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ON THE MECHANISM OF SAPONIN HEMOLYSIS—I

HYDROLYSIS OF THE GLYCOSIDIC BOND

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Abstract—The mechanism of saponin induced hemolysis was investigated by extracting the active hemolysing factor from ghost cells of saponin hemolysed blood. The fact that only the corresponding aglycones could be extracted, shows that hydrolysis of the glycosidic bond precedes hemolysis. It is suggested that lack of hemolytic activity in a saponin is due either to its non adsorbability to the red cell or to the lack of a proper membrane glycosidase necessary for hydrolysing the glycosidic bond.

THE HEMOLYTIC activity of plant saponins has been known for a long time. However, only after it had been proved that there exists no causal relationship between this characteristic and their capability to reduce surface activity[1–3] research on the factors endowing the saponins with hemolytic capability was started.

Some progress was achieved when it was shown that the hemolytic effect must be ascribed to the aglyconic part of the molecule, namely, the sapogenin.[3] This conclusion was drawn from the facts that all sapogenins by themselves are strongly hemolytic, and that minor modifications in the composition of the aglyconic part of the saponin may have enormous effects on the hemolytic potencies of both saponins and their corresponding sapogenins. Thus, elimination of the ester functions in aescin[4,5] and styrax saponin A[3]—both very potent hemolysins—yield completely inactive glycosides, and the corresponding active estersapogenins lose all their activity on basic hydrolysis.

It was further suggested that a common mechanism must underlie the hemolytic activity of saponins and sapogenins.[6] This was inferred from the observation that both saponins and sapogenins are rapidly and irreversibly absorbed by the red cell membrane, long before hemolysis commences.

Testing the hemolytic activity of a great variety of sapogenins and sapogenin derivatives, made it possible to deduce an accurate structure activity relationship for these compounds.[7–9] However, in spite of the great similarity between saponin and sapogenin induced hemolysis, no such relationship could be found for the saponins.

Although all hemolytically active saponins yield active sapogenins, no quantitative correlation could be found between hemolytic potency and the presence of the sugar moiety in the molecule.[3,10] In some cases it enhances the hemolytic capability of the sapogenin, while in others it inhibits or even annuls it. The highly active β-amyrin is converted to an inactive β-glucoside,[10] but in the case of digitonin the glycosidic bond enhances hemolysis a hundredfold.[3] Moreover, different glycosides of identical sapogenins may differ markedly in their hemolytic potencies. For instance α-hederin

is three times more active than hederacosid A.[10] It must therefore be assumed that the nature of the glycosidic part has some influence on the extent of hemolysis. Schlosser and Wulff,[10] having tested a great variety of saponins, suggest that relatively short and compact sugar chains with high polarity intensify hemolysis, while sugar branching has no effect on this property.

A plausible explanation for the diversifying effects of the glycosidic part must account for the fact that sapogenin induced hemolysis is always adversely affected by additional polar groups.[5,11] Therefore the problem arises how saponins bearing such strong polar functions as the glycosidic parts can be hemolytic at all.

It was the aim of this investigation to shed some light on the role of the glycosidic bond in saponin induced hemolysis. The possibility was considered that in their original form the highly polar saponins are non hemolytic and attain this property only after conversion to their corresponding aglycones by some hydrolytic process occuring on the erythrocyte membrane.

To test this hypotheses, membranes obtained from hemolysed blood were extracted, and the adsorbed hemolysing factor identified by chromatography. A mechanism for saponin induced hemolysis based on the results obtained in these experiments is discussed.

EXPERIMENTAL

Materials

Digitonin, solanin, solanidin, tomatin, tomatidin, ammoniated glycyrrhizin, glycyrrhetinic acid and smilagenin used in these investigations are commercial products. Digitogenin was obtained by acid hydrolysis of digitonin. Oleanolic acid was a gift of Dr. J. Kashman, Dept. Org. Chemistry, University of Tel-Aviv. Styrax saponin A was obtained from styrax officinalis L., as described.[12] Styrax saponin B was obtained by basic hydrolysis of styrax saponin A. Styrax sapogenin A and styrax sapogenin B were obtained by acid hydrolysis of the corresponding saponins. The isotonic buffer solution used had the following composition: $Na_2HPO_4\ 2H_2O$ 3·95 g; KH_2PO_4 0·76 g; NaCl 7·2 g aqua dist. ad. 1000 ml.

Methods

Acetylations were performed as usual (pyridin–acetic anhydride). The acetates obtained were purified by crystallization. Methylation was done with diazomethane as usual.

Hemolysis tests

Blood. Citrated rat blood was used. The erythrocytes were separated by centrifugation and washed with saline until the supernatant was colourless. The erythrocytes were then diluted with buffer to give a suspension of the desired concentration.

Solutions of saponins: All saponins were dissolved in buffer solution.

Solutions of sapogenins. The sapogenins were dissolved in dimethyl sulfoxide (DMSO). (Care was taken that no precipitation occurred on dilution with water.) Before use the DMSO solutions were diluted with distilled water in the proportion of DMSO solution: water 5 : 1. This avoids spontaneous hemolysis.

Determination of the hemolytic activity. The test solutions consisted of 1·4 ml of erythrocyte suspension, varying volumes of hemolysin solution and buffer (in case

of saponins), or DMSO water mixture 5:1 (in case of sapogenins) making up a total volume of 2 ml. The components were added in the following order: first the erythrocyte suspension, then the buffer or the DMSO water mixture and last the hemolysing agent. The mixtures were incubated for 2 hr at 37°, then centrifuged at 1500 rev/min for 10 min and the optical density of the supernatant determined at 540 nm. The percentage of hemolysis was determined by comparison with a sample in which 100% hemolysis was effected by treatment with digitonin.

Isolation of ghost cells. Ghost cells from hemolysed blood were obtained by centrifugation in a Beckman model L2–50 ultracentrifuge rotor R–30, 13,000 rev/min (20,000 g) at 4° for 20 min. The precipitated ghosts were twice washed with aqueous NaCl solution (0·05 M).

Ghost cells from saponin hemolysed blood: The mixtures described in Table 1 were incubated for 2 hr at 37°. The saponin concentrations were made up so that about

TABLE 1. INCUBATION MIXTURES FOR OBTAINING GHOST CELLS FROM SAPONIN HEMOLYSED BLOOD

Name of saponin	Blood suspension		Saponin solution		Final molar conen of saponin	Hemolysis obtained (%)
	Volume (ml)	Conen (%)	Volume (ml)	Conen (μg/ml)		
Styrax saponin-A	100	2	100	0·8	3×10^{-7}	75
Styrax saponin-B	100	2	100	100	4×10^{-5}	0
Digitonin	100	2	100	12	5×10^{-6}	78
Tomatin	100	2	100	20	1×10^{-5}	65
Solanin	2	100*	200	100	1×10^{-4}	60
Glycyrrhizin	1†	100*	200	5000	3×10^{-3}	30

* Undiluted blood was used to achieve maximal saponin concentration.
† The final blood concentration had to be reduced to 1 per cent to get an observable extent of hemolysis.

70% hemolysis was obtained after 2 hr incubation. With glycyrrhizin only 30% hemolysis could be obtained at maximal saponin concentration. After 2 hr incubation the non-hemolysed erythrocytes were separated by centrifugation at 1500 rev/min for 10 min. Ghost cells were obtained from the hemolytic supernatants as described.

Extractions of ghost cells

(a) *Extraction of aglycones.* The ghosts obtained by ultracentrifugation were collected and extracted with hot chloroform (reflux 20 min). The chloroform was decanted and extraction repeated twice. The combined extracts were concentrated to a small volume and analysed by t.l.c.

(b) *Extraction of glycosides*: The residual ghost cells (from the chloroform extraction) were thrice extracted with hot ethanol 70 per cent (reflux 20 min) and the combined extracts concentrated for t.l.c. analysis.

Thin layer chromatography was done on silica gel G coated plates. The following solvent systems were used: (a) chloroform 65, methanol 35, water 5; (b) chloroform 65, methanol 35, water 10; (c) butanol 60, acetic acid 30, water 10; (d) chloroform 90, methanol 10; (e) chloroform 90, methanol 5; (f) chloroform 90, methanol 0·1; (g) petroleum spirit (80–100°) 60, ethyl acetate 40.

Detection of the spots was done by spraying with chlorosulfonic acid and charring at 120°.

Thin-layer chromatographic analysis of saponins adsorbed on ghosts. Three spots were applied to the plate: (1) the concentrated alcoholic water extract of the ghost cells from erythrocytes after contact with saponin; (2) the particular saponin tested; (3) the concentrated alcoholic water extract of ghost cells from normal erythrocytes hemolysed by distilled water. The plates were developed as follows: styrax saponins A and B and tomatin, solvent system (a); digitonin and solanin solvent system (b); glycyrrhizin solvent system (c).

Thin-layer chromatographic analysis of sapogenins adsorbed on ghosts. Three spots were applied to the plate: (1) the concentrated chloroform extract of ghosts from saponin hemolysed erythrocytes; (2) the sapogenin corresponding to the saponin used for hemolysis; (3) the concentrated chloroform extract of ghost cells from normal blood. The plates were developed in the following solvent systems: styrax sapogenin A, styrax sapogenin B and digitogeninsystem (d); tomatidin and solanidin system (e); glycyrrethinic acid system (g).

TABLE 2. INCUBATION MIXTURES OF BLOOD AND SAPOGENINS FOR AGLYCONE EXTRACTIONS FROM HEMOLYSED BLOOD

Name of sapogenin	Volume of blood (2% suspension) (ml)	Sapogenin dissolved in DMSO water 5:1		Buffer (ml)	Final concn of sapogenin	Hemolysis obtained (%)
		Volume (ml)	Concn (μg/ml)			
Styrax sapogenin-A	50	6	10	44	7×10^{-5}	68
Smilagenin acetate	50	18	25	32	1×10^{-5}	70
Oleanolic acid acetate	50	8	100	42	$1{\cdot}6 \times 10^{-5}$	65
Oleanolic acid methyl ester	50	6	50	44	6×10^{-6}	60

Aglycone extractions from sapogenin hemolysed blood. The mixtures described in Table 2 were incubated for 2 hr at 37° and centrifuged at 1500 rev/min. The supernatant obtained was extracted three times with hot chloroform (reflux 20 min). The combined extracts were washed with water, dried and concentrated to a small volume for t.l.c. analysis.

A control extract of normal water hemolysed erythrocytes, was run on t.l.c. plate, together with the sapogenin-hemolysed blood extract and with the sapogenin used for the experiment. The solvent systems used were: (d)—for styrax sapogenin-A; (f)—for smilagenin acetate; (g)—for oleanolic acid acetate and methyl oleanate.

Determination of the extent of saponin hemolysis as a function of incubation time. Erythrocyte suspension—2% (150 ml) and saponin solution were incubated at 37°. Fifty ml samples were withdrawn at various periods, centrifuged in a cooled centrifuge 4° (3000 rev/min, 5 min). The optical density of the supernatant was determined at 540 nm and the percentage of hemolysis determined. Complete hemolysis was achieved by adding a few crystals of digitonin. The following saponin solutions were used: digitonin (150 ml) 12 μg/ml; styrax saponin-A (150 ml) 0·8 μg/ml; glycyrrhizin (450 ml) 5 mg/ml; tomatin (150 ml) 20 μg/ml.

Identification of adsorbed hemolysin after various incubation periods. Ghost cells were collected from the supernatants, and the remaining whole erythrocytes from the preceding experiment. Extractions and t.l.c. analysis were performed as described.

RESULTS AND DISCUSSION

Three types of saponins were chosen for the present investigations: spirostanols, triterpenes and alkaloids. The hemolytic activities of the glycosides and of their corresponding aglycones are summarized in Table 3.

TABLE 3. THE HEMOLYTIC POTENCY EXPRESSED AS H_{50} (THE CONCENTRATION GIVING 50% HEMOLYSIS) OF VARIOUS SAPONINS AND OF THEIR CORRESPONDING SAPOGENINS. HEMOLYSIS WAS TESTED ON 2% RAT BLOOD SUSPENSION, INCUBATED AT 37° FOR 2 hr

Name of compound	H_{50}
Styrax saponin-A	$2{\cdot}7 \times 10^{-7}$
Styrax sapogenin-A	$2{\cdot}5 \times 10^{-7}$
Styrax saponin-B	$>4 \times 10^{-5}$
Glycyrrhizin	$>3 \times 10^{-3}$
Glycyrrhetinic acid	2×10^{-4}
Digitonin	$3{\cdot}3 \times 10^{-6}$
Digitogenin	$2{\cdot}7 \times 10^{-4}$
Solanin	$1{\cdot}0 \times 10^{-4}$
Solanidin	$7{\cdot}0 \times 10^{-5}$
Tomatin	8×10^{-6}
Tomatidin	$5{\cdot}3 \times 10^{-5}$

Only in one case, that of styrax saponin-A, the presence of the sugar part does not effect hemolysis, and the activity of the saponin equals that of the aglycone. In all other saponins it has either an enhancing or an inhibiting effect. Thus digitonin and tomatin are more potent than their corresponding aglycones, while glycyrrhetinic acid and solanin are less active.

We started by trying to clarify whether the constitution of the saponin undergoes any changes after being adsorbed to the membrane. Three possibilities were considered—the glycosidic bond undergoes hydrolysis; the aglyconic part undergoes some chemical change; the saponin remains unchanged.

Ghost cells were collected from the hemolysed portion of blood which had been incubated for 2 hr with a saponin concentration giving 70% hemolysis. Partially hemolysed blood (70 per cent) was chosen to avoid an excess of saponin which might be adsorbed to the membranes by some secondary process. We extracted the ghost cells with 70% ethanol to remove glycosides and with chloroform to remove aglycones.

Table 4 summarizes the results obtained from testing the extracts by t.l.c. In all cases no saponin whatsoever remained adsorbed to the membrane, and only the corresponding aglycones could be detected.

Since the ghosts were collected only from the hemolysed part of the blood (approx 70 per cent), it seemed interesting to test whether sapogenins could also be extracted from the residual 30 per cent namely the non-hemolysed erythrocytes. The unhemolysed cells were disrupted by distilled water and the ghosts collected and extracted with chloroform and with ethanolic water. The results which are summarized in columns 4 and 5 of Table 4 show that sapogenins are present on the non-hemolysed

Table 4. Saponins and sapogenins extracted from ghost cells obtained from hemolysed and non hemolysed erythrocytes incubated with saponin (2 hr, 37°) at concentrations giving approx 70% hemolysis

Saponin examined	Ghosts from hemolysed cells		Ghosts from non-hemolysed cells	
	Saponin extracted with 70% ethanol	Sapogenin extracted with chloroform	Saponin extracted with 70% ethanol	Sapogenin extracted with chloroform
Digitonin		+	+	−
Tomatin	−	+	+	−
Solanin	−	+	+	−
Styrax saponin-A	−	+	−	+
Styrax saponin-B	−	−	−	−
Glycyrrhizin*	−	+	−	+

* Only 30% hemolysis could be obtained.

erythrocytes but that they are still accompanied by the intact saponins. The fact that in the hemolysed part of the blood only aglycones could be found on the membranes, gives rise to the assumption that hydrolysis of the glycosidic bond is a prerequisite for obtaining hemolysis. To test this assumption we incubated erythrocytes with saponins for various periods, thus obtaining a variety of hemolytic extents. The ghosts were collected from all samples, extracted and tested for the presence of both saponins and sapogenins. The results, which are summarized in Table 5, clearly show that even after very short incubation periods *those* membranes which were collected from the hemolysed part of the blood, had only sapogenins adsorbed to their membranes, while to the non-hemolysed membranes both saponins and sapogenins were adsorbed.

The fact that the non hemolysed erythrocytes have saponins adsorbed to their membranes, and in the case of glycyrrhizin, if the incubation periods were short enough, only saponins, shows that the first phase in the hemolytic process is that of the adsorption of the saponins to the erythrocytes. The hydrolysis which precedes the rupture of the the membrane, is probably the second phase.

Since the hydrolytic process occurs after adsorption to the membrane, we must assume the presence of a proper membrane glycosidase catalysing this reaction. Glycosidases are known to be of high specificity both to the sugar component and to the nature of the glycosidic bond, but the constitution of the aglyconic part has only influence on the rate of hydrolysis.[13]

In a previous communication we demonstrated that the process of the saponin adsorption is irreversible and completed within very short incubation periods, long before hemolysis commences.[6] This phase can therefore have no influence whatsoever on the rate of hemolysis. Consequently, if the proposed conception of the hemolytic mechanism is valid, and if the last step – the rupture of the cell membrane – is not rate determining, the rate of hemolysis should vary markedly from one saponin to the other. Figure 1 shows the time dependence of hemolysis for four different saponins, i.e. digitonin, tomatin, styrax saponin-A and glycyrrhizin. The great variations in the hemolytic rates are obvious, in digitonin and tomatin – both possessing a β-galactosidic bond[14,15] – the rate of hemolysis is too fast to be measured. In glycyrrhizin however the hemolytic process is so slow that it extends over a period of at least 12 hr, a period after which spontaneous hemolysis is too pronounced for

TABLE 5. SAPONINS AND SAPOGENINS EXTRACTED FROM GHOST CELLS OBTAINED FROM HEMOLYSED AND NON HEMOLYSED ERYTHROCYTES AFTER INCUBATION WITH SAPONINS FOR VARIOUS PERIODS

Time of incubation (min)	Styrax saponin-A (3×10^{-7} M)					Digitonin (3×10^{-6} M)					Glycyrrhizin (2×10^{-3} M)				
	Hemolysis (%)	Ghosts from hemolysed blood		Ghosts from non hemolysed blood		Hemolysis (%)	Ghosts from hemolysed blood		Ghosts from non hemolysed blood		Hemolysis (%)	Ghosts from hemolysed blood		Ghosts from non hemolysed blood	
		Saponin	Sapogenin	Saponin	Sapogenin		Saponin	Sapogenin	Saponin	Sapogenin		Saponin	Sapogenin	Saponin	Sapogenin
5	8	−	+	+	+	48		+	+	+					
10	26	−	+	+	+	52	−	+	+	+	0			+	
20	46	−	+	+	+	52	−	+	+	+	0			+	−
60	60	−	+	+	+						8	−	+	+	−
120	61	−	+	+	+	52	−	+	+	+	25	−	+	+	−
180											30	−	+	+	−

the experiment to be continued. Since glycyrrhizin is readily absorbed by the erythrocytes (Table 5), the reason for the low hemolytic activity of this saponin—especially when compared with that of the corresponding sapogenin (Table 3)—can be due either to a low activity, or to a low concentration of the proper glycosidase (β-glucuronidase).[16]

Our experiments also furnish an explanation for the the total lack of hemolytic potency in Styrax saponin B (Table 3). This saponin is for some as yet unexplicable reason not adsorbed to the erythrocytes (Table 4), and therefore the first stage obligatory for the hemolytic process does not occur.

It was mentioned above that some alterations in the aglyconic moiety of the saponins might occur during the hemolytic process. The most probable reactions to be considered are oxidations of hydroxyl groups or hydrolysis of ester bonds, yet t.l.c. examination of the membrane extracts always showed the presence of the corresponding sapogenin only. This holds true also for styrax saponin-A which is esterified in the aglyconic part of the molecule. This observation was strengthened by examining various sapogenins, including sapogenin esters, after hemolysis. Since

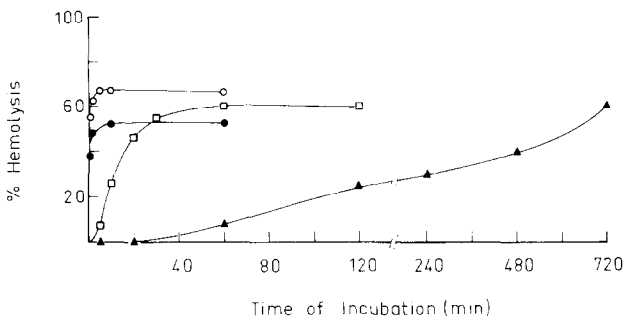

FIG. 1. Time dependence of saponin induced hemolysis. (●) tomatin (1×10^{-5}M); (□) styrax saponin A (3×10^{-7}M); (○) digitonin (3×10^{-6}M); (▲) glycyrrhizin (2×10^{-3}M).

in these tests dimethyl sulfoxide was used for achieving dissolution of the hemolysins, no ghosts were collected, but chloroform extractions were done on the hemolysed part of the blood. The sapogenins tested were: styrax sapogenin-A, smilagenin, smilagenin acetate, oleanolic acid acetate and oleanolic acid methyl ester. In all these compounds—sapogenins or sapogenin esters–only the original hemolysin used could be extracted, showing that no chemical change had occurred during hemolysis.

The results of our present investigations confirm our previous deductions[3] that the aglyconic part of the saponin molecule is responsible for its hemolytic activity. Two conditions must be fulfilled in order to bestow on a saponin hemolysing activity. It must be adsorbed to the erythrocyte membrane and the glycosidic bond must be hydrolysed. This is based on the assumption that all aglycones produced on the membrane are active in accordance with our observation that all steroids and triterpenes are hemolytic provided they can be dissolved.[6,7] It seems therefore reasonable to assume that the cause for the inactivity of some saponins must be looked for either in their non adsorbability to the erythrocyte membrane, or the lack of a proper membrane enzyme capable of hydrolysing the glycosidic bond.

Furthermore, we can now explain the fact that saponins, although they are characterized by the presence of the highly polar glycosidic function which generally inhibits hemolysis, are hemolytically active. As a matter of fact this function is no longer existent in the active hemolysin since hydrolysis precedes hemolysis. An explanation is also provided for the fact that some sapogenins are more potent than the saponins from which they are derived, as was exemplified by the case of glycyrrhizin. The results do not however furnish a plausible explanation for the observation that in certain cases the saponins are more active than their corresponding aglycones. Possibly two parallel hemolytic mechanisms operate in these cases, the one described above and a second by way of reducing the surface activity which has formerly been considered as the main factor causing hemolysis.

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