Saponins as Possible Factors of Resistance of Legume Seeds to the Attack of Insects

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A general method has been elaborated for preparing saponins from seeds of chickpeas, garden peas, broad beans, haricot beans, lentils, and peanuts. Paper chromatographic analyses revealed three to six fractions per preparation. Upon hydrolysis two to five aglycones per preparation were found. Further fractionation on Amberlite IRA 401 S resulted in isolation of a saponin fraction which upon hydrolysis gives a single aglycone but different sugars. Saponin preparations I (CaO-precipitable) inhibited development of the Azuki bean beetle (*Callosobruchus chinensis* L.), a major pest of stored legume seeds, when incorporated in diets, whereas saponin preparations II (which do not precipitate with CaO) hardly affected development. A mixture of saponin preparations I and II from chickpeas or lentils, seeds normally attacked by the Azuki bean beetle, had no adverse effect, whereas a similar mixture from a variety of garden peas resistant to this insect inhibited development.

Serious deterioration of legume seeds in storage is caused by infestation with bruchid beetles, of which the Azuki bean beetle (*Callosobruchus chinensis* L.) is a prominent example. This insect is regarded as a major pest of chickpeas, broad beans, lentils, azuki beans, and, to a lesser extent, garden pea seeds. It has been the subject of a series of investigations concerning the physical and chemical factors affecting its host specificity (Applebaum, 1964; Applebaum *et al.*, 1965, 1968; Avidov *et al.*, 1965a,b; Ishii, 1952; Podoler and Applebaum, 1968). Its inability to develop in soybeans is attributed mainly to the high protein-carbohydrate ratio of the seed (Applebaum *et al.*, 1968) and, in part, to its saponin content (Applebaum *et al.*, 1965). Its inability to develop in haricot beans is attributed partly to toxic pentosans present in these beans (Ishii, 1952).

Saponins occur in a wide variety of plants, but their role in the plant has not yet been elucidated (Birk, 1969). Their presence, distribution, and composition in soybeans have been investigated (Birk *et al.*, 1963; Gestetner *et al.*, 1963, 1966a,b; Meyer, 1950; Okano and Ohara, 1933; Sumiki, 1929; Willner *et al.*, 1964). Saponins are present and have been determined in peanuts as well (Dieckert and Morris, 1958), but very little is known about their presence and content in other legume seeds. Although different antibiological properties have been attributed to saponins, none of them was substantiated for legume seed saponins when ingested by humans or farm animals. Moreover, a recent investigation of soybean saponins (Ishaaya *et al.*, 1969) clearly proved the harmlessness of ingested soybean saponins for chicks, rats, and mice. Because of their triterpenoid and glycosidic dual nature, soybean saponins exhibit hydrophobic and, to a lesser extent, hydrophylic properties which seem to be involved in their biological activities. Indeed, the isolated soybean sapogenins in themselves lack effect on *C. chinensis*. Several questions were posed in the present investigation: Are saponins present in other edible legume seeds? If so, do they affect the development of *C. chinensis*? Is this effect correlated to what is known of the host specificity of the beetle, and to what extent may saponins be regarded as specific metabolic defense mechanisms evolved in legume seeds against insects?

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EXPERIMENTAL

Preparation of Saponins from Legume Seeds. The following legume seeds were used for saponin preparation:

Cicer arietinum L. (chickpeas, Californian variety)
Pisum sativum L. (garden peas, Hungarian variety)
Vicia faba L. (broad beans, Cyprus variety)
Phaseolus vulgaris L. (haricot beans, Polish variety)
Lens esculenta Moench. (lentils, variety unknown)
Arachis hypogaea L. (peanuts, Dixie Anak variety)

Seed coats were removed from broad beans and lentils. Dehulled peanuts were immersed for 12 hours in diethyl ether to facilitate removal of the testa. All the other seeds were used as received. The seeds were ground in a coffee mill and sieved through a 60-mesh sieve. The meals were then extracted with diethyl ether in a Soxhlet apparatus for 48 hours to remove lipids and pigments.

Saponins were extracted with 80% ethanol as described by Birk *et al.* (1963) for soybean saponins. The ethanolic extracts were treated with diethyl ether for removal of the residual pigments. The aqueous, ether-free solution was brought to pH 4.0 and refrigerated overnight. A precipitate was formed and removed by centrifugation at 105,000 × G for 30 minutes. The supernatant was treated with CaO, with constant stirring, for precipitation of the saponins (Ochiai *et al.*, 1937). The mixture was filtered through Whatman No. 1 filter paper. Saponins were present in most cases in both precipitate and filtrate; the supernatant saponins are a distinct fraction and do not precipitate with excess of CaO.

The precipitate, which contains the saponins as Ca salts, was dispersed in 20 ml. of absolute ethanol and the ethanolic suspension was neutralized with dilute H_2SO_4; the solution was then brought to a final concentration of 80% ethanol, and stirred overnight at 55° to 60° C. The filtrate was concentrated in vacuum to

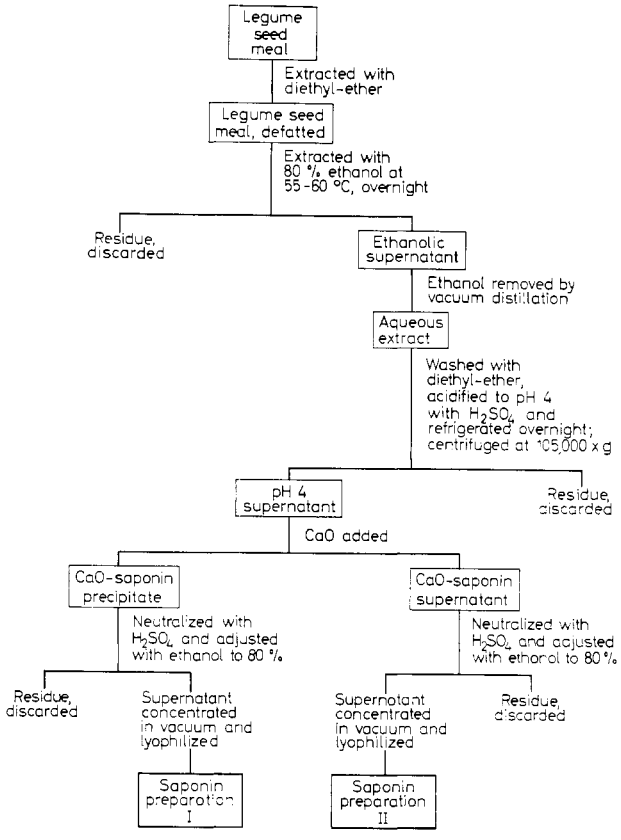

Figure 1. Schematic outline of procedure for preparation of legume seed saponins

a minimal volume of water and then lyophilized. The hygroscopic powder thus obtained was designated saponin preparation I.

The supernatant, obtained after the Ca-saponin precipitation, was treated in a similar manner—namely, extracted with ethanol, neutralized with H_2SO_4, concentrated in vacuum, and lyophilized. The hygroscopic powder was designated saponin preparation II. Both preparations were kept in a desiccator over P_2O_5. A schematic presentation of the procedure is given in Figure 1.

Chromatographic Identification and Characterization of Legume Seed Saponins. Paper chromatographic separations of the saponin preparations were performed on Whatman No. 3MM filter paper as described by Birk *et al.* (1963) for soybean saponins.

For identification of the aglycone components the different saponin preparations were hydrolyzed in 1*N* H_2SO_4 in dioxane-water (1 to 3) for 5 hours under reflux—conditions optimal for hydrolysis of soybean saponins (Gestetner *et al.*, 1966a). The cooled hydrolyzate was diluted with water and the sapogenins were extracted with diethyl ether and analyzed by circular paper chromatography according to Gestetner (1964).

Prior to determination of the carbohydrate moiety of the saponins, the mono-, di-, and oligosaccharides, accompanying the saponins and soluble in 80% ethanol, were removed by passing aqueous solutions of the saponin preparations (30 to 60 mg. per ml.) through an Amberlite IRA 401 S column (1.4 × 14.5 cm.). The column was washed with 50 ml. of water to elute the free sugars and nonadsorbed saponins, and then with 20 ml. of 2*N* NaOH to elute the adsorbed saponins.

The alkaline eluate was adjusted to a concentration of 1.33*N* H_2SO_4. Dioxane, in the ratio of 1 to 3, was added and the mixture was hydrolyzed for 5 hours under reflux as above. Sodium ions were removed by shaking the cooled hydrolyzate with beads of Amberlite IR 120 and the suspension was filtered through Whatman No. 1 filter paper. The filtrate was neutralized with $BaCO_3$ and the $BaSO_4$ removed by centrifugation followed by filtration.

The carbohydrate content of the acid hydrolyzate was determined by the paper chromatographic procedure of de Whalley *et al.* (1951). The chromatograms were stained with the $AgNO_3$ reagent described by Trevelyan *et al.* (1950). Glucose, galactose, xylose, rhamnose, arabinose, ribose, deoxyribose, fucose, glucuronic acid, and its lactone were used as markers.

The in vitro hemolytic activity was determined by the method of Büchi *et al.* (1950) and the hemolytic indices (H.I.) were calculated according to Wasicky and Wasicky (1961).

Effect of Saponin Preparations on Growth of *C. chinensis*. The effect of the various saponin preparations on the development of *C. chinensis* was assayed by incorporating these materials in artificial beans prepared with broad bean flour.

Stock cultures of *C. chinensis* were reared on chickpeas at a density not exceeding six larvae per seed. Cultures were held at a constant temperature of 28°C. and all experiments were performed at this temperature.

Newly emerged adult beetles from stock cultures were allowed to oviposit for several hours on these artificial beans and then removed. To avoid stress, no more than nine hatched larvae were left on each artificial bean. Starting from the thirtieth day after oviposition the vials were examined daily and adults that had emerged were counted and removed. Two months after oviposition the artificial beans were broken and all the adults which had developed, but did not succeed in emerging, were counted.

RESULTS AND DISCUSSION

The available methods for the extraction of legume saponins do not meet the requirements for a standard

Table I. Characterization of Saponin Preparations I and II from Several Legume Seeds

Seed	Saponin Preparation	Yield, %[a]	Hemolytic Index
Chickpeas	I	1.0	500
	II	5.0	None[b]
Garden peas	I	1.1	None[b]
	II	3.1	None[b]
Lentils	I	1.9	500
	II	4.7	None[b]
Broad beans	I	2.2	Not assayed
	II	4.1	Not assayed
Haricot beans	I	3.2	None[b]
Peanuts	I	1.6	500

[a] Calculated on basis of lipid-free meal as 100%.
[b] Even at concentration of 14 mg. saponin preparation per 2 ml. reaction mixture.

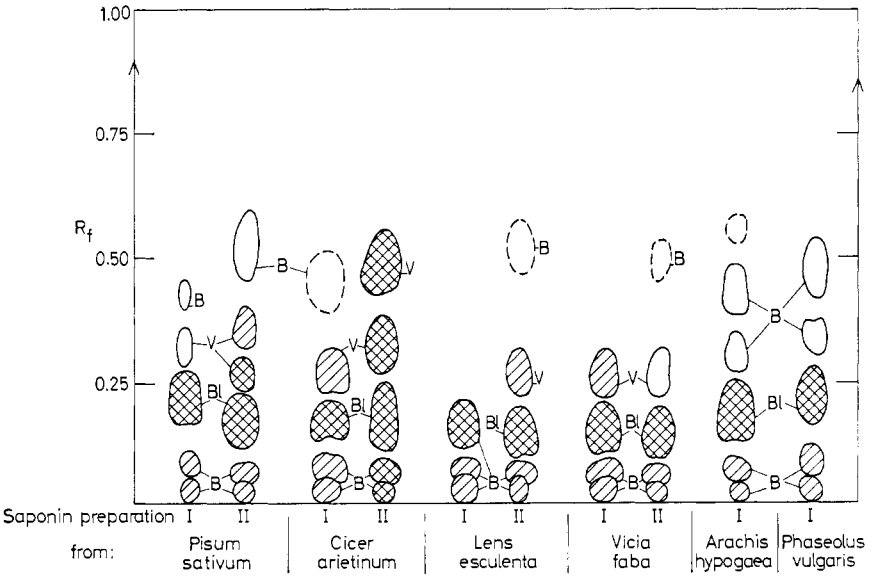

Figure 2. Ascending paper chromatography of saponin preparations on Whatman 3MM filter paper with butanol-ethanol-water (6:2:3) as solvent and $SbCl_3$ in chloroform as staining reagent

Colors obtained. Brown (B); blue (Bl); violet (V). Increasing intensity of spots marked as: ⬚ < □ < ▨ < ⊠

procedure of extraction, suitable for biological assay. Thus, for example, peanut saponins, precipitated as lead salts (Dieckert and Morris, 1958), cannot be used for growth experiments with insects. On the other hand, the butanol extraction of saponins from aqueous phase, suitable for preparation of soybean saponins (Birk *et al.*, 1963), gives very low yields with other legume seeds. In addition, the water-soluble legume seed saponin preparations do not precipitate upon addition of diethyl ether to their aqueous solutions, as do alfalfa and clover saponins (Thompson *et al.*, 1957).

A standard procedure which was finally elaborated is given in detail above. Table I indicates some properties of the saponin fractions obtained by this procedure. CaO almost completely precipitated the saponins from aqueous solutions of the peanut and haricot bean saponins, whereas in all the other seeds the saponin content of the supernatant fraction, saponin preparation II, was substantial. This preparation gives higher apparent yields than CaO-precipitated saponin preparation I; this is partly due to the presence of carbohydrates, amino acids, and peptides which are carried along in the initial extraction procedure.

Separation and Identification of Legume Seed Saponins. Ascending paper chromatography indicated the presence of at least three fractions in each prepara-

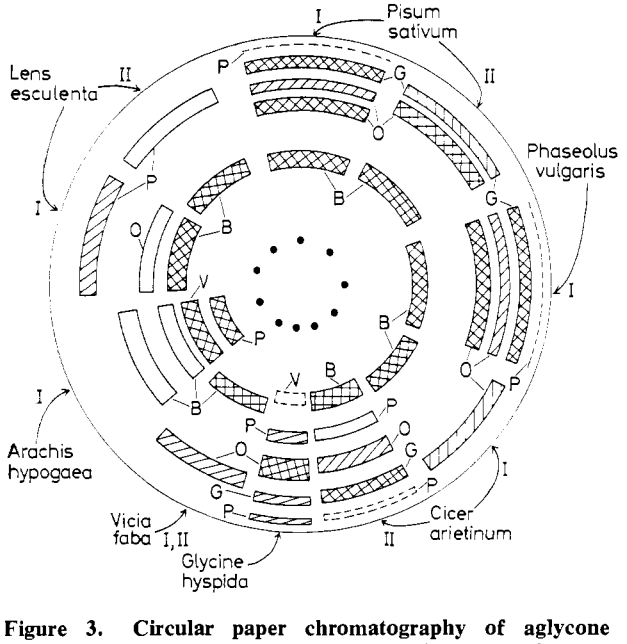

Figure 3. Circular paper chromatography of aglycone moiety of legume seed saponin preparations on Whatman 3MM filter paper with hexane–chloroform–acetic acid (100:10:2.5) as solvent and $SbCl_3$ in chloroform as staining reagent

Colors obtained. Brown (B); green (G); orange (O); pink (P); violet (V). Increasing intensity of spots is marked as: ⬚ < □ < ▨ < ⊠

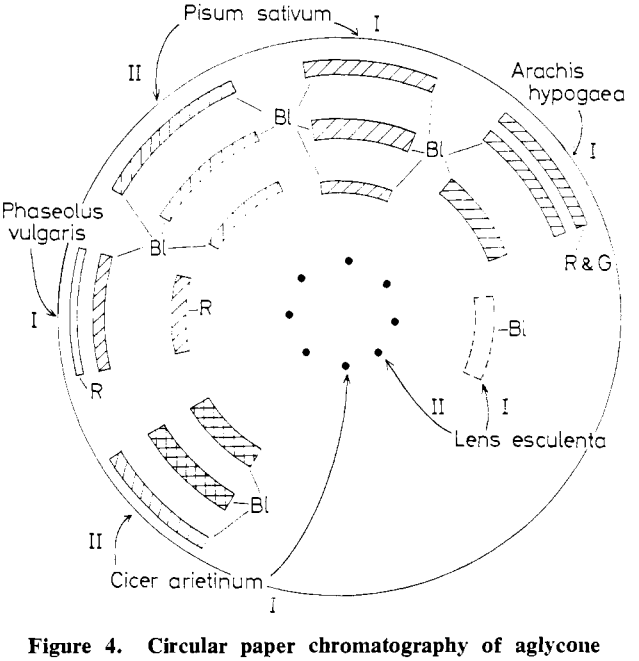

Figure 4. Circular paper chromatography of aglycone moiety of legume seed saponin preparations, which did not adsorb on Amberlite IRA 401 S, on Whatman 3MM filter paper with hexane–chloroform–acetic acid (100:10:2.5) as solvent and $SbCl_3$ in chloroform as staining reagent

Colors obtained. Blue (Bl); green (G); red (R). Increasing intensity of spots marked as ⬚ < □ < ▨ < ⊠

Table II. Chromatographic Separation of Carbohydrates in Acid Hydrolyzates of Amberlite IRA 401 S Fractions of Legume Seed Saponins

R_G Value	Corresponding to[a]	Saponin Preparation from: Garden Peas I	Garden Peas II	Chickpeas I	Chickpeas II	Lentils I	Lentils II	Broad beans, I	Peanuts, I	Haricot beans, I
0.06	Glucuronic acid	+								
0.15	x_1				+			+	+	
0.3	x_2	±								
0.45	x_3	+++		+				+		
0.8	Galactose	±	±	++	±	+	+	++	±	+
1.0	Glucose			++	++	++	++	+++	++	++
1.2	Arabinose	+++			+		+	±	+	
1.4	Xylose	++								
1.55	Deoxyribose	±								
1.7	Rhamnose						±	±		
2.2	x_4							+		+
2.3	x_5							+		+
3.0	x_6		+					+		
3.1	x_7					++	+			
3.6	x_8		++	±			+			++

[a] x_1 . . . . . x_8 indicate unidentified carbohydrates.

Table III. Effect of Legume Seed Saponin Preparations on Development of *Callosobruchus chinensis*

Saponin Preparation		No. of Larvae at Start	Development, %	Emergence, %	Av. Day of Adult Emergence
Chickpea	I	342	5.6	0	0
	II	289	35.2	13.2	33.4
	I + II	112	31.0	7.5	35.2
Garden pea	I	251	0	0	0
	II	297	15.0	0	0
	I + II	105	8.0	0	0
Lentil	I	324	3.5	0	0
	II	284	48.2	30.5	34.2
	I + II	97	28.0	18.2	35.4
Peanut	I	288	0	0	0
Haricot bean	I	233	20.0	11.3	36.2
Control	(no saponin)	296	41.1	21.8	30.6

tion (Figure 2), which are presumably saponins but may include nonsaponin accompanying materials which stain with the nonspecific antimony trichloride reagent. The similarity in R_f values of the resolved saponins does not necessarily indicate that they are identical in chemical composition. CaO precipitation separates different saponins, as can be seen from the differences in the chromatographic patterns of saponin preparations I and II.

Separation and Identification of Legume Seed Saponin Aglycones. The aglycones were separated and identified by circular paper chromatography (Figure 3). Comparison of their R_f values to those of the individual sapogenols indicates slight differences in some cases which are difficult to evaluate. Some legume aglycones clearly differ from the soybean sapogenols in their chromatographic behavior. Thus, for example, two additional aglycones, common to haricot beans and garden peas, were observed. In peanuts, two aglycones were observed, which differed chromatographically from the aglycones appearing in all the other legume seed saponin preparations. Finally, the aglycones clearly differ in saponin preparations I and II of the same seed.

Saponin preparations I and II of the various seeds were subjected to an additional fractionation on the anion exchanger IRA 401 S. Part of the saponins were not adsorbed and were eluted with distilled water. The chromatographic distribution of their aglycones is presented in Figure 4. The adsorbed saponins were eluted with 2*N* NaOH. Only one chromatographically identical aglycone was observed in the acid hydrolyzates of all seed preparations which adsorbed on the anion exchanger (Figure 5).

Separation and Identification of Legume Seed Saponin Carbohydrates. The original saponin preparations I and II of the various seeds are accompanied by ethanol-soluble free carbohydrates, which interfere with the possibility of identifying the intact carbohydrate components of the saponins. A fraction of each saponin preparation was freed of the accompanying carbohydrates, with the aid of the Amberlite IRA 401 S column, and then acid-hydrolyzed. The paper chromatographic analyses of the monosaccharides present in the acid hydrolyzates of these saponins are given in Table II; eight components were not identified.

The qualitative differences in carbohydrate content among the various saponin preparations from legume seeds relate to the single, chromatographically identical, aglycone (Figure 5). The carbohydrates observed in the acid hydrolyzate of the peanut saponin fraction therefore relate to only one minor component of the total saponin content of the peanut—that which is ad-

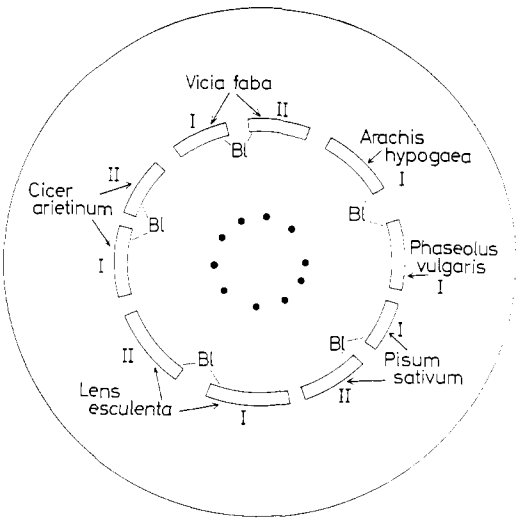

Figure 5. Circular paper chromatography of aglycone moiety of legume seed saponin preparations, adsorbed on Amberlite IRA 401 S and eluted with NaOH, on Whatman 3MM filter paper with hexane–chloroform–acetic acid (100:10:2.5) as solvent and $SbCl_3$ in chloroform as staining reagent

Color obtained. Blue (Bl)

sorbed on Amberlite IRA 401 S. The glucose, arabinose, and the unidentified carbohydrate may well be below the level of detection when the hydrolyzate is prepared from the total saponin preparation. The different carbohydrate content of peanut saponins, reported by Dieckert and Morris (1958), may be due to this fact or to varietal differences such as those reported for alfalfa saponins (Pedersen *et al.*, 1967).

Effect of Legume Seed Saponin Preparations on Development of *C. chinensis*. This was evaluated by incorporating the individual saponin preparations of each seed in artificial diets, either separately (saponin preparation I or II alone) or in combination (saponin preparations I and II together), at levels similar to their actual concentration in the seed (Table III). Almost all the saponin preparation I, except for that from haricot beans, strongly inhibited development, while most of the saponin preparation II hardly affected development.

The addition of saponin preparation II to artificial beans in addition to saponin preparation I overcome the deleterious effect of the latter in the case of chickpeas and lentils. These seeds normally serve as hosts for development of *C. chinensis* larvae. In this respect the results of all the biological growth experiments are in accord with the host compatibility of *C. chinensis*. It seems reasonable to attribute the beneficial effect of saponin preparation II to the fact that these are cruder preparations than saponin preparation I, and are accompanied by free amino acids, peptides, and carbohydrates of the seed. If, indeed, such accompanying components overcome the inhibitory effect of saponins, it would limit the importance of such saponins as mechanisms of resistance.

Nevertheless, viewing the biological action of saponins in their context as part of a complex of substances in the seeds, there are some cases wherein they could well serve as possible defense mechanisms against insects. To evaluate this possibility a more exact knowledge of the composition of the other ethanol-soluble components present in legume seeds is required.

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