Crystalline Saponins from Soybean Protein[1]

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ABSTRACT

Isolated soybean protein contains saponins which can be removed by a mild acid treatment and crystallized. The crystalline material is a mixture of at least three saponins. Hydrolysis of the saponins reveals soyasapogenols B, C, and D; soyasapogenol B is present in the highest concentration. According to paper and gas-liquid chromatography the crystalline saponins contain at least five different sugars: glucose, galactose, arabinose, rhamnose, and glucuronic acid. It is yet to be established whether or not the saponins are covalently bonded to the protein.

Muramatsu (1) in 1923 isolated from soybeans a compound believed to be a hydroxy acid that he called hispidic acid. Later, he isolated another material (2) which he assumed also to be a hydroxy acid. Sumiki (3) and Walz (4) later showed that the hydroxy acids were actually mixtures of saponins.

Ochiai et al. (5) separated crude saponins as calcium salts from ethanolic extracts of defatted soybeans. Acid hydrolysis of the saponins and purification of the resulting products by chromatographic adsorption yielded four sapogenins which were designated soyasapogenols A, B, C, and D. Later, selenium dehydrogenation studies indicated that the four sapogenins were triterpenoid alcohols (6).

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The triterpenoid nature of the four sapogenins was confirmed by Tsuda and Kitagawa (7) and by Tsuda and Ichikawa (8). Subsequently, Ruzicka and co-workers presented a series of structural formulas for soyasapogenols A, B, C, and D (9). In 1958, Smith et al. (10) re-examined the chemistry of soy sapogenins and proposed slightly different structures for soyasapogenols A, B, C, and D.

Studies by Bondi and co-workers have provided much new information about soybean saponins (11,12,13,14,15,16). Defatted soybean meal contains 0.6% saponins (16) which are glycosides of five triterpenoid alcohols. The fifth sapogenin, soyasapogenol E, was only discovered in 1964 (12). The sugars reported in soybean saponins are galactose, glucose, rhamnose, xylose, arabinose, and glucuronic acid (15).

Previously, all isolation procedures used aqueous alcohols to remove saponins from defatted meal. We have observed that when isolated soybean protein was heated in acid solution, saponins crystallized from the solution. This report describes the crystalline saponins we obtained from isolated soybean protein.

MATERIALS AND METHODS

Acid-precipitated soybean protein was prepared in the following manner: Dehulled, defatted soybean meal was extracted twice with water, first with a solvent-to-meal ratio of 10:1 and a second time at 5:1. After centrifugation to remove insolubles, the two extracts were combined and adjusted to pH 4.5 with hydrochloric acid; the precipitated curd was centrifuged, collected, and freeze-dried. The dry material was ground in a hammer mill containing a 100-mesh screen. The protein was then dissolved in potassium phosphate buffer, pH 7.6, containing 10% sodium chloride, and dialyzed at 4°C. for one week against several changes of buffer. The second week the protein was dialyzed in the cold against water, and then reprecipitated at pH 4.5 with acetic acid, thoroughly washed with water, and again freeze-dried.

Washed protein was suspended in 10 to 15 volumes of 0.1N HCl (pH~2) and heated with stirring at 95°-98°C. for 1/2 hr., whereupon crystals formed. After cooling, the suspension crystals were isolated by centrifuging at 12,000 × g at 1°-4°C. The recovered crystals were washed several times by centrifuging with 0.1N HCl to remove extraneous protein. The crude crystals were then dissolved in hot 40% ethyl alcohol, which was treated with activated carbon and Celite, filtered hot, and cooled slowly to yield crystals. Acetate derivatives of the saponins were prepared with acetic anhydride-pyridine (10).

The isolated saponins were hydrolyzed with 1.7N methanolic-HCl for 4 hr. at 100°C. in sealed tubes. After hydrolysis the contents were dried and then dispersed in water. Sapogenins were removed by extraction with benzene and the water solubles were analyzed for carbohydrate. The sapogenins were easily crystallized from benzene or methanolic hydrochloric acid to form long needles, which were soluble in chloroform or pyridine.

The carbohydrate portion of our crystalline soybean saponins was identified by two procedures. First, the water-soluble methyl glycosides of the sugars obtained by methanolysis of the saponins were hydrolyzed with hydrochloric acid to give the free sugars. The hydrolysate was neutralized with silver carbonate, filtered, and desalted to prevent streaking of the chromatograms. The carbohydrates were

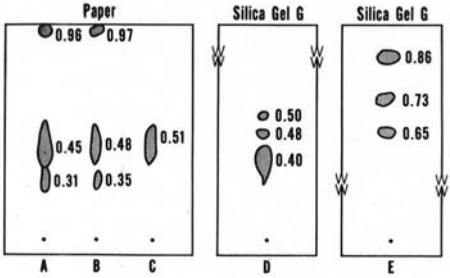

Fig. 1. Diagrams of paper and thin-layer chromatograms. Sample A, soybean saponins prepared by Birk et al. (11); B, soybean saponins prepared in our laboratory by the Birk procedure; C and D, crystalline saponins; and E, acetate derivatives of crystalline saponins. Solvent system was butanol:ethanol:water (6:2:3).

analyzed on Whatman No. 1 in butanol:pyridine:water (6:4:3) using two descensions. The carbohydrates were detected by spraying with ammoniacal silver nitrate (17) or with *o*-aminobiphenyl (18).

Secondly, the methyl glycosides of the sugars were analyzed by gas-liquid chromatography with the procedure of Sloneker (19). The methyl sugars were dispersed in 0.4 ml. pyridine and then 0.2 ml. bis(trimethylsilyl) acetamide and 0.1 ml. trimethylchlorosilane was added. After reacting ~ 1 hr. the resulting trimethylsilyl derivatives were separated on a 3% SE-30, 80- to 100-mesh acid-washed Chromosorb W column operated isothermally at 170°C. An F & M 810 instrument, manufactured by Hewlett-Packard and equipped with a flame ionization detector, was used.

RESULTS

The crystalline material obtained from acid solutions of isolated soybean proteins melted at ~220°C. and analyzed as follows: carbohydrate by the phenol-sulfuric acid procedure (20), 25-30%; ash, <0.1%; N, <0.05%; C, 56.81%; H, 8.82%.

Birk et al. (11) chromatographed soybean saponins from defatted soybean meal on Whatman 3MM filter paper with butanol:ethanol:water (6:2:3) and found four major spots that stained with antimony trichloride. Figure 1 shows the results when our crystalline saponins were chromatographed by Birk's procedure. In Fig. 1, A is a sample of saponins prepared in Professor Y. Birk's laboratory, B is a sample of soybean saponins isolated by Birk's procedure in our laboratory, and C is our crystalline saponin preparation from isolated soybean protein. Samples A and B were very similar and gave three to four antimony trichloride-positive areas which indicated the presence of saponins. Sample C gave only one elongated spot at R_f 0.51.

When the crystalline saponins were chromatographed on Silica Gel G with butanol:ethanol:water (6:2:3) (Fig. 1, sample D), three spots appeared after the plate was sprayed with antimony trichloride and heated. Acetate derivatives of the saponins were chromatographed on thin-layer plates using the same solvent system. Three spots were again observed (Fig. 1, sample E), all having R_f's higher than the original material.

After hydrolysis of saponins with methanolic hydrochloric acid, the sapogenins

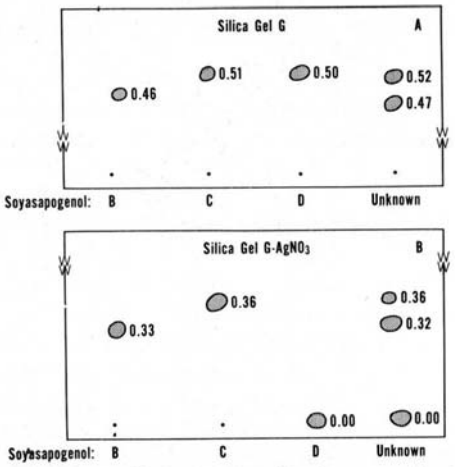

Fig. 2. Diagrams of thin-layer chromatograms for known and unknown soyasapogenols. A, Silica Gel G; B, Silica Gel G impregnated with silver nitrate. Solvent system was benzene:ethyl acetate (50:50).

were extracted with benzene and crystallized from a small volume of benzene. The sapogenins had a specific rotation of +81, C = 1 in pyridine, and were identified by Silica Gel G thin-layer chromatography. Benzene:ethyl acetate (50:50) as the developing solvent (21) gave the results shown in Fig. 2.

Soyasapogenols B, C, and D gave R_f's of 0.46, 0.51, and 0.50, respectively, on Silica Gel G when sprayed with antimony trichloride and warmed. The crystalline sapogenins gave R_f's of 0.47 and 0.52, suggesting the presence of soyasapogenol B plus either C or D, or both. We were able to distinguish between soyasapogenols C and D by using Silica Gel G impregnated with silver nitrate and benzene:ethyl acetate (50:50) as the developing solvent. Soyasapogenols B, C, and D had R_f's of 0.33, 0.36, and 0.00, respectively. The crystalline sapogenins gave R_f's of 0.00, 0.32, and 0.36 under the same conditions. These results suggested that the sapogenins we isolated were soyasapogenols B, C, and D. We were unable to obtain an authentic sample of soyasapogenol A. Examination of sapogenins from Birk's sample (Fig. 1, sample A) and from the alcohol-extractables of soybean protein (21) showed the presence of a spot with an R_f of 0.30 on Silica Gel G in benzene:ethyl acetate (50:50) in the absence of $AgNO_3$. This additional spot probably corresponded to soyasapogenol A.

Paper chromatography of the free sugars obtained by methanolysis of the saponins revealed glucose, galactose, arabinose, rhamnose, and glucuronic acid. When the methyl glycosides of the sugars were analyzed by gas-liquid chromatography the same sugars were detected.

DISCUSSION

Soybean meal has been reported to contain five soybean sapogenins. After crystallization of the saponins that are liberated from the acid-precipitated protein with 0.1N HCl, we observed only three saponins, three saponin acetates, or three

sapogenins. This finding suggests that fractionation of the saponins occurs when they are isolated by our procedure.

Comparison of soyasapogenols B, C, and D with our sapogenins leads us to conclude that we have the same three compounds in our saponin mixture. However, we did not have any authentic soyasapogenol A or E to compare with our samples. The possible presence of these soyasapogenols in our sample is therefore not ruled out, although soyasapogenol E reportedly gives a green color with antimony trichloride (12). We did not observe any green spots when we chromatographed our sapogenin fraction.

Gestetner et al.(15) found glucose, galactose, arabinose, rhamnose, xylose, and glucuronic acid in the saponins isolated from soybean meal. In the crystalline saponins isolated from acid-precipitated protein, we found the same sugars by paper and gas-liquid chromatography except for xylose. This absence also suggests fractionation of the saponins when the protein is isolated from soybean meal. Alternatively, the absence of xylose in our crystalline saponins may indicate lability of xylose residues in the oligosaccharide portion of the saponin molecule to the acid treatment used in the fractionation procedure.

Crystalline saponins were isolated from commercially prepared soy proteins, as well as from other purified laboratory samples. Heating the proteins in acid appears necessary for the formation of crystals. Although the requirement for acid treatment suggests that a mild hydrolysis of the protein may be necessary to release the saponins, no direct evidence was found for covalent linkage between the saponins and the protein.

Previous work (21) showed that a portion of the saponins in soybean proteins is extractable with aqueous ethanol. Obviously the alcohol treatment does not remove all the saponins from the protein, since subsequent treatment of alcohol-washed protein with acid liberates crystalline material. The function of the saponins in the protein is not understood. Since soybean saponins are surface-active (11), a detergent-protein-type interaction may occur during isolation of the proteins.

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