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INTERACTION OF ALFALFA SAPONINS WITH COMPONENTS OF THE ERYTHROCYTE MEMBRANE IN HEMOLYSIS*

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SUMMARY

The interactions of saponins with erythrocyte membrane components involved in the hemolytic mechanism were studied. It was found that the alfalfa saponin, medicagenic acid 3-β-*O*-triglucoside, forms interaction products of different stability with membrane cholesterol, proteins and phospholipids. The resulting structural changes affect only slightly the membranous enzyme activities examined. It is suggested that breakdown of the structure of the membrane stems from a combination of non-specific interactions of saponins with membrane proteins, phospholipids and cholesterol leading consequently to hemolysis.

INTRODUCTION

Alfalfa saponin extracts have been characterized by their thin-layer chromatographic distribution patterns, hemolytic and foam-forming activities and sapogenin and carbohydrate composition[1]. Alfalfa saponin preparations from tops and roots differ in the carbohydrate content which is higher in top extracts than in root extracts[1]. Both the top and root saponin extracts contain medicagenic acid[2], hederagenin[3] and soyasapogenols A, B, C, D and E as their sapogenins[2]. The higher hemolytic activity of the root extract as compared to that of the top extract results from the relatively higher content of medicagenic acid as well as from the higher sapogenin: carbohydrate ratio in alfalfa root saponin extract[4]. Medicagenic acid has been found to be the saponin component responsible for *Tribolium castaneum* larval growth depression[5] and for fungistatic[4] and hemolytic[4] activities of alfalfa saponins. The inability of certain alfalfa saponins, which contain soybean sapogenols A, B, C, D and E as their sapogenins, to lyse red blood cells, seems to disagree with previous findings that soybean saponins do exert a considerable hemolytic activity[4]. This discrepancy can be explained by the lower sapogenin: carbohydrate ratio (1:5) in alfalfa saponins as compared to the 1:1 ratio in soybean saponins.

Alfalfa saponins and steroid saponins such as digitonin form water-insoluble complexes with various sterols[6,7]. It has been shown earlier that only sterols of the cholestanol series are able to interact with digitonin and alfalfa saponins[7]. But,

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unlike complexes formed with digitonin, the sterol–alfalfa saponin addition products are not dependent on the presence of an equatorial 3-β-hydroxyl group, in the absence of which digitonin does not form complexes with sterols.

It was also found that cholesterol, as well as plant sterols, can successfully counteract larval growth impairment caused by alfalfa saponins[5]. However, in the case of fungal growth only cholesterol and 7-dehydrocholesterol are effective, whereas plant sterols are not[4]. Attempts to counteract the inhibitory effect of soybean saponin on the growth of *Tribolium castaneum* larvae were not successful[1]. On the other hand, preincubation of alfalfa and soybean saponins with proteins such as casein, hemoglobin and soybean proteins could prevent the non-specific inhibitory effect of these saponins on various enzymes[1].

The present work deals with the comparative hemolytic activity of alfalfa saponins, soybean saponins and digitonin, namely with their interactions with the membrane of the red blood cell. It has been the aim of our study to find out which of the membrane elements are involved in the hemolytic mechanism. As a major tool we used a recently characterized alfalfa saponin, medicagenic acid 3-β-*O*-triglucoside[8], since medicagenic acid has been found to be the major sapogenin actively involved in the different biological properties exerted by alfalfa saponins.

MATERIALS AND METHODS

Preparation of saponins

Alfalfa saponin extracts were prepared from alfalfa, Hairy Peruvian variety, tops or roots by the method of Shany *et al.*[2]. Cholesterol-precipitable alfalfa root saponins were prepared from alfalfa saponin extracts according to Gestetner *et al.*[4]. Medicagenic acid 3-β-*O*-triglucoside was prepared by thin-layer chromatographic separation of cholesterol-precipitable alfalfa root saponins on Kieselgel HR plates[8]. Soybean saponin extract was prepared from diethyl ether-extracted soybean flour, as described by Birk *et al.*[9].

Determination of hemolytic activity

Citrated ram blood was used. The blood was withdrawn from the jugular vein, washed by repeated centrifugation with isotonic sodium phosphate buffer (pH about 7.4), until the supernatant was colorless. The degree of hemolysis was determined spectrophotometrically[10] in a 4% suspension of erythrocytes in the same buffer.

The hemolytic activities of the various saponin preparations were determined also in the presence of plasma, serum albumin, cholesterol and egg lecithin. Blood plasma was obtained by centrifugation of ram blood; it was diluted 25-fold with the above isotonic buffer and aliquots were added to the reaction mixture. A solution of 0.5% crystalline bovine serum albumin (NBCo) and suspensions of 0.1% cholesterol (Fluka) or 0.4% egg lecithin (Sigma) were prepared in isotonic buffer and various amounts were added to the reaction mixture. When hemolysis was performed in the presence of bovine serum albumin the latter was preincubated for 30 min with the saponin before the addition of the red blood cells. The mixtures of cholesterol and saponin and of lecithin and saponin were heated at 80 °C for 30 min and cooled to room temperature prior to the addition of the red blood cells.

Preparation of erythrocyte ghosts

Erythrocyte ghosts (hemoglobin free) were prepared by gradual hemolysis in hypotonic aqueous solution from 10 ml blood according to Danon *et al.*[11]. After completion of hemolysis the preparation was freeze-dried. Alternatively, the ghosts were prepared by hemolysis according to Kofler[12], with medicagenic acid 3-β-*O*-triglucoside or with digitonin in concentrations causing hemolysis after 45–60 min. The hemoglobin and excess of saponin were removed by washing the ghosts as described by Danon *et al.*[11].

Extraction and identification of lipids from erythrocyte ghosts

Lipids from 25 mg of freeze-dried ghosts were gradually extracted using different solvents consecutively. The solvent mixture chloroform–methanol, employed conventionally for this purpose, could not be used because methanol released saponins from their interaction product with cholesterol. Therefore, the first extraction was performed with 20-ml portions of benzene in an ultra-turax disintegrator, until no lipids (*e.g.*,free fatty acids, triglycerides, phospholipids and cholesterol) could be identified in the extract by thin-layer chromatography in chloroform–96% ethanol–water (70:28:2, by vol.). The extraction was then continued with 20-ml portions of 96% ethanol and followed with boiling pyridine. Each of the extracts was concentrated to 5 ml and a sample of 0.25 ml was taken for quantitative determination of phospholipids by measurement of inorganic phosphorus according to Ames and Dubin[13]. The residual 4.75 ml were saponified with 6% KOH in ethanol for 60 min and saved for identification and determination of cholesterol and free fatty acids. Cholesterol was determined by gas–liquid chromatography in a Packard No. 7300/7400 gas chromatograph, equipped with flame ionization detector, on a 2 m × 0.32 cm glass column of 5% OV-101[14] on GCQ[14] at an operating temperature of 260 °C (ref. 14). Retention time was quoted relative to a commercial sample of cholesterol (Sigma Chemicals Co.). Fatty acids, originally either free or bound, were identified as methyl esters in the above saponified solution which had been acidified with dilute HCl and refluxed with H_2SO_4 in methanol for esterification. The methyl esters were determined by gas–liquid chromatography on a 2.44 m × 0.32 cm glass column packed with 15% diethyleneglycol succinate polyester on GCW[15] at an operating temperature of 215 °C (ref. 15). Reference compounds were donated by Dr D. Sklan.

Isolation of protein from erythrocyte ghosts

The ghosts were suspended in cold distilled water containing 5 mM EDTA + 5 mM 2-mercaptoethanol adjusted to pH 7.5 and treated as described by Marchesi *et al.*[16]. The soluble proteins were separated by a Sephadex G-200 column[16], and the acid hydrolysate fraction designated "spectrin" gave a similar amino acid analysis to that isolated by Marchesi *et al.*[16].

Interaction of root alfalfa saponin extract with proteins

A sample of 20 mg root saponin extract and 100 mg of protein was suspended in 10 ml water, stirred overnight at 37 °C and suction-filtered through 2.5 g Celite-535 filter aid, on sintered-glass filter 5 cm diameter. The saponins and proteins which did not interact, were removed from the cake by leaching with five 100-ml portions

of water. The protein–saponin interaction product was dissociated by passing five 25-ml portions of 96% ethanol through the cake. The interacting saponins were identified by thin-layer chromatography in ethylacetate–acetic acid–water (7:2:2, by vol.). Further characterization of these saponins was carried out by subjecting them to acid hydrolysis in 2.0 M H_2SO_4 according to Tsukamoto *et al.*[17]; the released sapogenins were analysed by thin-layer chromatography in a solvent system of isopropyl ether–acetone (73:30, by vol.) to 100 ml of which two drops of glacial acetic acid have been added. The plates were stained with conc. H_2SO_4 (ref. 5) and the saponins and sapogenins were identified, comparing their mobilities to appropriate markers.

The interactability of the following proteins and polypeptides was examined: polylysine, polyphenylalanine, polyalanine (Miles Yeda, Rehovot, Israel), trypsin- and chymotrypsin-inhibitor AA from soybeans[18] and proteins prepared from erythrocyte ghosts.

Interaction of medicagenic acid 3-β-O-triglucoside, and digitonin with cholesterol

10 mg of crystalline cholesterol (Sigma Co.) and 10 mg of either medicagenic acid 3-β-*O*-triglucoside or digitonin in 10 ml water were allowed to interact as described by Gestetner *et al.*[7]. The unbound cholesterol was extracted with successive portions of 25 ml of benzene until no cholesterol could be identified by thin-layer chromatography of the extract. The bound cholesterol was primarily extracted with successive portions of 25 ml 96% ethanol and finally with several portions of 25 ml of boiling pyridine. The cholesterol content of each extract was determined by gas–liquid chromatography as described above.

Assay of enzyme activities in erythrocyte ghosts

The ghosts were suspended in 0.15 M Tris buffer (pH 7.4) and brought to a concentration of $5 \cdot 10^9$ ghosts/ml. The suspension was used as enzyme solution. The following enzyme activities were determined: $(Na^+–K^+)$-ATPase (EC 3.6.1.3.) according to Hamaguchi and Cleve[19]; acetylcholinesterase (EC 3.1.1.7) according to Miller[20]; aldolase (EC 4.1.2.13), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) and lactate dehydrogenase (E.C. 1.1.1.27) according to Wu and Racker[21].

RESULTS AND DISCUSSION

Preliminary experiments showed that when the hemolytic activities of the saponin preparations were assayed in the presence of various amounts of plasma, the hemolysis by alfalfa root saponin and alfalfa top saponin was fully prevented, whereas that by soybean saponin was partially retained. This difference may stem from preferential affinity and specificity of the different saponins for plasma constituents. Attempts have therefore been made to examine the hemolytic activities of the different saponin preparations in the presence of various plasma constituents such as albumin, lecithin and cholesterol, which have been shown already to counteract the hemolytic activity of commercial saponin[22]. The results in Fig. 1 show that the hemolytic activity of alfalfa root saponin extract is inhibited to a very small extent and only by high concentrations of serum albumin, whereas the

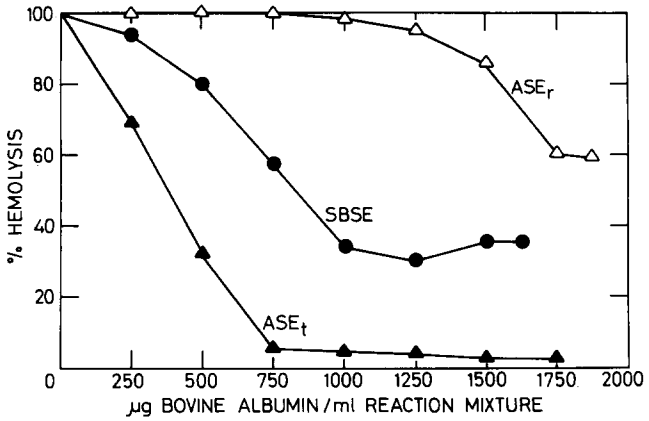

Fig. 1. Effect of different amounts of bovine serum albumin on the hemolytic activity o : △—△ alfalfa saponin extract from roots (ASEr); ▲—▲, alfalfa saponin extract from tops (ASEt); ●—●, soybean saponin extract (SBSE).

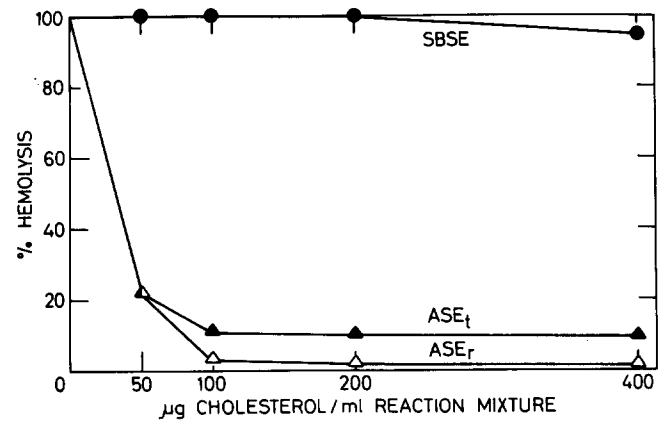

Fig. 2. Effect of different amounts of cholesterol on the hemolytic activity of :△—△, alfalfa saponin extract from roots (ASEr); ▲—▲, alfalfa saponin extract from tops (ASEt); ●—●, soybean saponin extract (SBSE).

activities of alfalfa top saponin and soybean saponin extracts are more susceptible to the presence of albumin in the reaction mixture. No quantitative estimation could be carried out in lecithin-containing reaction mixtures because of turbidity; it was however obvious that the hemolytic activities of alfalfa top saponin and soybean saponin extracts, but not of alfalfa root saponin extract, were fully prevented. On the other hand, addition of cholesterol to the reaction mixture (Fig. 2) resulted in full inhibition of the hemolytic activities of alfalfa top and root saponin extracts, while the activity of soybean saponin extract was unaffected even when much higher concentrations of cholesterol were used.

The results of the experiments with the various plasma constituents lead to the assumption that similar interactions of saponins might occur with erythrocyte membrane constituents. For this purpose an alfalfa saponin, medicagenic acid

TABLE I

PERCENTAGE OF FATTY ACIDS, PHOSPHOLIPIDS AND CHOLESTEROL EXTRACTED WITH VARIOUS SOLVENTS FROM ERYTHROCYTE GHOSTS OBTAINED BY GRADUAL HEMOLYSIS WITH EITHER NaCl, MEDICAGENIC ACID 3β-*O*-TRIGLUCOSIDE OR DIGITONIN

Ghosts extracted with	*Gradual hemolysis obtained with*	*Lipid extracted as % of control*								
		NaCl (Control)			*Medicagenic acid 3β-O-triglucoside*			*Digitonin*		
		Fatty acids*	*Phospho-lipids*	*Chole-sterol*	*Fatty* acids*	*Phospho-lipids*	*Chole-sterol*	*Fatty* acids*	*Phospho-lipids*	*Chole-sterol*
Benzene		100	100	100	98.0	60.3	88.5	99.0	43.1	83.5
Ethanol 96%		None	None	None	None	34.5	2.5	None	47.4	9.5
Boiling pyridine		None	None	None	None	None	4.0	None	None	5.0

* Originally either free or bound.

TABLE II

PERCENTAGE OF CHOLESTEROL CONSECUTIVELY EXTRACTED WITH VARIOUS SOLVENTS FROM REACTION MIXTURE CONTAINING CHOLESTEROL *PLUS* EITHER MEDICAGENIC ACID 3β-*O*-TRIGLUCOSIDE OR DIGITONIN

Cholesterol extracted with	*Aqueous suspension of cholesterol with*	*Cholesterol extracted as % of control*		
		Control	*Medicagenic acid 3β-O-triglucoside*	*Digitonin*
Benzene		100	88.0	79.0
Ethanol, 96%		None	2.0	3.0
Boiling pyridine		None	2.0	11.0

3-β-*O*-triglucoside, was chosen. The stability of its interaction products was compared to those of another saponin, digitonin, which is known to form unstable complexes with cholesterol which are dissociable in ethanol, and stable ones — which can only be dissociated by boiling pyridine[23].

The interactability of medicagenic acid 3-β-*O*-triglucoside and digitonin with the various lipids present in the erythrocyte membrane is shown by the results presented in Table I. The fatty acid, either free or bound as triglycerides, are fully extracted with benzene. Since fatty acids could not be found in the subsequent ethanolic and pyridine extract, when hemolysis was effected with either of the saponins (Table I), it has been concluded that neither the alfalfa saponin nor digitonin can interact with the fatty acids of the erythrocyte membrane. On the other hand, 30–50% of the membranal phospholipids do interact with the saponins (Table I), but the products of this interaction are dissociable in ethanol. Although no full recovery of the phospholipids was obtained after extraction with ethanol, subsequent extraction with boiling pyridine did not liberate any more phospholipids.

Examination of the interaction products of saponins with membranal cholesterol showed (Table I) that although 83–88% of the cholesterol could be removed with benzene, the rest of the cholesterol had formed both an "unstable" (dissociable in ethanol) and a "stable" (dissociable in hot pyridine) addition product with either medicagenic acid 3-β-*O*-triglucoside or with digitonin. Similar results were obtained *in vitro*, by boiling each of the saponins with commercial cholesterol (Table II).

TABLE III

EFFECT OF SAPONIN-INDUCED HEMOLYSIS ON ENZYMIC ACTIVITIES OF ERYTHROCYTE GHOSTS

Enzymic activities of ghosts prepared by gradual hemolysis served as control.

Enzyme assayed	*Activity as % of control*
$(Na^+ - K^+)$-ATPase	80
Acetylcholinesterase	70
Aldolase	100
Lactate dehydrogenase	100
Glyceraldehyde-3-phosphate dehydrogenase	100

The interaction of alfalfa saponins with membranal proteins and with other proteins and polypeptides was investigated as well. When alfalfa root saponin extract was mixed with either of the proteins or polypeptides examined, only part of the saponins could be recovered by washing the celite-cake with water. Whereas in the absence of proteins or polypeptides, the root saponin extract was fully removed by washing the celite-cake with water. It has therefore been concluded that membranal and other proteins and polypeptides of different charge and nature are able to form water-insoluble interaction products with alfalfa saponins. The thin-layer

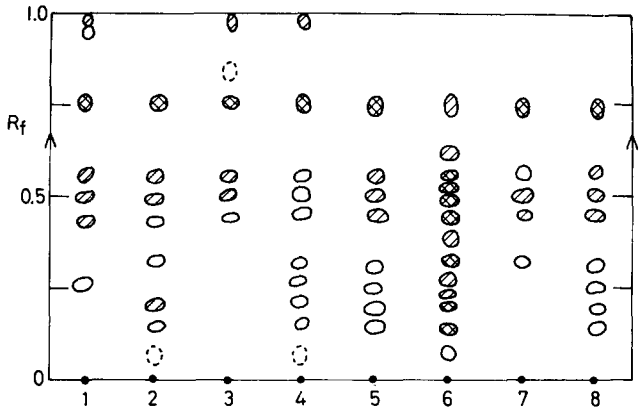

Fig. 3. Thin-layer chormatographic separation of saponins released from interaction products between alfalfa root saponins and polypeptides, proteins and cholesterol. 2 mg of the saponins were dissolved in 0.5 ml of 96% ethanol and 25-μl aliquots were spotted on the chromatoplate. (1) polylysine; (2) polyphenylalanine; (3) trypsin and chymotrypsin inhibitor, amino acids, from soybeans; (4) polyanaline; (5) cholesterol; (6) alfalfa saponin extract from roots; (7) "spectrin": protein fraction from erythrocyte ghosts; (8) pellet III: protein fraction from erythrocyte ghosts. Intensity of colors is marked as follows: dottled circle > circle > diagonal hatching > cross hatching.

chromatographic pattern of the saponins (Fig. 3) released from these addition products is in most cases similar to that of the dissociated saponin-cholesterol addition product; all these saponins contain only medicagenic acid in their aglycone moiety. The material with R_F of 0.75 is medicagenic acid 3-β-*O*-triglucoside, showing that this alfalfa saponin is also involved in the interaction with membranal protein. The finding that only medicagenic acid containing saponins have interacted with the various proteins indicates a preferential affinity of these saponins for proteins, but does not exclude the possibility of interaction of other saponin preparations, *i.e.* soybean saponin extract, which do not contain medicagenic acid, with proteins (Fig. 1)

In order to demonstrate the interaction of saponins with membranal protein, the enzymatic activities of a suspension of erythrocyte ghosts obtained by hemolysis with medicagenic acid 3-β-*O*-triglucoside were examined. As shown in Table III only a partial inhibitory activity of the saponin was noted. The interaction of alfalfa saponins with membranal proteins is probably a non-specific surface–surface interaction, enhanced by the presence of medicagenic acid in the saponin. It is assumed that the resulting conformational change affects only slightly the enzymic activity but may enhance the damage to the structure of the membrane. Although the interaction of alfalfa saponins with cholesterol is much more specific than the interactions which occur with proteins or phospholipids, the hemolysis should not be attributed solely to the interaction of saponin with membranal cholesterol, but rather to a concerted attack on the various membrane constituents. It is assumed that the interactions of the saponin with the different membrane components breaks up the association of cholesterol with phospholipids, which is thought to be responsible for the "strength" of the lipid phase[24,25] and consequently hemolysis occurs. Thus it seems feasible that interaction of only a small portion of membranal

cholesterol (about 6.5%) with the saponin is sufficient to cause hemolysis, contrary to lysolecithins, which are thought to lyse red blood cells by causing massive losses of cholesterol[26].

The course of events occurring during hemolysis caused by medicagenic acid 3-β-*O*-triglucoside applies probably to other medicagenic acid saponins and also to other saponins, which display similar affinities for membranal cholesterol. But it is certainly not valid for soybean saponin extract, which is unable to interact with cholesterol[1].

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