Soybean Saponins

Fate of Ingested Soybean Saponins and the Physiological Aspect of Their Hemolytic Activity

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Biological properties of soybean saponins were studied on chicks, rats, and mice kept on soybean saponin-containing diets. Neither soybean saponins nor soybean sapogenins could be found in the blood of these organisms. Ingested soybean saponins were hydrolyzed into sapogenins and sugars by the cecal microflora of chicks, rats, and mice. Saponin-hydrolyzing enzyme(s) from the cecal microflora of rats was partially purified by successive column chromatography on DEAE-cellulose and Ca phosphate (hydroxyl apatite) in the presence of 2-mercaptoethanol. The optimal activity of the crude enzyme(s) was at pH 6.5; of the purified preparation, at pH 6.1. It has shown a low degree of specificity, indicated by the numerous glycosides, including alfalfa saponins, that it hydrolyzes. The in vitro hemolytic activity of soybean saponins on red blood cells was fully inhibited in the presence of plasma or its constituents.

The growing nutritional interest in legume seeds, especially soybeans, as a rich potential source for plant proteins drew attention also to minor soybean constituents—such as saponins—to which various antinutritional properties have been attributed. One of the most striking activities of saponins in general is their in vitro hemolytic activity. Birk *et al.* (1963) have shown that heat processing, the common beneficial process for elevating the nutritional value of raw soybean meal, does not affect the hemolytic activity of soybean saponins. Soybean saponins, added to diets of chicks, rats, and mice, do not affect the growth of these animals (Ishaaya *et al.*, 1968). The aim of the present investigation was to follow up the passage of ingested soybean saponins through the various parts of the digestive tracts of chicks, rats, and mice and to find whether or not they are absorbed into the blood stream. The effect of other blood constituents on the hemolytic activity of soybean saponins was studied as well.

MATERIALS AND METHODS

Soybean saponin extract (SBSE) was prepared from ether-extracted soybean flour, Lincoln Variety, by the method of Birk *et al.* (1963).

Follow Up of SBSE in Digestive Tract of Animals Kept on Soybean Meal Diets. Groups of 10 male albino mice (S.W.R./J.), three male rats (Wistar × Glasgow, Nes-Ziona strain), and three male Leghorn chicks (all one month old) were kept for 10 days on standard diets containing 20% heated soybean flour (Izhar Oil Industry, Ltd., Israel). The animals were killed with chloroform, and their digestive tracts were excised and divided into three parts: small intestine (duodenum included), cecum, and colon. The respective parts of the digestive tracts of the animals of each group were pooled and cut into small pieces. For extracting soybean sapogenins, 100 ml. of ether were added, the mixture was stirred for 30 minutes, and the supernatant was collected. The soybean saponins were extracted from the residue by stirring with 100 ml. of 80% ethanol for 30 minutes. The etheric and ethanolic extracts were evaporated to dryness. The residue of the etheric extract was dissolved in 0.5 ml. of chloroform and analyzed for soybean sapogenins by horizontal paper chromatography as described by Gestetner (1964). This method is sensitive to >4 μg. of sapogenin. The residue of the ethanolic extract was dissolved in 5 ml. of 80% ethanol and the soybean saponins were identified by ascending paper chromatography (Birk *et al.*, 1963), by which >40 μg. of saponin can be detected.

Saponins and Sapogenins in Blood. These determinations were performed on blood drawn from groups of 10 mice, three rats, and three chicks. The plasma was collected by centrifugation and added, dropwise, to 25 volumes of petroleum ether (60° to 80°) plus ethanol (1 to 1) for deproteinization according to Cook (1958). The proteins were removed by centrifugation and washed with chloroform and 80% ethanol to remove possibly adsorbed sapogenins and saponins. The supernatant was evaporated to dryness, and the dry residue was treated first with 5 ml. of chloroform, for extraction of soy-

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bean sapogenins, and then with 5 ml. of 80% ethanol for the extraction of saponins. The chloroformic and ethanolic extracts were analyzed for their constituents as described above. The chloroformic and ethanolic washings of the protein residue were examined as well.

In Vitro Incubation of SBSE with Parts of Digestive Tracts of Rats, Mice, and Chicks. Two-month-old rats and mice and one-month-old chicks, kept for 14 days on standard diets devoid of soybean flour, were killed with chloroform and their digestive tracts were excised and divided into three parts: small intestine, cecum, and colon. The various parts of the digestive tract of each test animal were cut into small pieces and added to 5-ml. solutions of 0.1% SBSE in 0.1*M* phosphate buffer, at pH's corresponding to the respective parts of the digestive tracts examined—i.e., pH 7.5 to 7.7 for the small intestines and pH 6.5 to 6.7 for the ceca and colons. After stirring for 5 minutes the reaction mixtures were incubated for 3 hours at 37°C. The liberated soybean sapogenins were then extracted with 30 ml. of ether by shaking for 20 minutes. The etheric phase was separated by centrifugation at low speed, concentrated to dryness, taken up in 0.5 ml. of chloroform, and analyzed for soybean sapogenins by paper chromatography (Gestetner, 1964). The aqueous phase of the reaction mixture was also evaporated to dryness; the residue was taken up in 5 ml. of 80% ethanol and analyzed for soybean saponins (Birk *et al.*, 1963).

Isolation and Purification of Soybean Saponin-Hydrolyzing Enzyme(s) from Cecal Microflora of Rats. Preparation of Crude Enzyme Solution. Ten 3-month-old rats, kept on a standard diet, were killed with chloroform, and their ceca were excised and cut into small pieces. The cecal contents were extracted with water (10 ml. per cecum) by stirring for 15 minutes and the resulting suspension was filtered through glass wool. The bacterial cells in the filtrate were submitted to sonic disruption, using 60-ml. aliquots in a Raytheon sonic oscillator at 8 kc., 0°C., for 5 minutes. The nondisrupted cells and the debris were removed by centrifugation at 27,000 g, 2°C., for 30 minutes. The supernatant, designated "crude enzyme solution," was supplemented with 2-mercaptoethanol to the concentration of 0.01*M* and stored at −18°C., prior to purification. A solution of $E^{1\text{ cm}}_{280\text{ m}\mu} = 1.0$ was defined to possess one extinction unit per ml. (E.U. per ml.).

Chromatography of "Crude Enzyme Solution" on a DEAE-Cellulose Column. DEAE-cellulose (C. Schleicher & Schüll) was equilibrated with 0.01*M* phosphate buffer, pH 7.6, containing 0.01*M* 2-mercaptoethanol (the starting buffer). The suspension of the adsorbent was then poured into a column (2.2 × 30 cm.) and allowed to settle under gravity. Forty milliliters of the crude enzyme solution ($E^{1\text{ cm}}_{280\text{ m}\mu} = 12.5$) was adjusted with phosphate buffer, pH 7.6, to a final buffer concentration of 0.01*M* and applied to the column. Elution was performed with the starting buffer, followed by stepwise increases of NaCl concentration (0.15, 0.35, and 0.5*M*) in the same buffer. The rate of flow was 300 to 500 ml. per hour and 6-ml. fractions were collected. The various fractions were assayed for soybean saponin-hydrolyzing activity as described below. The active fraction was dialyzed against 0.01*M* 2-mercaptoethanol for 16 hours in the cold, adjusted to pH 6.8 with 0.01*M* phosphate buffer, and designated "DEAE-effluent."

Chromatography of "DEAE-Effluent" on Calcium Phosphate (Hydroxyl Apatite) Column. Calcium phosphate (hydroxyl apatite) was prepared by the method of Tiselius *et al.* (1956). The adsorbent was poured into a column (2.2 × 30 cm.) and washed with 2 liters of 0.001*M* phosphate buffer, pH 6.8, in 0.01*M* 2-mercaptoethanol. DEAE effluent (100 E.U.) was then applied to the column and elution was performed with four stepwise increases of buffer concentrations (0.001, 0.05, 0.1, and 0.3*M*). The rate of flow was about 300 ml. per hour and 6-ml. fractions were collected and assayed for soybean saponin-hydrolyzing activity as described below. The active fraction, designated "purified enzyme solution," was subjected to polyacrylamide gel electrophoresis according to Ornstein and Davis (1964). The electrophoresis was performed in 15% gel at pH 8.9 for 45 minutes at 200 volts, 7.5 ma. per tube.

Determination of Enzymatic Activity. Five milliliters of 0.1% SBSE solution in 0.1*M* phosphate buffer, pH 6.2 (the substrate solution), were incubated with appropriate amounts of the enzyme solution for 16 hours at 37°C. Three 25-ml. portions of chloroform were added for extraction of the liberated soybean sapogenins while the sugars remained in the aqueous phase. The two phases were separated in a separatory funnel and evaporated to dryness. The chloroformic residue was dissolved in 5 ml. of chloroform and the soybean sapogenins were identified by horizontal paper chromatography. For quantitative determination the modified Lieberman-Burchard reagent was used (Gestetner *et al.*, 1966). The aqueous residue was taken up in 1 ml. of water and the liberated sugars were separated by paper chromatography with benzene-butanol-pyridine-water (1:5:3:3) as solvent (de Whalley *et al.*, 1951). The chromatograms were stained with the silver nitrate-NaOH reagent (Trevelyan *et al.*, 1950). One enzyme unit was defined as the amount of enzyme required for the liberation of 1 μg. of soybean sapogenin under the above described experimental conditions. Specific activity was expressed as enzyme units per extinction unit.

Effect of Plasma Constituents and Soybean Proteins on Hemolytic Activity of SBSE. Hemolytic Activity was assayed on washed ram red blood cells by the method of Büchi *et al.* (1950) and the hemolytic indices were calculated according to Wasicky and Wasicky (1961). When the hemolytic activity was determined in the presence of plasma constituents or soybean proteins, aliquots of isotonic solutions of these materials replaced the buffer in the reaction mixture.

Preparation of Plasma and Its Constituents. Plasma was prepared from ram red blood by centrifugation, followed by dilution with isotonic phosphate buffer, pH ~7.4, to the concentration of 20 μl. per ml. The plasma proteins were precipitated by dropwise addition of one volume of undiluted plasma to 25 volumes of petroleum ether (60° to 80°C.) plus ethanol (1 to 1). The mixture was centrifuged, the supernatant was decanted, and the precipitate was washed twice with petroleum ether plus ethanol (1 to 1). The combined supernatants were shaken with water in a separatory funnel, the aqueous ethanolic phase was separated, and the petroleum ether phase was

Table I. Paper Chromatographic Detection of Soybean Saponins and Sapogenins in Digestive Tracts and Blood of Mice, Rats, and Chicks Kept for 10 Days on Diets Containing 20% Soybean Flour

	Small Intestine		Cecum		Colon		Blood	
	Saponins	Sapogenins	Saponins	Sapogenins	Saponins	Sapogenins	Saponins	Sapogenins
Mice	+	−	−	+	−	+	−	−
Rats	+	−	−	+	−	+	−	−
Chicks	+	−	−	+	−	+	−	−

washed twice with water and allowed to settle. The petroleum ether and aqueous ethanolic phases and their washings were combined and concentrated almost to dryness in a rotary vacuum evaporator and dried finally in a desiccator over P_2O_5. The resulting three fractions—i.e., precipitated proteins, petroleum ether extract (lipids), and aqueous ethanolic extract—were then dissolved in isotonic phosphate buffer pH ~7.4.

PREPARATION OF SOYBEAN PROTEINS. Water-soluble soybean proteins were prepared by suspending defatted (ether-extracted) soybean flour in water (1 to 10), stirring for 30 minutes, and collecting the supernatant by centrifugation. Soybean whey proteins were prepared as above from an acidified (HCl to pH 4.6) aqueous extract of defatted soybean flour and the supernatant was neutralized, dialyzed against distilled water, and freeze-dried. The acid-precipitated proteins were dried in a desiccator over P_2O_5 and then dissolved in isotonic phosphate buffer.

RESULTS AND DISCUSSION

In vivo experiments with mice, rats, and chicks showed that ingested soybean saponins are not absorbed into the blood as saponins or sapogenins. Paper chromatographic analyses of the various parts of the digestive tracts of these animals indicated the presence of sapogenins in the ceca and colons, whereas in the small intestines only soybean saponins could be detected (Table I).

The absence of soybean saponins from the chromatograms of the ceca and colons may be due to the initially low concentration of the saponins and does not necessarily imply total decomposition of soybean saponins in these parts of the digestive tract. As a matter of fact, when the contents of the cecum and colon were incubated in vitro with SBSE, both saponins and sapogenins could be identified chromatographically (Table II). Moreover, the quantitative determination of soybean sapogenins in the collected feces accounted for only 60 to 65% of the total amount of soybean saponins ingested by these animals. As shown in Table II, the results of the in vitro experiments with different parts of the digestive tract agree with those obtained in vivo.

The fact that soybean saponins are decomposed by the microflora present in the cecum and colon of mice, rats, and chicks has been ascertained by the following steps:

The cecum and colon were repeatedly washed with isotonic NaCl until no microorganisms could be identified microscopically in the washings.

The microorganisms were separated by centrifugation and then washed three times with isotonic NaCl to remove possible accompanying enzymes in the solution.

The washed cecum and colon as well as the washed microorganisms were incubated with SBSE and the latter

Table II. Chromatographic Detection of Soybean Saponins and Sapogenins in 3-Hour Digests of SBSE with Parts of Digestive Tract of Mice, Rats, and Chicks

	Small Intestine		Cecum		Colon	
	Saponins	Sapogenins	Saponins	Sapogenins	Saponins	Sapogenins
Mice	+	−	+	+	+	+
Rats	+	−	+	+	+	+
Chicks	+	−	+	+	+	+

was hydrolyzed only in the presence of the washed microorganisms.

The soybean saponin–hydrolyzing enzyme(s) from the crude extract of cecal microflora of rats was partially purified by subsequent chromatography on DEAE-cellulose and calcium phosphate columns (Figure 1, *a*, *b*). Preliminary experiments indicated the —SH dependence of this enzyme(s), as the various preparations lost most of their activity when prepared in the absence of 2-mercaptoethanol. The purification steps are summarized in Table III. By successive fractionation on DEAE-cellulose and calcium phosphate columns an approximately 50× purification of the enzyme(s) was achieved. The purification procedure did not, however, result in one homogeneous material, since disc electrophoresis in polyacrylamide gels showed the presence of at least two bands.

The pH dependence of the enzyme(s) is shown in Figure 2. The optimal activity of the crude enzyme is at pH 6.5, whereas the purified preparation shows a slight shift to pH 6.0 to 6.1. Enzymatic breakdown of soybean saponins proceeds during 16 hours of incubation at a linear rate.

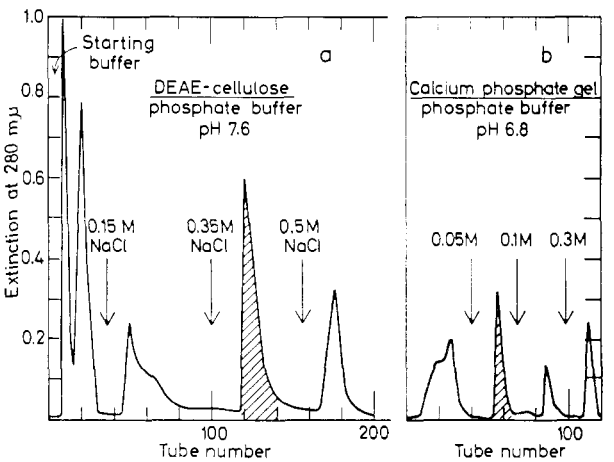

Figure 1. Column chromatography of soybean saponin–hydrolyzing enzyme(s) from cecal microflora of rats

A. On DEAE-cellulose
B. Active peak on calcium-phosphate
Shaded area indicates enzymatic activity

Table III. Purification of Soybean Saponin-Hydrolyzing Enzymes(s) Isolated from Cecal Microflora of Rats

Enzyme Preparation	Total Vol., Ml.	Total Protein, E.U.	Enzyme, Units per Ml.	Total Enzyme Units	Specific Activity, Enzyme Units per E.U.	Coefficient of Purification
Sonic extract	70	1060	311.5	21805	20.6	...
DEAE-cellulose effluent	220	58.4	89.0	19580	335.3	16.3
Calcium phosphate effluent	60	2.4	40.0	2400	1000	48.6

To detect its specificity toward different glycosidic bonds, the enzyme preparation was incubated with various substrates.

The sugars liberated by the purified soybean saponin-hydrolyzing enzyme from soybean and alfalfa saponins and from natural, defined glycosides are given in Table IV. Except for inulin and the 1,2-α-glycosidic linkage between the glucose and fructose residue of raffinose, all the examined substrates were susceptible to the action of the enzyme. The liberation of several sugars from soybean and alfalfa saponins and from various glycosides indicates that the soybean saponin-hydrolyzing enzyme is a glycosidase(s) of a low degree of specificity rather than a specific saponin-hydrolyzing enzyme. An association of several glycosidases has also been suggested for emulsin (Heyworth and Walker, 1962; Shibata and Nisizawa, 1965). In view of the activity of the soybean saponin-hydrolyzing enzyme on various glycosidic bonds of saponins from soybeans and from alfalfa, it should be tried as a possible tool for structure determination of these compounds as well as for the elucidation of their structure-activity relationship.

Ingested soybean saponins or sapogenins are not chromatographically detectable in the blood of chicks, rats, and mice even when the analyses are performed on blood pooled from several animals (Table I). It may therefore be assumed that no considerable amounts of saponins (<40 μg.) or sapogenins (<4 μg.) are absorbed per total blood volume of 10 mice or three rats or three chicks. It was of interest, however, to find whether soybean saponins can hemolyze red blood cells in their natural medium—namely, in the presence of plasma. The results presented in Table V clearly demonstrate that even minute amounts of plasma depress considerably the hemolytic activity of SBSE. The finding that the inhibition of hemolysis is due to the protein and lipid constituents of plasma agrees with the results of similar experiments with other triterpenoid and steroid saponins (Griminger and Fisher, 1958; Schmidt-Thomé, 1942; Vogel and Marek, 1962). The interaction between saponins and plasma proteins and lipids may arise from the surface activity of the involved materials. It simulates the nonspecific SBSE-protein interaction which accounts for the inhibition of certain enzymes by SBSE and for the counteraction of this inhibition by preincubation of SBSE with proteins (Ishaaya and Birk, 1965).

The inhibition of the hemolytic activity of SBSE is not limited to plasma lipids and proteins; soybean proteins, the natural accompanying materials of SBSE, and particularly the acid-precipitable soybean proteins, also depress the hemolytic activity of SBSE (Table VI). But in this case complete inhibition of the hemolytic activity of SBSE could not be achieved as with plasma proteins, since at concentrations higher than 1.20 mg. per reaction mixture the soybean proteins show a slight hemolytic activity, probably due to a change in the osmotic pressure. However, since the saponin content of soybeans is rather low (Gestetner *et al.*, 1966) and a 75 to 1 protein per saponin ratio exists *in situ*, it may be assumed that in the digestive tract the hemolytic activity of the saponin is fully masked by accompanying proteins. SBSE, although hemolytic in vitro, does

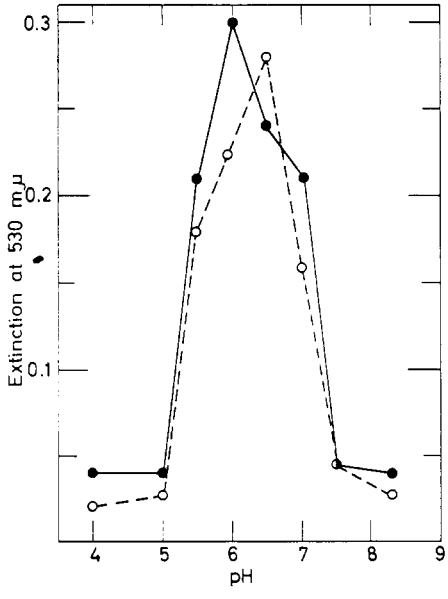

Figure 2. pH dependence of soybean saponin-hydrolyzing enzyme(s)

○ Crude enzyme solution
● Purified enzyme solution

Table IV. Paper Chromatographic Identification of Carbohydrates Liberated after 3-Hour Incubation of Purified Soybean Sapon-Hydrolyzing Enzyme with Various Substrates

Substrate	Carbohydrates Liberated
Soybean saponins	Glucose, galactose, arabinose, rhamnose, glucuronic acid
Alfalfa saponins[a]	Glucose, galactose, arabinose, rhamnose, glucuronic acid
Starch	Glucose, oligosaccharides
Glycogen	Glucose, oligosaccharides
Inulin	None
Lactose	Glucose, galactose
Trehalose	Glucose
Raffinose	Galactose
Salicin	Glucose
Rutin	Glucose, rhamnose

[a] Donated by E. D. Walter, Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, Calif.

Table V. Impairment of Hemolytic Activity of SBSE by Plasma or Its Constituents

Plasma or Fractions Added per Test Tube	Minimal SBSE Needed for Full Hemolysis, Mg. per Test Tube	Hemolytic Index
None (control)	0.200	10000
Plasma		
1 μl.	0.200–0.275	7300–8000
2 μl.	0.300–0.325	6150–6600
3 μl.	0.350–0.375	5300–5700
4 μl.	0.400–0.425	4700–5000
5 μl.	0.500	4000
10 μl.	0.700–0.750	2650–2850
20 μl.	2.00	100
Plasma proteins, 0.45 mg.	3.00	No hemolysis[a]
Plasma lipids, 0.35 mg.	3.00	No hemolysis[a]
Ethanolic extract, 1.00 mg.	0.200	10000

[a] Even after addition of maximal soluble amount of SBSE to reaction mixture.

Table VI. Effect of Soybean Proteins on Hemolytic Activity of SBSE

Protein Added to Reaction Mixture	Minimal SBSE Needed for Full Hemolysis, Mg. per Test Tube	Hemolytic Index
...	0.20	10,000
Aqueous extract of soybean protein, 1.2 mg.	0.57	3,500
Acid-precipitated proteins, 1.2 mg.	0.55	3,600
Whey proteins, 1.2 mg.	0.20	10,000

not impair growth of chicks, rats, and mice even when ingested in a 10× concentration of that in soybean meal *in situ* (Ishaaya *et al.*, 1968). Thus it may be concluded that soybean saponins, although hemolytic in vitro, do not possess detrimental physiological effects in vivo, since they pass intact through the small intestine, are decomposable by the cecal microflora, and do not seem to be absorbed into the blood stream, but, if absorbed, should lose their hemolytic power by interaction with plasma constituents.

LITERATURE CITED

Birk, Y., Bondi, A., Gestetner, B., Ishaaya, I., *Nature* **197,** 1089 (1963).
Büchi, J., Hippenmeyer, F., Dolder, R., *Pharm. Acta Helv.* **25,** 143 (1950).
Cook, R. P., "Cholesterol," p. 484, Academic Press, New York, 1958.
de Whalley, H. C. S., Albon, N., Gross, D., *Analyst* **76,** 287 (1951).
Gestetner, B., *J. Chromatog.* **13,** 259 (1964).
Gestetner, B., Birk, Y., Tencer, Y., Bondi, A., *Phytochemistry* **5,** 803 (1966).
Griminger, P., Fisher, H., *Proc. Soc. Exptl. Biol. Med.* **99,** 424 (1958).
Heyworth, R., Walker, P. G., *Biochem. J.* **83,** 331 (1962).
Ishaaya, I., Birk, Y., *J. Food Sci.* **30,** 118 (1965).
Ishaaya, I., Birk, Y., Bondi, A., Tencer, Y., Hebrew University, Rehovot, Israel, unpublished results, 1968.
Ornstein, L., Davis, B. J., *Ann. N. Y. Acad. Sci.* **121,** 321, 404 (1964).
Schmidt-Thomé, J., *Z. Physiol. Chem.* **275,** 208 (1942).
Shibata, Y., Nisizawa, K., *Arch. Biochem. Biophys.* **109,** 516 (1965).
Tiselius, A., Hjerten, S., Levin, O., *Arch. Biochem. Biophys.* **65,** 132 (1956).
Trevelyan, W. E., Procter, D. P., Harisson, J. S., *Nature* **166,** 144 (1950).
Vogel, G., Marek, M. L., *Arzneimittel-Forsch.* **12,** 815 (1962).
Wasicky, R., Wasicky, M., *Qual. Plant. Mater. Veg.* **8,** 5 (1961).

Received for review March 4, 1968. Accepted July 8, 1968. Eighth in a series on soybean saponins. The seventh paper is Gestetner B., et al., Phytochem. **5,** *803 (1966). Investigation supported by Grant FG-IS-112 from the U. S. Department of Agriculture.*