Acylated Steryl Glucosides from Soybean Globulins: Isolation and Characterization[1]

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ABSTRACT

Previous studies at the Northern Laboratory showed that isolated soybean proteins contain materials extractable with aqueous alcohols consisting of phosphatides, saponins, β-sitosteryl glucoside, genistein, triglycerides, and unidentified compounds. We have now identified acylated steryl glucosides as an additional fraction of the materials extractable with 86% (v./v.) ethanol from freeze-dried acid-precipitated soybean globulins. The dry solids extracted from soy proteins with alcohol were leached with hexane, and the portion soluble in hexane was chromatographed on an activated magnesium silicate column. Yields of acylated steryl glucosides were 0.06% of the weight of the protein. The acylated steryl glucosides were characterized by thin-layer chromatography, hydrolysis to steryl glucosides, and analyses of fatty acids, sterols, and glucose. Although the fatty acids included 26% linoleic and 2% linolenic acid, the intact acylated steryl glucosides were not oxidized by the Theorell preparation of soybean lipoxygenase.

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Occurrence of nonprotein constituents in isolated soybean proteins was first reported by Smiley and Smith in 1946 (1). They found that the nonprotein materials were extractable with ethanol, and their preliminary studies suggested that these impurities were phospholipids. Subsequently, it was shown that the alcohol-extractable materials inhibit foaming of soybean protein isolates (2,3). Fractionation and characterization of the alcohol extractables from dialyzed soybean globulins revealed a complex mixture—phosphatidyl choline, phosphatidyl ethanolamine, saponins, β-sitosteryl glucoside, and genistein plus unknown compounds (4). We have now identified acylated steryl glucosides as an additional group of compounds in the alcohol-extractable mixture. Here we describe our isolation and characterization of this class of sterol derivatives found in acid-precipitated soybean globulins.

MATERIALS AND METHODS

Soybean Proteins

Dialyzed globulins were prepared from hexane-defatted soybean flakes as described by Nash et al. (4). Extraction of the freeze-dried globulins with hexane was omitted, since negligible amounts of material are removed with this solvent.

Alcohol-Extractables

The procedure described earlier (4) was followed with minor modifications: extraction time was limited to 4 hr., and no precautions were taken to exclude light during concentration to dryness on a rotary evaporator at 40° to 50°C. The dry solids were fractionated by leaching with hexane until colorless extracts resulted. The portion soluble in hexane was concentrated for further fractionation on a chromatographic column.

Thin-Layer Chromatography (TLC)

Commercial, precoated silica gel plates (Type F-254, Brinkmann Instruments, Inc., Westbury, N.Y.) were developed (5) with chloroform:methanol:acetic acid:water (90:8:1:1). Compounds were detected nonspecifically by spraying the TLC plates with 50% sulfuric acid and then heating to 90° to 95°C. Phosphatidyl choline was detected with Dragendorf reagent (6).

Column Chromatography

In a modification of the method of Kiribuchi et al. (5), 30 g. of 60–100-mesh Florisil (activated magnesium silicate purchased from Floridin Co., Hancock, W. Va.) was washed successively with methanol (two 300-ml. portions), diethyl ether (two 300-ml. portions), and finally with *n*-hexane (two 150-ml. portions). After residual hexane was evaporated by air-drying, the washed Florisil was heated at 110°C. for 4 hr. It was then deactivated by adding 2.1 ml. water and equilibrating overnight. The Florisil was packed into a 0.9-cm. (i.d.) glass column filled with hexane by adding portions of dry powder and allowing it to settle until the column bed was 76 cm. deep.

The portion of the alcohol-extractables that dissolved in hexane was

concentrated to a small volume in hexane and applied to the column. A stepwise elution schedule followed was:

ml.	*Solvent*
60	*n*-Hexane
75	2% Methanol in diethyl ether
90	12% Methanol in diethyl ether
90	55% Methanol in diethyl ether
200	Methanol

Flow rate was about 180 ml. per hr., and 15-ml. fractions were collected, evaporated to dryness, and weighed in tared beakers.

Gas-Liquid Chromatography (GLC) of Fatty Acids

Acylated steryl glucosides and soybean oil were saponified, and the resulting fatty acids were esterified with methanolic hydrochloric acid as described by James (7). The methyl esters were analyzed with a Packard Model 7409 chromatograph equipped with a Model 804 oven. Samples were injected directly onto glass columns (6 ft. long and 1.5 mm. i.d.) packed with 20% diethylene glycol succinate coated on 80–100-mesh Chromosorb W. The oven was operated isothermally at 155°C. with the inlet at 210°C. and the detector (hydrogen flame ionization) at 200°C. Helium carrier gas flow was 36 to 43 ml. per hr. Values are expressed as percentages of total area of GLC peaks.

GLC Analysis of Sterols

From 6 to 10 mg. acylated steryl glucosides was added to 3 ml. amyl alcohol, 5 ml. 15% hyrochloric acid, and 25 ml. of methanol, and then refluxed for 6 hr. (8). The reaction mixture was taken to near dryness on a rotary evaporator (40° to 50°C.) and extracted with diethyl ether. The free sterols in the diethyl ether extract were analyzed directly on a 6 ft. × 1.5-mm. i.d. glass column packed with 3% OV-101 on 100–110-mesh Anakrom SD in the Packard Model 7409. Oven temperature was 240°C. with inlet and detector (hydrogen flame ionization) temperatures of 260°C. Helium carrier gas flow was 31 ml. per hr. Values are expressed as percentages of total area of sterol peaks as identified by comparison of retention times with known sterols.

Glucose Analysis

The glucose content of acylated steryl glucosides was determined by the phenol-sulfuric acid procedure of Dubois et al. (9). Glucose served as the standard. The sugar constituent of acylated steryl glucosides was identified and quantitated by the alditol acetate GLC procedure of Sloneker (10), as modified for soybean saponins (11). Samples of 8 to 10 mg. of acylated steryl glucosides were analyzed. A known weight of glucose was similarly treated to determine the molar response factor. The alditol acetates were analyzed in the Packard Model 7409 gas chromatograph by injection onto a 6 ft. × 1.5-mm. glass column packed with 3% ECNSS-M coated on 100–110-mesh Gas-Chrom Q. The column was operated isothermally at 195°C. with inlet and detector (hydrogen flame ionization) temperatures at 210°C. Helium carrier gas flow was 25 ml. per hr.

Stability to Lipoxygenase Action

Stability of acylated steryl glucosides to oxidation by lipoxygenase (E.C. 1.13.1.13) was determined by measuring oxygen uptake with a Clark oxygen electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) in an Oxygraph, Model K-1C (Gilson Medical Electronics, Middleton, Wis.). The reaction volume was 2.2 ml. consisting of 1.7 ml. 0.05M potassium borate buffer (pH 9.0), 0.3 ml. substrate, and 0.2 ml. lipoxygenase. The substrate was prepared by emulsifying 10 mg. of purified acylated steryl glucoside with 5 μl. of Tween 20, 0.1 ml. water, and 0.05 ml. of 0.05M borate buffer and then diluting to 1 ml. with water. The test solution of soybean lipoxygenase (Theorell enzyme from Nutritional Biochemical Corp., Cleveland, Ohio) had an activity of 0.11 μmole O_2 per min. per ml. (against potassium linoleate as substrate).

RESULTS

Yields of Alcohol-Extractables

A total of 14 preparations of alcohol-extractables were made in the course of our studies with yields ranging from 2.09 to 4.10% and a mean of 2.65% based on weight of protein (moisture-free basis). In previous work from a different batch of soybean globulins, yield of alcohol-extractables was 3.7% (4).

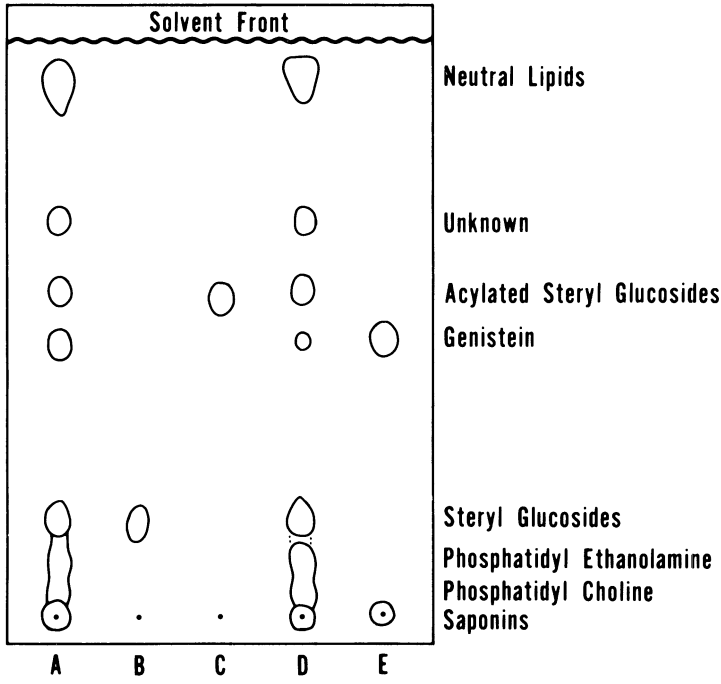

Fig. 1. Silica gel thin-layer chromatography (TLC) of alcohol-extractables (A); steryl glucosides (B); 6-*O*-palmitoyl-β-D-glucosyl-β-sitosterol (C); alcohol-extractables soluble in hexane (D); and alcohol-extractables insoluble in hexane (E).

Preliminary TLC Studies

Our objective was to isolate and identify the compound(s) previously designated as fraction G when alcohol-extractables were analyzed by silica gel G TLC with chloroform:methanol:water (65:25:4) as the developing solvent (4). Preliminary analyses with this developing solvent and TLC plates commercially prepared showed that fraction G migrated nearly to the solvent front and was poorly separated from fraction H, believed to contain glycerides. Results were more satisfactory with chloroform:methanol:acetic acid:water (90:8:1:1) as used by Kiribuchi et al. (5); under these conditions fraction G migrated with an R_f of 0.6 to 0.7 and was clearly separated from the neutral lipids. Fraction G gave a purple color on the TLC plate when sprayed with 50% sulfuric acid and heated. This color reaction resembled that of steryl glucosides, although the R_f of the steryl glucosides was lower than for fraction G. These observations suggested that fraction G consisted of acylated steryl glucosides, which were isolated from soybean phosphatides by Kiribuchi et al. (5) and Lepage (12). This tentative conclusion was strengthened by TLC of the alcohol-extractables and reference compounds (Fig. 1). Fraction G moved with an R_f slightly higher than synthetic 6-*O*-palmitoyl-β-D-glucosyl-β-sitosterol but gave the same color reaction with 50% sulfuric acid. Genistein occurred immediately below this region and was readily identified by its yellow color on spraying the TLC plate with 50% sulfuric acid (4). Steryl glucosides were found at an R_f of about 0.2; whereas saponins remained at the origin with this solvent (11). The spots between steryl glucosides and the origin consisted of phosphatidyl choline and phosphatidyl ethanolamine, which were identified by comparing their R_f values with those of authentic samples. An unknown occurred at R_f 0.7, and the neutral lipids were at R_f 0.9.

Fractionation of Alcohol-Extractables

To characterize conclusively the compounds tentatively identified as acylated steryl glucosides, they were isolated from alcohol-extractables by extraction with hexane followed by chromatography on a Florisil column.

Hexane Extraction. When the dry alcohol-extractables were leached with hexane, 20 to 30% of the starting solids remained as a white residue. TLC analysis (Fig. 1) showed that the fraction insoluble in hexane consisted of saponins and genistein, plus traces (not shown in figure) of almost all the other components in the alcohol-extractables except neutral lipids. The fraction soluble in hexane closely resembled the starting material but contained reduced amounts of saponins and genistein.

Florisil Chromatography. The alcohol-extractables that dissolved in hexane were chromatographed on a Florisil column and separated into seven fractions (Fig. 2). In a typical experiment, yields of the various fractions from 807 mg. of material soluble in hexane were as follows:

Fraction	*mg.*
A	10
B	194
C	23
D	30
E	20
F	104
G	97

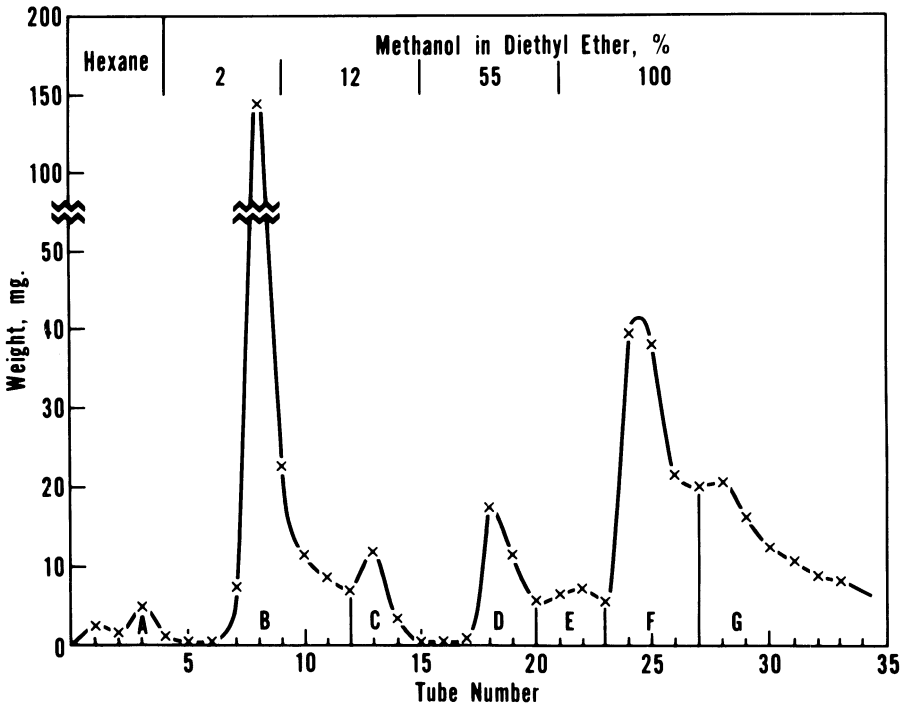

Fig. 2. Florisil chromatography of alcohol-extractables soluble in hexane (807 mg.) from 38.7 g. protein (dry basis). A stepwise elution schedule appears at top of figure.

Fractions recovered accounted for 59% of the sample applied to the column.[2] TLC analysis (Fig. 3) showed fractions A and B to be neutral lipids with traces of genistein and acylated steryl glucosides in B. Fraction C, which eluted with 12% methanol in diethyl ether, contained the acylated steryl glucosides. Steryl glucosides eluted in fractions D and E. Fraction F contained the unknown compound of R_f 0.7 noted earlier (Fig. 1) while fraction G consisted of material remaining at the origin (probably saponins) and a major spot at R_f 0.1.

Yields of acylated steryl glucosides in nine chromatographic separations ranged from 0.04 to 0.08% with a mean of 0.06% based on the weight of the starting protein; yields of steryl glucosides varied from 0.05 to 0.13% with an average of 0.09%. By comparison, Kiribuchi et al. (5) obtained 0.033% acylated steryl glucosides and 0.037% steryl glucosides from ground soybeans.

Characterization of Purified Acylated Steryl Glucosides

Fraction C from Florisil chromatography had an R_f on TLC corresponding to synthetic 6-*O*-palmitoyl-β-D-glucosyl-β-sitosterol as noted earlier for the alcohol-extractables (Fig. 1). It also gave a positive Liebermann-Burchard test for

[2]Presumably solvents more polar than those used are needed to elute the remainder of the alcohol-extractables.

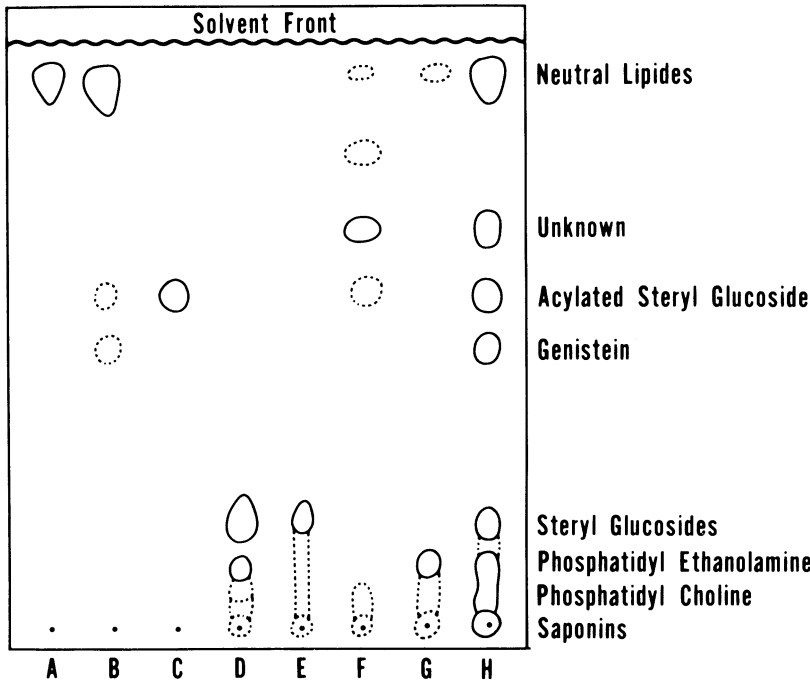

Fig. 3. Silica gel TLC of fractions (A–G) isolated by Florisil column chromatography of alcohol-extractables (H).

sterols. When treated with phenol-sulfuric acid (9), fraction C formed an orange color with an absorption maximum at 488 nm., which is characteristic of hexoses (9,13). The infrared spectrum of fraction C (in carbon tetrachloride) resembled spectra for acylated steryl glucosides published by Kiribuchi et al. (5) and Lepage (12).

Conversion to Steryl Glucosides and Free Sterols. A 2-mg. sample of fraction C was refluxed for 2 hr. in 0.4 ml. 10% alcoholic potassium hydroxide, neutralized, and concentrated to dryness. When the residue was extracted with amyl alcohol and the extract analyzed by TLC, no acylated steryl glucosides remained. Instead, spots corresponding to steryl glucosides and free sterols were observed. Hydrolysis for 4 hr. converted steryl glucosides to free sterols extensively.

Fatty Acid Analysis. A 15-mg. portion of fraction C was saponified and the fatty acids were converted to methyl esters with methanolic hydrochloric acid (7). Analysis of the methyl esters by GLC yielded the results contained in Table I. Values for acylated steryl glucosides from soybean phosphatides reported by Kiribuchi et al. (5) and Lepage (12) are included in Table I. For comparative purposes a sample of commercially refined soybean oil was also analyzed. Our fatty acid analysis closely agrees with the Japanese workers' values, but differs from Lepage's results.

Sterol Analysis. Acid hydrolysis of fraction C by the method of Morris and Lee (8) yielded free sterols, which were analyzed by GLC. Acid hydrolysis by other

methods (5,12) appeared to extensively degrade the sterols. Three GLC peaks were observed that had retention times corresponding to campesterol, stigmasterol, and β-sitosterol. Compositions of the sterols are compared with those reported by earlier workers, as follows:

Sterol	*NRRL results* %	*Kiribuchi et al. (5)* %	*Lepage (12)* %
Campesterol	25.2	20	22.4
Stigmasterol	19.9	29	18.9
β-Sitosterol	54.9	51	58.7

Our sterol analyses agree most closely with those reported by Lepage and provide additional evidence that fraction C does, indeed, consist of acylated steryl glucosides.

Identification of Glucose. Although the phenol-sulfuric acid test, described earlier, suggested the presence of glucose in our acylated steryl glucosides preparation, GLC analysis provided more definitive evidence. The chromatogram of the alditol acetates of the sugar fraction revealed only two peaks: 2-deoxyglucitol (internal standard) and glucitol (Fig. 4). The glucose content of our acylated steryl glucosides preparation was 5.3% by GLC analysis, whereas the phenol-sulfuric acid method gave us 8.6% glucose. Both of these values are considerably below 22.2%, the theoretical value for 6-*O*-palmitoyl-β-D-glucosyl-β-sitosterol. Our low glucose values may have resulted from incomplete acid hydrolysis since acylated steryl glucosides are poorly soluble in such aqueous media as those used in both analyses.

Stability of Acylated Steryl Glucosides to Lipoxygenase

Lipoxygenase has been implicated as a cause of undesirable flavors in soybean products because it oxidizes linoleic and linolenic acids to hydroperoxides which, in turn, decompose to form a large number of compounds (14,15). Since acylated steryl glucosides contain linoleic and linolenic acids (Table I), we tested fraction C as a substrate for lipoxygenase (Theorell preparation). In a preliminary test with an impure preparation of acylated steryl glucosides (TLC analysis showed material migrating in the region of the neutral lipids), there was an initial uptake of oxygen that slowly decreased until about one-half of the oxygen was consumed. When a pure preparation of acylated steryl glucoside was tested, however, there was no uptake of oxygen. Apparently the linoleic and linolenic acid moieties in acylated steryl glucoside are not oxidized by the lipoxygenase isozyme we tested. Small amounts of free linoleic or linolenic acid could account for the oxygen uptake noted in the impure acylated steryl glucoside preparation.

TABLE I. FATTY ACID COMPOSITION OF ACYLATED STERYL GLUCOSIDES AND SOYBEAN OIL

Fatty Acid	Acylated Steryl Glucosides			Soybean Oil
	NRRL Results %	Kiribuchi et al. (5) %	Lepage (12) %	%
Palmitic	42.3	42.7	33.7	10.2
Stearic	11.3	9.6	7.0	4.5
Oleic	18.1	13.7	8.8	21.8
Linoleic	26.3	31.2	47.4	54.0
Linolenic	2.0	2.8	2.2	8.8

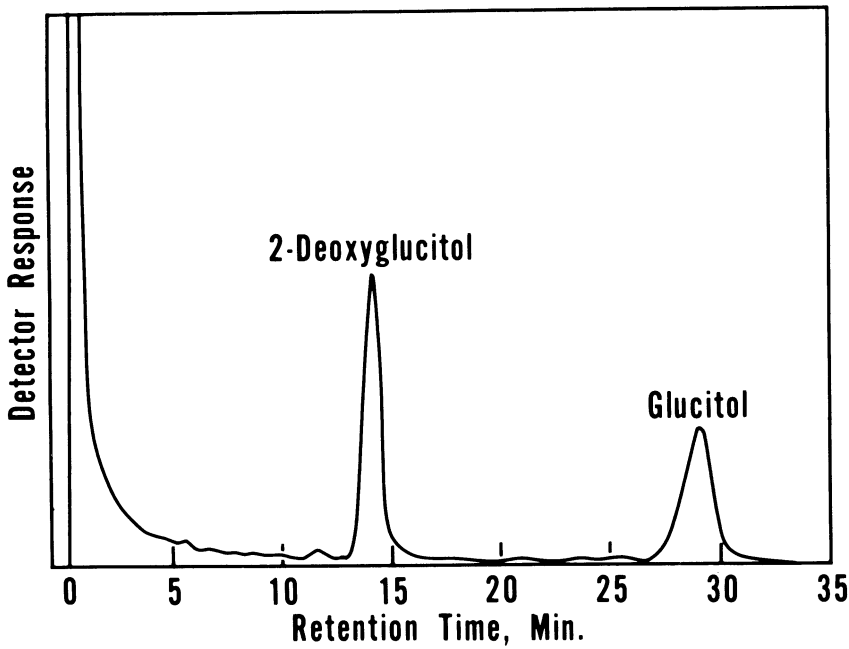

Fig. 4. Gas-liquid chromatogram of alditol acetates from acid hydrolysate of acylated steryl glucosides. 2-Deoxyglucitol was derived from 2-deoxy-D-glucose, which served as the internal standard.

DISCUSSION

While our work was in progress, Maga and Johnson (16) reported that a commercial soybean protein isolate contained traces of acylated steryl glucoside, but they presented no characterization data to support their claim. As a result of earlier work by Nash et al. (4) and our latest studies, there is now little doubt that these sterol derivatives occur in isolated soybean proteins.

Kiribuchi et al. (5) and Lepage (12) separated acylated steryl glucosides from soybean phosphatides, which are obtained from crude soybean oil; both Honig et al. (17) and Maga and Johnson (16) reported that acylated steryl glucosides also occur in defatted soybean meal. Our results confirm existence of acylated steryl glucosides in defatted meal; apparently hexane extraction of soybean flakes, while separating the oil, removes only part of the acylated steryl glucosides present.

The observed differences in sterol and fatty acid composition between our acylated steryl glucosides and those of other workers are of questionable significance since starting materials and separation methods were different. It is clear, however, that the fatty acids in sterol derivatives are more saturated than in soybean oil. The palmitic acid content of acylated steryl glucosides is about four times that of soybean oil (Table I).

Since defatted soybean flakes used to prepare protein isolates are likely to contain lipoxygenase activity, the linoleic and linolenic acid moieties of acylated steryl glucoside are potential sites of attack by the enzyme. We found, however, that acylated steryl glucosides are stable to the Theorell preparation of

lipoxygenase but cannot exclude the possibility that other isozymes of lipoxygenase (18) may attack the polyunsaturated acid residues. Their apparent stability to lipoxygenase, and their relatively low levels in isolated protein, suggest that acylated steryl glucosides are not an important source of flavor compounds resulting from lipoxygenase action. Nonetheless, the unsaturated fatty acids in these sterol derivatives must be considered as potential sources of flavor compounds via the slower and less specific mechanism of autoxidation. Further studies are needed to determine the effects of acylated steryl glucosides on the physical and functional properties of soybean proteins.

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