Thin Layer and Anion Exchange Chromatography of Soybean Saponins

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Abstract

Twenty-two solvent systems were evaluated for TLC of soybean saponins on silica gel. A maximum of four fractions separated by single development with the different solvents. Six successive developments with chloroform-methanol-water (65:25:4) separated soybean saponins into 10 or more fractions. Column chromatography of soybean saponins on an anion exchange resin with a linear gradient of acetic acid yielded seven fractions. Multiple development TLC of the saponins separated by column chromatography showed that a definite fractionation occurred. Several column chromatographic fractions contained components with identical TLC R_f values, but the components were clearly different on the basis of colors detected with sulfuric acid and on the basis of their elution positions from the ion exchange resin. Soybean saponins are more complex mixtures than previously recognized but can be fractionated by chromatography on thin layer plates and anion exchange resins.

Introduction

Recent work in Israel indicates that soybean saponins are a complex mixture (1–4). Hydrolysis of the saponins yields five sapogenins and six sugars, including glucuronic acid (3). Chromatography of soybean saponins on alumina columns and on paper gave four fractions (2). One of these contained only soyasapogenol A plus glucose, but the other three contained all five of the soyasapogenols and five to six different sugars (3). Assuming that the sugars are present as oligosaccharides and that each alumina column chromatographic fraction contains only one oligosaccharide moiety, the minimum number of different saponins in soybeans must be 16. If variations occur in the composition or structure of the oligosaccharide moiety, an even greater number of saponins is possible.

The presence of glucuronic acid residues in soybean saponins suggested that anion exchange chromatography might be useful in fractionating the saponin mixture. Although our preliminary experiments confirmed the possibility of separating soybean saponins on anion exchange resin columns, these pointed out the need for assessing the extent of separation achieved. Previous work showed that soybean saponins can be chromatographed on silica gel thin layers but with limited separations (5). We therefore evaluated 22 solvent systems and multiple development to improve separations of saponins by TLC.

Experimental Procedures

Preparation of Soybean Saponins

Soybean saponins were prepared by the procedure of Birk et al. (1) with the following minor modifications: The 80% ethanol extracts of defatted meal were taken to almost a syrup and then diluted with water to facilitate the subsequent ether extraction step. After extraction into butanol, the saponins were taken to dryness in a rotary evaporator, redispersed in water, and freeze-dried to obtain the fraction which we refer to as crude saponins. In subsequent purification steps, centrifugation was substituted for filtration to reduce the time required. We refer to the final saponin preparations as purified saponins.

Crystalline saponins were prepared from isolated soybean proteins as described elsewhere (6).

TLC

Merck precoated analytical silica gel plates (Brinkmann Instruments Inc.) were used. About 150–200 μg of sample in 80% ethanol were applied per spot. After development of the plates with solvent, the spots were detected by spraying the plates with 50% sulfuric acid and then heating to 90–95 C. For multiple development, the plates were allowed to air-dry between developments.

Ion Exchange Chromatography

In 2 ml of water were dissolved 20 mg of purified saponins and 10 mg of ethylenediaminetetraacetic acid (EDTA) disodium salt by adjusting to pH 7–8 with 0.1 N NaOH. We found that in the absence of EDTA the saponins were not completely soluble in water, presumably because of residual calcium ion in the preparations. The saponin solution was applied to a 0.6 × 63 cm column of −400 mesh AG1-X4 (Bio-Rad Laboratories) in acetate form. The column was eluted at 25 C with a linear gradient of acetic acid from 2.5 to 5 N. The gradient was generated with a Beckman Model 131 gradient pump (Beckman Instruments, Inc.) set to deliver a ½ liter gradient. The pump was prepared by pumping only limiting solution for several minutes to ensure that the tubing from the reservoir to the rotary valve was filled with 5 N acetic acid. The program cam was then placed in the prestart position and water was pumped to flush the mixing cylinder of acetic acid. Before engaging the gears of the pump at the start of elution, the program cam was advanced to position 5 (halfway point on the cam) so that the gradient increased abruptly from 0 to 2.5 N acetic acid and then increased linearly to 5 N acetic acid during the run. The pumping rate was 7.5 ml/hr and 2 ml fractions were collected with timed flow in a Technicon fraction collector (Technicon Chromatography Corporation). Drop counting was unsatisfactory because of a gradual decrease in drop size during the run. The column was prepared for reuse by washing with water until the effluent was free of acetic acid.

The acetic acid gradient was determined by titrating effluent fractions with 0.1 N sodium hydroxide. Saponins in the effluent were estimated by determining sugar content as glucose with phenol-sulfuric acid (7). Control analyses showed that EDTA did not give a color and did not interfere with color formation from

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TABLE I

R_f Values of Soybean Saponins on Silica Gel With Different Solvents

Solvent	Solvent composition[a]	Literature reference to solvent	R_f values	Comments
1	Butanol-benzene-formic acid-water (100:19:10:25)—upper phase	8	0.01[b], 0.06, 0.09, 0.17	Compact spots
2	Butanol-acetic acid-water (6:1:2)	.....	0.24, 0.26[b], 0.31, 0.35	.....
3	Butanol-acetic acid-water (4:1:1)	9	0.20, 0.35	Streaking
4	Butanol-acetic acid-water (2:1:1)	10	0.51[b], 0.71	Streaking
5	Butanol-acetic acid-diethyl ether-water (9:6:3:1)	10	0.12[b], 0.27, 0.32	Streaking
6	Butanol-ethanol-water (7:2:5)	9	0.26[b], 0.31	.....
7	Butanol-ethanol-water (6:2:3)	1,6	0.37, 0.43	.....
8	Butanol-ethanol-15 N ammonium hydroxide (7:2:5)	9	0.24[b], 0.29, 0.34, 0.55	Compact spots
9	Butanol-ethanol-1 N ammonium hydroxide (60:13:30.5)	11	0.20, 0.22, 0.24	Compact spots
10	Ethyl acetate-acetic acid-water (70:16:15)—upper phase	12	0.02[b], 0.07, 0.22, 0.25	
11	Ethyl acetate-acetic acid-water (3:1:3)—upper phase	13	0.02, 0.06, 0.15, 0.17	Streaking
12	Ethyl acetate-pyridine-water (5:1:4)—upper phase	14	0.00, 0.03	
13	Ethyl acetate-pyridine-water (3:1:3)—upper phase	13	0.00, 0.07	Streaking
14	Chloroform-methanol (3:1)	15	0.00	
15	Chloroform-methanol-water (65:25:4)	5	0.05[b], 0.10, 0.14, 0.18	Streaking
16	Chloroform-methanol-water (65:35:10)	16	0.26[b], 0.30, 0.34, 0.37	Streaking
17	Chloroform-methanol-acetic acid-water (90:8:1:1)	17	0.00	
18	Chloroform-methanol-acetic acid-water (65:25:8:4)	.....	0.05, 0.10, 0.15, 0.22	Streaking
19	Chloroform-tetrahydrofuran-pyridine (10:10:2)—saturated with formamide	18	0.06	
20	Benzene-ethanol-water-ammonium hydroxide (200:47:15:1)—upper phase	10	0.00	
21	Benzene-ethanol-water-acetic acid (200:47:15:1)—upper phase	10	0.00	
22	Methylene dichloride-methanol-formamide (80:19:1)	19	0.00, 0.01	

[a] All solvent ratios are on a volume basis.
[b] R_f decreased with increase in concentration of saponin.

glucose. The elution position of EDTA from the ion exchange column was not determined. Saponins were recovered by pooling tubes for each fraction and then freeze drying.

Results and Discussion

Twenty-two different solvent mixtures were evaluated for TLC of soybean saponins on silica gel (Table I). Several of these solvents have been used for chromatography of other saponins and related compounds. Little, if any, migration of the saponins was observed with eight of the solvents, apparently because the solvents were not polar enough. These solvent systems may, however, be useful for analysis of soybean extracts that contain saponins plus less polar compounds, such as isoflavones, sterols, phospholipids and triglycerides (5). The effect of polarity on R_f is shown by the chloroform-methanol solvent systems. No migration occurred with solvent 14, but decreasing the amount of chloroform and adding water (solvents 15 and 16) caused the saponins to move. Although R_f values increased as solvent polarity was enhanced, separations were not improved as noted in comparing solvent 2 with 4 and solvent 15 with 16.

Five of the best solvent systems were 1, 2, 8, 10 and 15. Separation of saponins with these solvents is diagrammed in Figure 1. Results of use of solvent 8 for analysis of various preparations of crude and purified saponins, plus a crystalline sample, are shown in Figure 2. After spraying with 50% H_2SO_4 and heating, the plate showed that the crude saponins separated into four to five spots as compared to four spots for the purified saponins and three spots in the crystalline saponin sample. The two minor fast-migrating spots for the crude saponins were brown, while the other spots were magenta. The purified and crystalline saponins gave only magenta spots. The brown spots occurring with the crude saponins may be impurities. It is also apparent that the R_f values for the purified saponins do not agree with R_f values for the crude or the crystalline saponins. This discrepancy between migration rates of the crude and purified saponins suggests that modification of the saponins may have occurred during the final steps of purification. The cause or nature of the modification is unknown. Modification of the crystalline saponins is not unexpected since they are prepared by heating soybean proteins in 0.1 N HCl (6).

The maximum number of fractions obtained from purified saponins by TLC (Fig. 1, 2) was four, the same number as separated by paper chromatography (1). Since multiple development will often improve

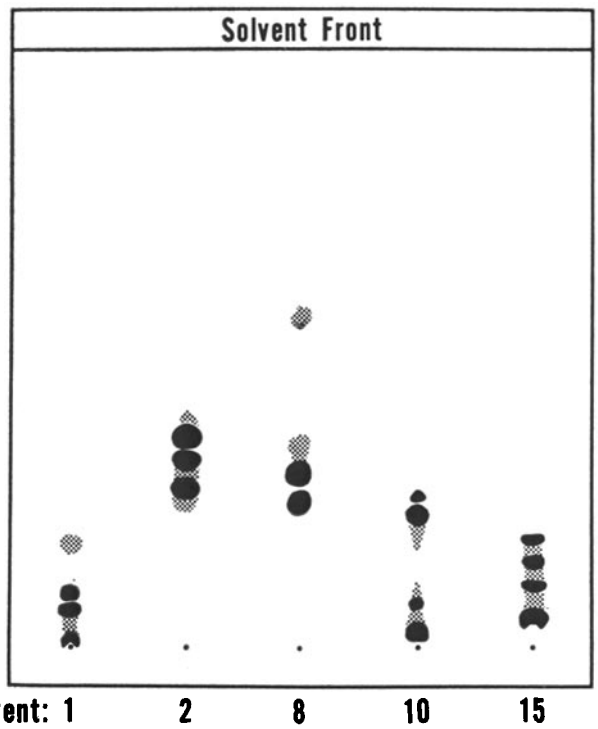

FIG. 1. Schematic diagram of TLC of soybean saponins with Solvent Systems 1, 2, 8, 10 and 15 (Table I). Spots were visualized with 50% H_2SO_4 and heat.

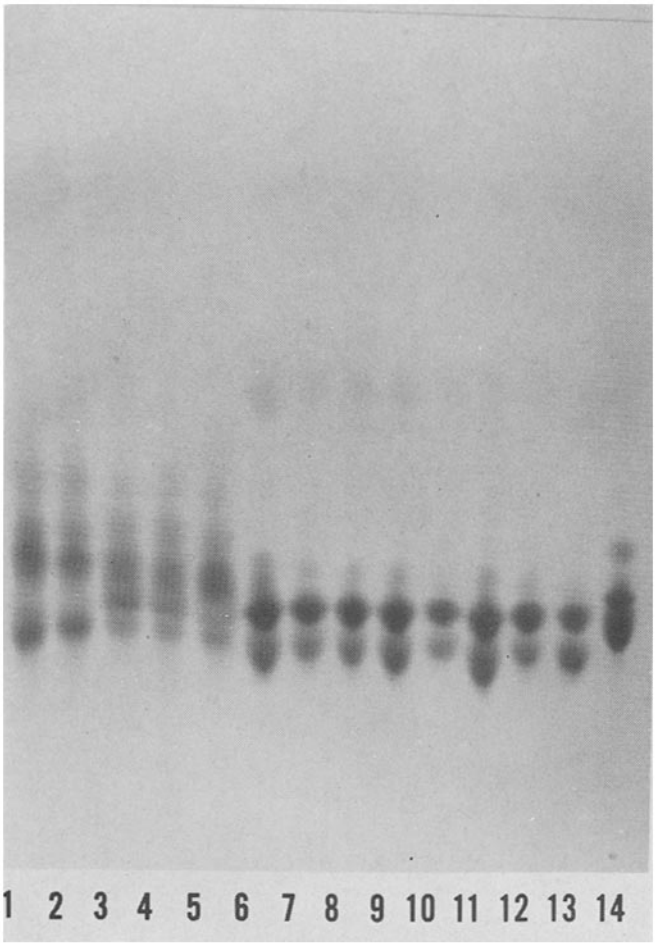

FIG. 2. TLC of crude (1–5), purified (6–13) and crystalline (14) soybean saponins. Plate was developed with butanol-ethanol-15 N NH_4OH (Solvent 8).

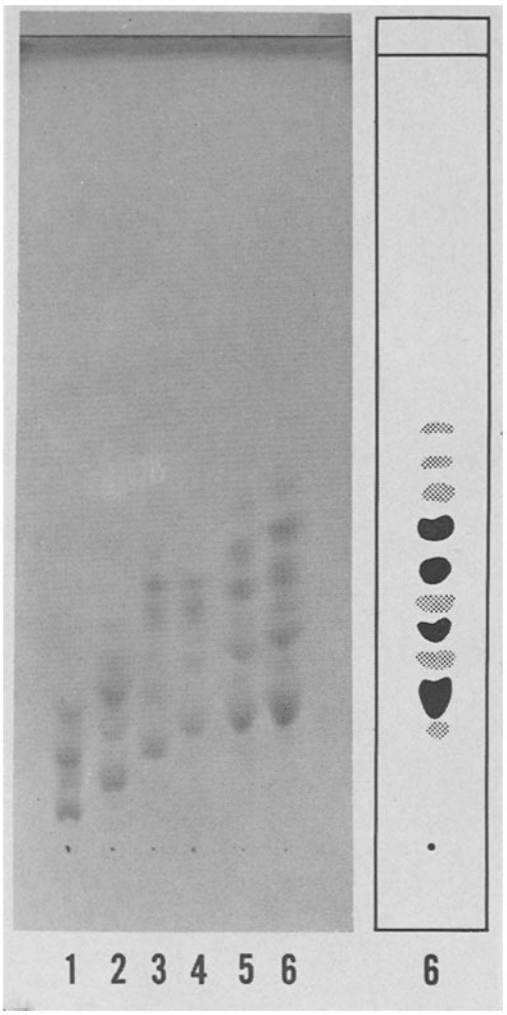

FIG. 3. TLC after multiple development of purified soybean saponins with chloroform-methanol-water (Solvent 15). Numerals indicate the number of developments given each sample. Resolution from six developments is diagrammed on right-hand side. A single sample was spotted before each development beginning with Sample 6 to show the effect of successive developments on one plate.

separations of closely migrating compounds (20–22), we tried this technique with solvents 1, 2, 8, 10 and 15. (An earlier experiment with solvent 15 by A. M. Nash of this Laboratory suggested the potential usefulness of this technique for TLC of soybean saponins.) Six developments with solvents 1 and 8 failed to increase the separation obtained with a single development. Multiple development with solvents 2 and 10 increased separations of the saponins as compared to single development, but the spots became more diffuse. Solvent 15 gave the most satisfactory results on multiple development of thin layer plates. After six successive developments with solvent 15, four major fractions and six to seven minor fractions were apparent (Fig. 3). Soybean saponins are obviously more complex than is indicated by single development TLC (Fig. 1, 2) or by paper chromatography (1).

The presence of glucuronic acid in soybean saponins (3) suggested that fractionation might be achieved by anion exchange chromatography. Figure 4 shows the elution diagram when soybean saponins were chromatographed on AG1-X4 (analytical grade Dowex 1-X4) resin with a linear gradient of acetic acid from 2.5 to 5 N. Preliminary experiments indicated that little, if any, carbohydrate-containing material was eluted at acetic acid concentrations below 2.5 N. Seven major fractions designated A-G were obtained. All the fractions gave a typical carbohydrate color with phenol-sulfuric acid, except fraction E. The phenol-H_2SO_4 reaction mixture of this fraction was pinkish-yellow and gave an absorption spectrum with peaks at 485 and 535 mμ. In the absence of phenol, the reaction mixture was still pink but only the 535 mμ peak was observed in the absorption spectrum.

Analysis of column fractions A-G by six developments on silica gel thin layers (solvent 15) is diagrammed in Figure 5. The fractions show an increase in R_f value with an increase in the normality of acetic acid needed to achieve elution from the anion exchange resin. Previous studies with silica gel thin layers and solvent 15 indicated that R_f values of soybean nonprotein components increased as polarity of the compounds decreased (5). The saponin fractions may, therefore, be eluting from the resin in order of decreasing polarity. Fraction A, which is the first fraction eluted from the column, has the lowest R_f. Similarity in R_f of Fraction B with the slowest spot of Fraction C indicates incomplete separation of Fractions B and C. Surprisingly, the spot of R_f 0.17 in Fraction C appears to be a major component; thus it may not be a contaminant from Fraction B. The peaks for Fractions D and E are incompletely separated from each other (Fig. 4) but do not appear cross-contaminated when analyzed by TLC. The two fastest moving spots (R_f 0.41 and 0.44) in Fraction E (Fig. 5) were purple (after

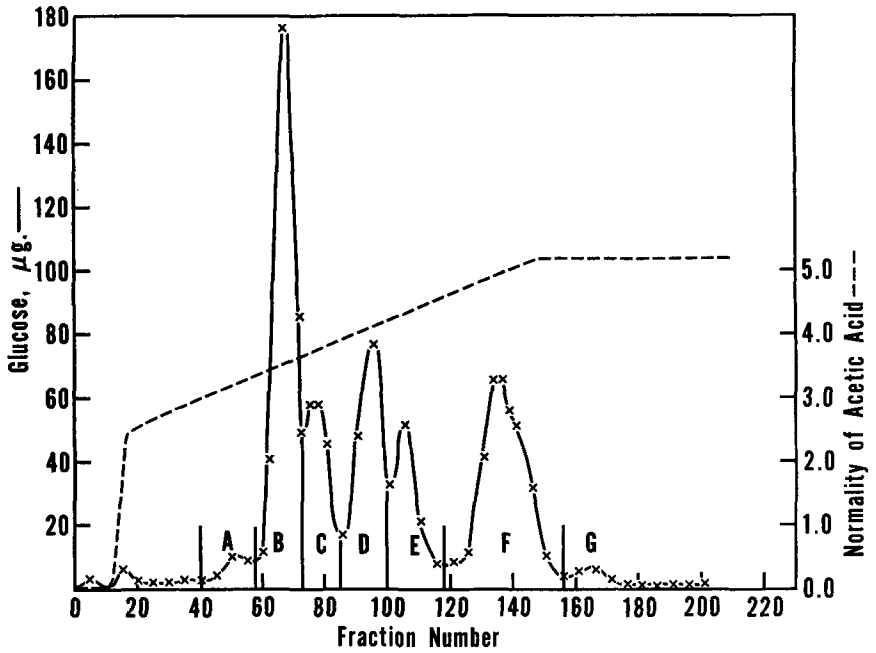

FIG. 4. Gradient elution diagram for chromatography of purified soybean saponins on AG1-X4 anion exchange resin. Dashed curve is acetic acid concentration and solid curve is glucose content determined by phenol-H_2SO_4.

spraying with 50% sulfuric acid and heating) in contrast to the spots of all other fractions, which were magenta. Thickening of the peak for Fraction F (Fig. 4) suggests that two components are present; multiple development TLC confirmed this interpretation. The fastest migrating spot (R_f 0.41) in Fraction F corresponds in R_f to one of the spots in Fraction E, but these compounds are obviously different because of dissimilarities in color. The major component in Fraction G appears to correspond to the spot of R_f 0.44 in Fraction E, but the differences in elution position from the column and in color (with 50% sulfuric acid plus heat) make it doubtful that the two components are identical.

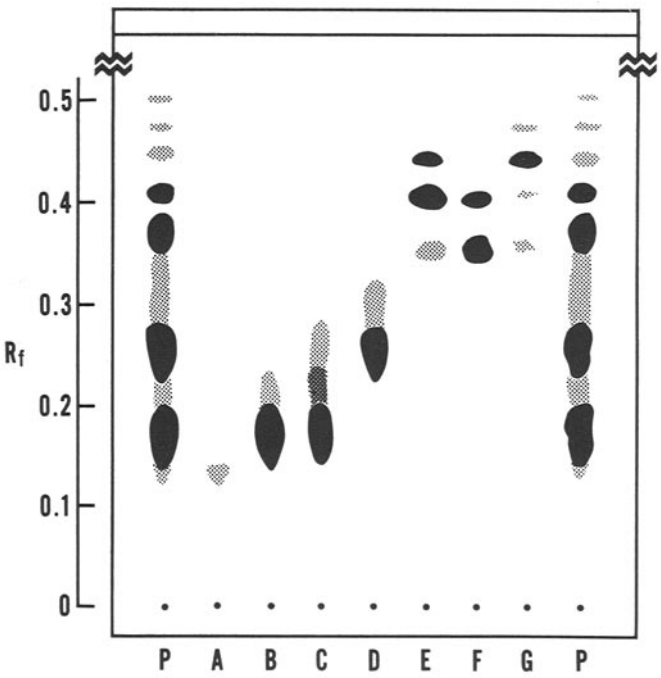

FIG. 5. Diagram of TLC of purified soybean saponins P (extreme left and right) and anion exchange column fractions A-G. Plate was developed six times with chloroform-methanol-water (Solvent 15).

Our results show that multiple development with chloroform-methanol-water (solvent 15) greatly increases resolution of soybean saponins by TLC. A disadvantage of this technique is the time required for analysis. We have found, however, that a single development requires about 4 hr; thus, two developments can be made in a normal working day. Although 10 or more saponins were resolved by TLC, analysis of the fractions obtained by chromatography on the anion exchange resin showed that several saponins have identical R_f values even after multiple development.

In the past, anion exchange resins have been used to separate acidic saponins from neutral compounds such as sugars (23). Our experiments now demonstrate that acidic saponins such as those of soybeans can also be fractionated by anion exchange chromatography. We are studying the ion exchange process further in an attempt to obtain greater resolution of the saponin mixture by this procedure.

ACKNOWLEDGMENT

J. H. Sloneker suggested that we try AG1-X4 resin for this work and contributed helpful discussions related to the handling of the resin.

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[Received July 2, 1969]