of soybean seeds were extracted with a 10-fold volume of 70% aqueous ethanol overnight. The supernatant was collected by filtration. The residue was extracted again. Three extracts were combined and concentrated under reduced pressure. The concentrates were dispersed in butanol−water (1:1). The butanol layer was evaporated and lyophilized to afford a soyasaponin fraction.

**Immunodiffusion.** Ouchterlony's analysis (Ouchterlony, 1949) was performed using 1% agarose in PBS buffer (0.01 M phosphate buffer, pH 7.2, with 0.15 M NaCl) containing 0.1% $NaN_3$.

**ELISAs.** Microtiter plates were coated with 100 μL of BSA solutions (10 μg/mL) in 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl diluted serially. After overnight incubation at 4 °C, the solutions were removed and the wells were washed three times with 0.01 M phosphate buffer, pH 7.2, containing 0.5 M NaCl and 0.05% Tween 20. The wells were coated with 150 μL of 1% ovomucoid solution in PBS buffer to block the active surface area remaining after immobilization of the BSA solution. After a 90 min incubation at 37 °C, the solutions were removed, washed, and replaced with 100 μL of rabbit anti-bovine albumin (Organon Teknika N.V.-Cappel Products) whole serum solutions. After a 30 min incubation at 37 °C, the solution was removed, washed, and incubated with 100 μL of HRP−anti-rabbit IgG solutions for 30 min at room temperature. The solutions were removed, washed, and replaced with 100 μL of substrate solutions [0.4 mg/mL *o*-phenylenediamine dihydrochloride in 0.2 M $Na_2HPO_4$−0.1 M citric acid−$H_2O_2$ 25:25:0.1 (v:v:v)]. After incubation for 30 min at room temperature, reactions were stopped with 100 μL of 3 N $H_2SO_4$, and the plates were read at 490 nm in a Bio-Rad Model 450 microplate reader.

**Circular Dichroism (CD).** Protein samples were dissolved in 0.01 M phosphate buffer, pH 7.6. A cell having a path length of 0.1 cm was used. The CD spectra of sample solutions were measured using a JASCO J-600. Spectra were recorded from 250 to 200 nm. Calculation of the secondary structure from CD spectra was conducted according to the method of Chen et al. (1974).

**Protease Digestion.** Protease digestion was carried out as follows. Four milliliters of 0.1% protein solution in 0.01 M phosphate buffer, pH 7.6, was mixed with 250 μL of 0.1% α-chymotrypsin solution in the same buffer. The enzymatic reaction was carried out at 38 °C for a given period. Then, 4 mL of 4% aqueous trichloroacetic acid was added, and the resulting precipitate was removed by centrifugation. The hydrolyzed products in the supernatants were estimated according to the method of Lowry et al. (1951).

**α-Chymotryptic Activity.** α-Chymotryptic activity was examined using succinyl-Ala-Ala-Pro-Phe-MCA (Peptide Institute). α-Chymotryptic activity was measured with the UV

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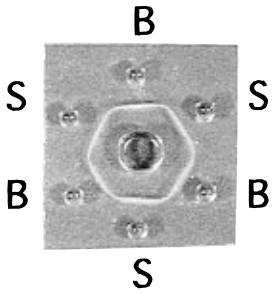


**Figure 1.** Immunodiffusion of BSA and BSA−soyasaponin complex. Antiserum was allowed to diffuse from the center well against BSA (B) and BSA−soyasaponin complex (BSA: soyasaponin = 1:1 w:w) (S).

absorbance at 340 nm of the products of 7-amino-4-methylcoumarin after incubation at 25 °C for 10 min in the mixture of 0.1 mM substrate solution (5 mL) in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ and 0.1% α-chymotrypsin solution (50 μL).

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE).** SDS−PAGE was performed using 15% gels according to the method of Laemmli (1970). Gel sheets were stained with Coomassie Brilliant Blue R-250.

**Gel Filtration.** Gel filtration chromatography was carried out at 4 °C with a Sephadex G-75 column (2.5 × 60 cm). The samples (100 mg) were dissolved in 5 mL of 0.2 M phosphate buffer, pH 7.2, and eluted with the same buffer. The eluted fractions were monitored with UV absorbance at 280 nm. Each fraction obtained was dialyzed against deionized water and lyophilized.

**Amino Acid Sequence.** After electrophoresis, the gel was blotted onto PVDF membrane according to the procedure of Matsudaira (1987). The PVDF membrane was washed in deionized water for 5 min and stained with Coomassie Brilliant Blue R-250.

The band corresponding to the desired peptide was cut out and applied to an automatic protein sequencer (ABI Model 471A/140B) to estimate the N-terminal amino acid sequence.

## RESULTS AND DISCUSSION

**Effect of Soyasaponin on Heat Stability of BSA Studied by ELISAs.** To carry out the ELISA of BSA−soyasaponin complex, the effect of soyasaponin on antigen−antibody reaction of BSA was estimated according to the method of Ouchterlony (1949). The result is shown in Figure 1. The complete fusion of precipitation lines between BSA and BSA−soyasaponin complex showed that soyasaponin did not affect the antigen−antibody reaction of BSA. The ELISA of BSA−soyasaponin complex gave results comparable to those of free BSA.

ELISAs of BSA heated with or without soyasaponin are shown in Figure 2. The reactivity of anti-BSA to BSA without soyasaponin heated at 60 °C was almost equal to that of the native BSA, but those heated at 80 and 100 °C were decreased significantly. On the other hand, the reactivity of BSA with soyasaponin was maintained at 60 and 80 °C and then decreased at 100 °C. At 80 °C, BSA with soyasaponin reacts with anti-BSA stronger than BSA without soyasaponin.

These results indicated that BSA was stable at 60 °C against heat treatment but partially denatured at temperatures >80 °C. On the other hand, BSA−soyasaponin complex was stable at 80 °C. In short, the heat stability of BSA was increased by the addition of saponin.

Furthermore, changes in the secondary structure of BSA were examined by CD spectra. Examining the effect of soyasaponin on BSA at room temperature

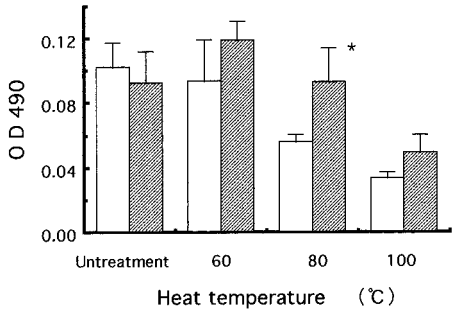


**Figure 2.** ELISAs of reactivity of BSA and BSA−soyasaponin complex: (open bar) BSA; (shaded bar) BSA−soyasaponin complex (BSA:soyasaponin = 1:1/w:w). BSA concentration was 0.039 μg/mL. An asterisk above a point means it is significantly different between BSA and BSA−soyasaponin complex ($P < 0.05$).

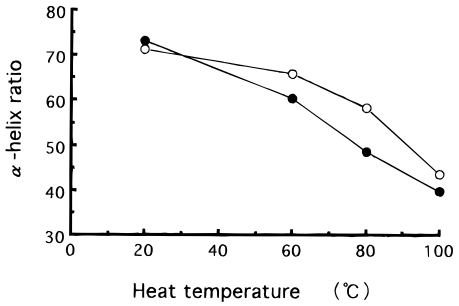


**Figure 3.** Changes in α-helix ratio of BSA and BSA−soyasaponin complex by heat treatment: (●) BSA; (○) BSA−soyasaponin complex (BSA:soyasaponin = 1:1/w:w).

indicated that soyasaponin did not affect the CD spectrum of BSA (data not shown). The relative ratio of the secondary structure of BSA was calculated to be 73% α-helix, 12% β-structure, and 15% random coil, which is in good agreement with the findings of Reed et al. (1975). Then BSA and BSA−soyasaponin complex were heated at various temperatures for 10 min. After the samples had cooled to room temperature, the CD spectra were measured and the α-helix contents were calculated according to the method of Chen et al. (1974). The α-helix content of BSA was gradually lowered with an increase in the heating temperature (Figure 3). With heating at 80 °C, the α-helix content of BSA was lowered by about 50%. However, the α-helix content of the BSA−soyasaponin complex was relatively higher than that of free BSA at each temperature tested. These phenomena also suggested that soyasaponin allows BSA to retain greater stability under heat treatment, coinciding with the ELISAs. This result is similar to that for BSA−fatty acid complex. The thermal stability of BSA reportedly increases with the addition of SDS or NaCl, and this was considered to be an effect of those anions (Gumpen et al., 1979). Soyasaponins have sugar chains containing one glucuronic acid. We supposed that the glucuronate anion moiety in soyasaponins might also be related to interaction with BSA.

**Effect of Soyasaponin on Protease Digestion of BSA and Protease-Resistant Fragment.** Free BSA and BSA containing 0.1% soyasaponin were applied to α-chymotrypsin digestion. BSA was hydrolyzed with the reaction time (Figure 4). The digestibility of the BSA−soyasaponin complex was much lower than that of free BSA. After 60 min of protease reaction, the acid-unprecipitable products reacting with BSA−soyasaponin were less than half of those with free BSA. At first, the effect of soyasaponin on α-chymotryptic activity was examined using synthetic substrate. α-Chymotrypsin

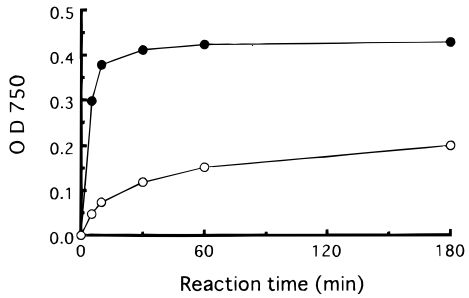


**Figure 4.** Protease digestibility of BSA and BSA−soyasaponin complex: (●) BSA; (○) BSA−soyasaponin complex (BSA: soyasaponin = 1:1/w:w).

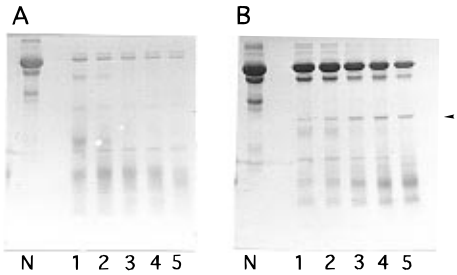


**Figure 5.** SDS−PAGE patterns of the α-chymotrypsin-digested BSA and BSA−soyasaponin complex for 0−180 min: (A) BSA; (B) BSA−soyasaponin complex (BSA:soyasaponin = 1:1/w:w). Lanes: (N) native BSA; (1) 5 min, (2) 10 min, (3) 30 min, (4) 60 min, (5) 180 min of reaction time; (arrow) unknown fragment.

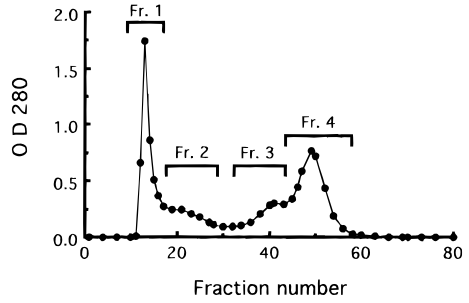


**Figure 6.** Gel filtration chromatography of the α-chymotrypsin-digested BSA−soyasaponin complex. The BSA−soyasaponin complex digested for 180 min was applied to a Sephadex G-75 column (2.5 × 60 cm) and eluted with 0.2 M phosphate buffer, pH 7.2.

was preincubated with 0.01% soyasaponin for 10 min, and then the α-chymotryptic activity was measured. Soyasaponin appears to slightly activate α-chymotrypsin, but there was no remarkable effect (data not shown).

Protease digestive fragments derived from BSA and BSA−saponin complex were analyzed by SDS−PAGE (Figure 5). The band corresponding to BSA disappeared with reaction time. In the case of BSA−soyasaponin complex, however, BSA was detected for 180 min of reaction time, and an unknown fragment was detected. This fragment was derived from the peptide moiety, which was resistant to the interaction of α-chymotrypsin with soyasaponin.

Next, the hydrolyzing mixture of BSA−soyasaponin complex which reacted for 180 min was separated into four fractions (fractions 1−4) by gel filtration chromatography (Figure 6). Figure 7 shows SDS−PAGE patterns of each fraction. Fraction 2 contained this unknown fragment, which was suggested to be a 26 kDa peptide by SDS−PAGE. The 26 kDa peptide of fraction 2 was blotted onto a PVDF membrane by semidry

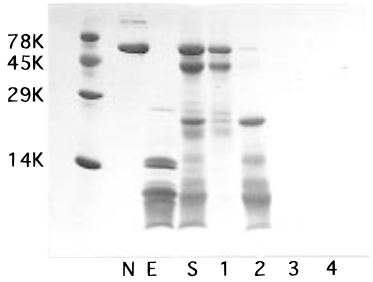


**Figure 7.** SDS−PAGE patterns of gel filtrated fractions from the α-chymotrypsin-digested BSA−soyasaponin complex (15% acrylamide gel was used for SDS−PAGE). Lanes: (N) native BSA; (E) α-chymotrypsin; (S) sample applied to gel filtration chromatography; (1−4) fractions 1−4 obtained from gel filtration.

western blot technique (Matsudaira, 1987) and then subjected to amino acid sequence analysis. The N-terminal amino acid sequence was determined as Asp-Thr-His-Lys-Ser-Glu-Ile and was exactly equal to the N-terminal amino acid sequence of native BSA (Hirayama et al., 1990). The N-terminal peptide fragment of BSA was shown to interact with soyasaponin to form a protease-resistant moiety with low sensitivity to α-chymotrypsin. The protease probe method on α-chymotrypsin, which examines the surface hydrophobicity of proteins (Kato et al., 1986), suggested that the aglycon moiety of saponin interacted with the surface hydrophobic region of BSA. As a consequence of this interaction, α-chymotrypsin was not able to approach the saponin-interacting region because of the hydrophilicities of the sugar chain, and the hydrolyzed ratio of BSA decreased.

These results appear to indicate that soyasaponin interacts with BSA and affects some properties of BSA. We considered that the interaction between BSA and soyasaponin might include electrostatic and hydrophobic interactions. Study of the interaction between protein and saponin is thought to be important in characterizing the physiological properties of protein−saponin complex in food materials such as soybeans.

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