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Effects of Soybean Saponins on Chymotryptic Hydrolyses of Soybean Proteins

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The effects of saponin fraction on chymotryptic hydrolysis of soybean acid precipitated protein and glycinin and β-conglycinin fractions were examined. Endogenous saponin affected the chymotryptic hydrolysis of soybean protein. Further addition of saponin suppressed the hydrolysis of soybean protein fraction. The effect of saponin on chymotryptic hydrolysis of glycinin was greater than on that of β-conglycinin. There were some differences in the effect of saponin on the subunits constituting the soybean globulins. Glycinin acidic polypeptides and β-conglycinin β-subunit became more resistant to chymotryptic hydrolyses by addition of saponin. Changes of CD spectra of glycinin and β-conglycinin by saponin reflected the sensitivity changes of soybean protein against chymotrypsin.

Keywords: *Soybean saponin; chymotrypsin hydrolysis; glycinin; β-conglycinin*

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 (Price et al., 1987). Because the aglycon is hydrophobic and the sugar chains are hydrophilic, these molecules have some excellent foaming and emulsifying properties.

Saponins are now expected to serve as functional components in food because of their physiological properties. Saponins from soybean, quillaja, alfalfa, soapwort, etc., have been reported 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.

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In a previous paper (Ikedo et al., 1996), soybean saponins were reported to interact with bovine serum albumin (BSA) and to decrease the sensitivities against chymotrypsin hydrolysis. BSA became thermodynamically more stable by interacting with saponins. To clarify the mechanisms of the physical and physiological functionalities of food materials, it is also important to characterize the interaction between proteins and saponins in food materials such as soybean, which contains a large amount of saponins.

Soybean seed also contains a large amount of proteins and is well-known as a nutritionally valuable protein. Soybean protein fraction was reported to show cholesterol-lowering activity in rats (Sugano et al., 1990). Soybean proteins are composed of two major globulins, namely, glycinin and β-conglycinin. Glycinin has a molecular weight of ~320 kDa and is composed of six subunits, each of which consists of an acidic and a basic polypeptide. An acidic polypeptide and a basic polypeptide are linked by a disulfide bridge and form a subunit (Badley et al., 1975; Staswick et al., 1981; Barton et al., 1982). β-Conglycinin has a molecular weight of ~150

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kDa and is composed of three kinds of subunits (α, α′, and β) in varying proportions (Koshiyama and Fukushima, 1976; Thanh and Shibasaki, 1978). In the present study, we estimate the effect of soybean saponin fraction on chymotryptic hydrolysis of soybean globulins and consider the interaction between soybean saponin and soybean protein.

MATERIALS AND METHODS

Preparation of Acid Precipitated Protein Fraction (APP) from Soybean Seeds. Soybean seeds were milled and extracted with *n*-hexane. Defatted soybean seed flour was mixed with 20 volumes of 0.03 M Tris-HCl buffer (pH 8) and stirred for 2 h at room temperature. The supernatant was collected by centrifugation (9000*g*, 30 min) and adjusted to pH 4.5. The resulting precipitation was dispersed in water, dialyzed, and lyophilized to afford soybean APP.

Removal of Saponin from Soybean Protein Fraction. An aliquot of Tris-HCl buffer extract from defatted soybean was mixed with an almost equal volume of Sumitomo Duolite S-861 resin, which was washed successively in ethanol and distilled water using a suction filter. Nonadsorbed fraction was collected and adjusted to pH 4.5, and the saponin-less APP was prepared as described above.

Preparation of Glycinin and β-Conglycinin Fractions from Soybean. Soybean glycinin and β-conglycinin fractions were prepared according to the method of Nagano et al. (1992). Defatted soybean meal was extracted with 15 volumes of water, adjusted to pH 7.5 with sodium hydroxide, and stirred for 1 h at room temperature. The supernatant was collected by centrifugation (9000*g*, 30 min), mixed with 10 mM 2-mercaptoethanol, adjusted to pH 6.4, and allowed to stand overnight at 0 °C. The precipitate was collected, dialyzed, and lyophilized to crude glycinin fraction. The supernatant was mixed with NaCl (0.25 M), adjusted to pH 5, and centrifuged (9000*g*, 30 min). The supernatant was diluted with an equal volume of water and adjusted to pH 4.8. The precipitate was collected by centrifugation (6500*g*, 30 min), dialyzed, and lyophilized to afford crude β-conglycinin fraction.

Purification of Glycinin and β-Conglycinin Fraction. Purification of glycinin and β-conglycinin fraction was carried out according to the method of Kitamura et al. (1974). The crude glycinin and β-conglycinin fractions were separately subjected to a concanavalin A−Sepharose column (10 × 60 mm) equilibrated with phosphate buffer ($\mu = 0.5$, pH 7.6) with 0.04% sodium azide. The adsorbed fraction was eluted with 0.01 M phosphate buffer containing 0.2 M methyl mannoside. The nonadsorbed fraction was used as glycinin and the adsorbed fraction as β-conglycinin.

Preparation of Soybean Saponin Fraction and Isolation of Soyasaponin I. Soybean saponin fraction was prepared from soybean seen hypocotyls in a manner reported in a previous paper (Ikedo et al., 1996). Seventy percent ethanol extract from soybean hypocotyl was dispersed in butanol/water (1:1, v/v). The upper layer was collected, concentrated, and lyophilized to afford saponin fraction. Saponin fraction was subjected to Sephadex LH-20 column chromatography and divided into group A and B saponins. Soyasaponin I was isolated from group B saponin using high-performance liquid chromatography. The column was a YMC-Pack ODS-AM-323-7, and the mobile phase used was methanol/2-propanol/water/acetic acid (70.0:6.0:24.0:0.1, v/v). Eluent was monitored by reflective index.

Determination of Saponin Content in Soybean Protein Fraction. Soybean protein fraction was mixed with 20 volumes of methanol and incubated for 5 h at 80 °C. The mixture was centrifuged (3000 rpm, 30 min). Saponin content in the supernatant was determined with HPLC according to a previous paper (Shimoyamada et al., 1990; Shimoyamada and Okubo, 1991) with some modifications. The column was a YMC-Pack ODS-AM-303, and the mobile phase used was methanol/2-propanol/water/acetic acid (70.0:6.0:24.0:0.1, v/v). Eluent was monitored by the absorbance at 210 nm.

Chymotryptic Hydrolysis of Soybean Protein Fraction. Chymotryptic hydrolysis 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. Four millilters of 4% aqueous trichloroacetic acid (TCA) solution was then added, and the resulting precipitate was removed by centrifugation. The TCA-soluble fractions in the supernatants were determined according to the method of Lowry et al. (1951).

Chymotryptic hydrolyses of isolated glycinin and β-conglycinin were carried out similarly. The reaction was stopped by adding 5% SDS solution. For glycinin, the resulting solution was subjected to HPLC to determine the remaining glycinin polypeptides. For HPLC separation, the column was a Waters Protein Pak 300 and the mobile phase was 0.03 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.1% SDS. In the case of β-conglycinin, the reaction mixture was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the degree of hydrolysis was calculated by densitograms.

SDS−PAGE. SDS−PAGE was performed using 12.5% gels according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 and subjected to a densitometer (Advantec Toyo DM-303-CMR).

Circular Dichroism (CD). Protein samples (0.20 mg/mL) were dissolved in 0.01 M phosphate buffer (pH 7.6). A cell having a path length of 0.1 cm was used. CD spectra of sample solutions were measured using a JASCO J-600 and recorded from 250 to 200 nm.

RESULTS AND DISCUSSION

Effects of Soybean Saponin on Chymotryptic Hydrolyses of Soybean Proteins. Previously, BSA was reported to decrease its sensitivities against chymotrypsin by interacting with soybean saponin fraction (Ikedo et al., 1996). Soybean APP was also used to estimate the effect of saponin fraction. To determine the effect of saponin on soybean protein, it is necessary to determine the endogenous saponins in the protein fraction, so the methanol extract from APP was analyzed by HPLC. The APP contained 0.4% of soyasaponin I per dry weight of protein. Kudou et al. (1987) reported that resin treatment decreased saponin content in soy milk, so the water extract from soybean was directly subjected to Sumitomo Duolite S-861 resin. Nonadsorbed fraction, which consisted of soybean proteins, contained a very small amount of saponin (data not shown). From these chromatograms, ∼92% of intact soybean saponin was removed from APP by resin treatment. Kudou et al. (1987) reported that ∼70% of saponin could be removed from soy milk. Almost complete removal of saponin from protein fraction may be due to low oil content in soybean protein fraction differing from soy milk.

APP, saponin-less APP, and saponin readded (protein fraction/saponin = 10:1, w/w) APP were subjected to chymotryptic hydrolyses. The hydrolysis level was estimated by determining the protein content in TCA-soluble fraction from reaction mixtures by using the method of Lowry et al. (1951). As a result (Figure 1), the hydrolysis level of APP significantly increased by removal of saponins. Endogenous saponin was shown to be able to affect the chymotryptic hydrolysis or digestibility. When the saponin fraction from soybean hypocotyl was added to saponin-less APP again (protein/saponin = 10:1, w/w), the chymotryptic hydrolysis level of soybean protein significantly decreased to ∼75% of the saponin-less APP. These reversible changes of chymotryptic hydrolysis levels may also show that the

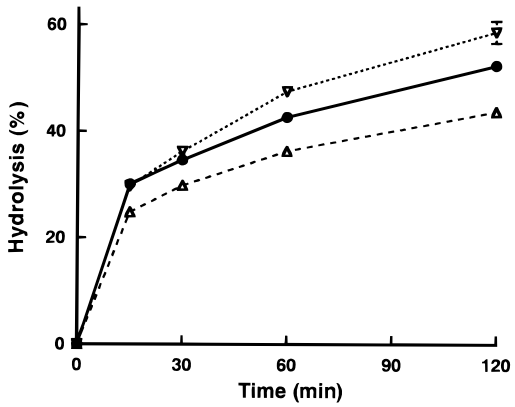

Figure 1. Effect of soybean saponin fraction on chymotryptic hydrolysis of soybean protein fraction: soybean APP (●); saponin-less APP (▽); saponin-added APP (△). Each data point is the average of triplicate measurements. The vertical bars depict the standard deviation. Data points without the bars show that the deviations are smaller than the symbol size.

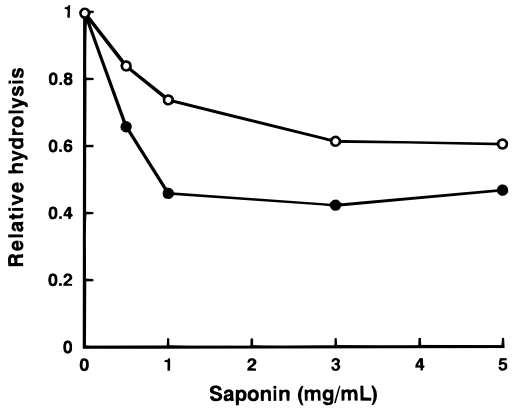

Figure 2. Chymotryptic hydrolysis levels of crude glycinin and conglycinin fraction with soybean saponin. Each protein [glycinin (●), β-conglycinin (○); 1 mg/mL] was incubated with chymotrypsin for 180 min. Each data point is the average of duplicate measurements.

removal of saponin by resin treatment has little effect on the high-order structure of soybean protein.

Ikedo et al. (1996) reported that chymotryptic hydrolysis of BSA was much decreased by the addition of soybean saponin fraction when the equal amount of saponin (w/w) was added to BSA. However, a relatively small amount of saponin, which was 10% of the protein weight to soybean saponin-less APP, was able to affect the chymotryptic hydrolysis of APP.

Soybean protein fraction was then divided into crude glycinin and β-conglycinin fractions according to the method of Nagano et al. (1992) and subjected to chymotryptic hydrolysis. Chymotryptic hydrolysis ratios of glycinin and conglycinin (protein concentration = 1 mg/mL) were about 75 and 60%, respectively, after 180 min of incubation. Each fraction decreased its hydrolysis level with the increase in saponin fraction added (Figure 2). The relative hydrolysis level of glycinin initially decreased greatly to 43% at 1 mg/mL of saponin and then remained almost constant. This level was almost equal to that of BSA (Ikedo et al., 1996). On the other hand, the hydrolysis level of β-conglycinin gradually decreased and reached 61% at 3 mg/mL of saponin. The effect of saponin on glycinin especially was stronger than on β-conglycinin and almost equal to that on BSA. Glycinin and β-conglycinin were

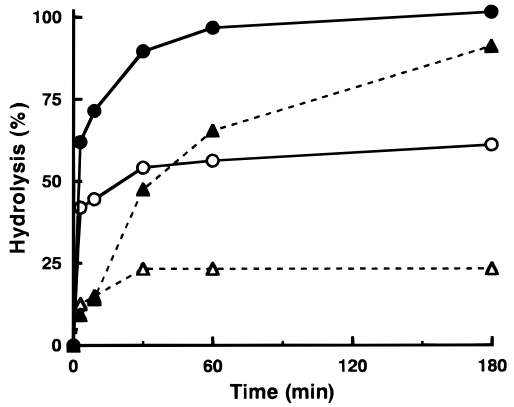

Figure 3. Chymotryptic hydrolysis levels of glycinin with soybean saponin: acidic polypeptide (●, ○); basic polypeptide (▲, △); without soybean saponin (●, ▲); with soybean saponin (○, △). Each data point is the average of duplicate measurements.

reported to have some structural differences; for example, glycinin is thought to be less flexible (Badley et al., 1975) and the N-terminal regions of α'-polypeptide of β-conglycinin are more surface probable (Nielsen et al., 1988). The differences in hydrolysis levels among proteins are considered to reflect these structures of the proteins.

Effect of Soybean Saponin on Chymotryptic Hydrolysis Levels of Each Subunit Constituting Glycinin and β-Conglycinin. Crude soybean glycinin and β-conglycinin fractions were next separately subjected to a Con-A−Sepharose column and purified. Glycinin and β-conglycinin obtained from the Con-A column, which contained no detectable saponins by HPLC, were hydrolyzed by chymotrypsin with or without saponin fraction. The reaction mixtures of glycinin and chymotrypsin were mixed with SDS solution to stop the reactions. The resulting mixtures were subjected to gel filtration HPLC, and the hydrolysis levels of the polypeptides were determined (Figure 3). Acidic polypeptides were rapidly hydrolyzed, and the hydrolysis level reached 100% after 60 min. Basic polypeptides were gradually hydrolyzed and reached 90% at 180 min. The hydrolysis levels were higher than those of crude proteins, but these differences were considered to be due to a difference in the methods of estimating the hydrolysis levels, namely, the TCA-soluble fraction and the subunits themselves. When the saponin fraction was added, the hydrolysis levels of acidic polypeptides greatly decreased and were almost constant at ~60% after 30 min of incubation. Basic polypeptides were also much less hydrolyzed, and ~80% of the constituents were maintained at 180 min of incubation without chymotryptic hydrolysis.

In previous papers (Kamata and Shibasaki, 1978; Nielsen et al., 1988; Kamata et al., 1991), the acidic polypeptides were first hydrolyzed by proteases and considered to locate on surfaces or some flexible regions of the protein. In this study, the acidic polypeptides were more rapidly hydrolyzed than the basic polypeptide with or without saponin. However, the acidic polypeptides became more resistant to chymotryptic hydrolysis, and ~40% of the native constituents remained during 180 min of incubation with saponin fraction, although they were completely hydrolyzed without saponin fraction. From the above data, saponin may interact with the acidic polypeptides, which locate on the surface of

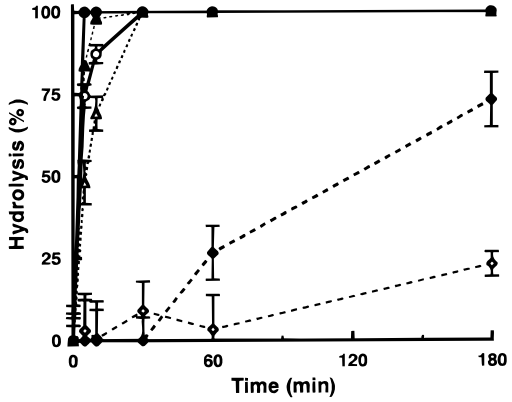

Figure 4. Chymotryptic hydrolysis levels of β-conglycinin with soybean saponin: α-subunit (●, ○); α′-subunit (▲, △); β-subunit (◆, ◇); without soybean saponin (●, ▲, ◆); with soybean saponin (○, △, ◇). Each data point is the average of triplicate measurements. The vertical bars depict the standard deviation. Data points without the bars show that the deviations are smaller than the symbol size

the glycinin molecule, and decrease the total sensitivity of this molecule to chymotrypsin by preventing chymotrypsin attack on the basic polypeptide.

On the other hand, β-conglycinin subunits were not satisfactorily separated by the gel filtration HPLC, so the hydrolysis levels were calculated using the densitograms from SDS−PAGE patterns. The hydrolysis level estimated by SDS−PAGE/densitogram was less accurate, but the result clearly showed that α- and α′-subunits were rapidly and completely hydrolyzed by chymotrypsin and that β-subunit was gradually hydrolyzed; the hydrolysis level reached ∼75% at 180 min of reaction time (Figure 4). Similar results were reported in a previous paper (Nielsen et al., 1988). By addition of saponin, hydrolyses of α- and α′-subunits were delayed slightly, but the subunits were almost completely hydrolyzed (Figure 4), so there was only a little effect of saponin fraction on chymotryptic hydrolyses of α- and α′-subunits. On the other hand, the hydrolysis level of β-subunit with saponin fraction was much more suppressed during incubation, and ∼75% of native β-subunit remained for 180 min of incubation in contrast to the case of β-subunit without saponin fraction, for which ∼25% of the protein remained. These results may suggest two hypotheses: one is that saponin selectively interacts with β-subunit in conglycinin subunits, and the other is that β-subunit is sensitive to the interaction with saponin and more easily changes its high-order structure than α- or α′-subunits. A more detailed study is in progress.

CD Spectra of Glycinin and β-Conglycinin Containing Soybean Saponin Fraction. Previously, BSA/saponin mixture reportedly became more thermodynamically stable than saponin-free BSA by CD spectra and ELISA (Ikedo et al., 1996) and differential scanning calorimetry (Shimoyamada et al., 1997). BSA did not change the CD spectrum by interacting with saponins (Ikedo et al., 1996), so thermodynamic stabilization of BSA by saponin was shown to determine the α-helix content from CD spectra of the BSA/saponin mixture. On the other hand, soybean whey protein fraction CD spectrum was changed by interaction with saponin (Shimoyamada et al., 1997). In the present study, CD spectra of soybean glycinin/saponin and β-conglycinin/saponin mixtures were compared with those of glycinin and β-conglycinin without saponin

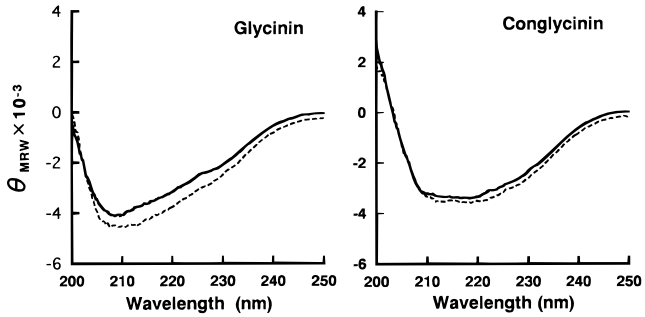

Figure 5. CD spectra of glycinin and β-conglycinin with saponin: without soybean saponin (solid line); with soybean saponin (broken line).

(Figure 5). CD spectra of glycinin were changed by addition of saponin in the wavelength range from 205 to 230 nm. However, the CD spectra of β-conglycinin, of which the decrease of sensitivity against chymotrypsin was smaller than that of glycinin, did not significantly change by addition of saponin fraction. These data may show that the inhibitory effect of saponin fraction on chymotryptic hydrolysis is related to the ability to modify the structures of proteins.

Quillaja saponin was reported to bind covalently to β-casein molecule and to lower serum cholesterol in gerbils (Potter et al., 1993). Soybean saponin is not thought to bind covalently to protein, because there is no heat treatment and no change in SDS−PAGE patterns of protein/saponin mixture (data not shown). Soybean saponin is perhaps bound noncovalently, for example, through hydrophobic interaction, hydrogen bond, ionic bond, etc., to protein. CD spectra of soybean protein with saponin may show changes of high-order structure by noncovalent interaction.

In conclusion, soybean glycinin and β-conglycinin, especially glycinin and β-conglycinin β-subunit, changed their sensitivity to chymotrypsin when soybean saponin fraction was added. Endogenous saponin may interact with soybean proteins, and their lower chymotryptic hydrolysis suggested some potential functionalities of soybean protein such as hypocholesterolemic effect.

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