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ON THE MECHANISM OF SAPONIN HEMOLYSIS—II

INHIBITION OF HEMOLYSIS BY ALDONOLACTONES

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Abstract—Saponin-induced hemolysis is inhibited by aldonolactones, which are specific glycosidase inhibitors. These results confirm our previous suggestion that hydrolysis of the glycosidic bonds of saponins is essential to their hemolytic activity. The aldonolactones tested exhibited little inhibitory specificity towards various saponins, suggesting the presence of non-specific glycosidases on the erythrocyte membrane.

In a recent publication [1] we pointed out that in all hemolytically active saponins the glycosidic bond is hydrolysed and the sapogenin is obtained before the hemolytic process sets on. This deduction was based on the finding that although saponins are rapidly absorbed by red blood cells, no glycosides could ever be extracted from ghost cells obtained from saponin-hemolysed erythrocytes. In all these cases only the corresponding aglycones were detected.

The hydrolytic processes occurring on the membrane must be enzyme-catalysed and it was suggested [2] that the sugars are split off successively by appropriate membrane glycosidases. We have found the presence of glycosidase activity associated with erythrocyte membranes [3]. Most glycosidases are known to be highly specific both to the sugar component and to the nature of the glycosidic bond [4]. However less specific glycosidases capable of hydrolysing several glycosidic bonds were described recently [5, 6].

Among the various specific glycosidase inhibitors known are the aldonolactones, which were studied extensively and found to inhibit effectively the hydrolysis of glycosides with corresponding structure and bond configuration.

It is the aim of our present investigation to determine whether saponin induced hemolysis can be inhibited by aldonolactones, thus testing our previous deduction that hydrolysis of the glycosidic bonds must precede hemolysis.

EXPERIMENTAL

Hemolysins. Digitonin, tomatin, glycyrrhizing ammonium salt, and tomatidine were commercial products. Aescin and α-hederin were gifts of Professor G. Wulff from the Institute of Organic Chemistry, the University of Bonn. Pariphyllin was a gift of Professor T. R. Seshadri from the Department of Chemistry, the University of Delhi. Digitogenin was obtained by acid hydrolysis of digitonin. All saponins were dissolved in buffer solution. The sapogenins were dissolved in dimethylsulfoxide–water (5:1).

Inhibitors. D-Galactono-1,4-lactone, a commercial sample, was crystallized from ethanol to constant m.p., 134–5°; $[\alpha]_D$-76° (H_2O). D-Glucono-1,5-lactone, a commercial sample, was used without purification. The inhibitor solutions of both lactones were freshly prepared before use as described by Levvy *et al.* [7] for "the most inhibitory solution of galactonolactone". The isotonic buffer solution used contained in 1000 ml distilled water: $Na_2HPO_4 \cdot 2H_2O$, 3·95 g; KH_2PO_4, 0·76 g; NaCl, 7·2 g. The pH was adjusted to 7·4.

Substrates. *o*-Nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-glucoside, were commercial samples. The glycosides were dissolved shortly before use in isotonic buffer solution to give a concentration of 1×10^{-1} M.

Blood. Citrated bovine blood was used in all experiments. The erythrocytes were separated by centrifugation and washed with saline until the supernatant was colorless. A 1 per cent suspension of erythrocytes in buffer was used for all experiments, except for those with pariphyllin and glycyrrhizin, where 0·07 and 0·5 per cent suspensions were used, respectively.

The inhibitory tests. Two sets of experiments were run in parallel.

(a) Erythrocyte suspension (2 ml) was incubated with inhibitor solutions of various concentrations (0·1 ml) at 37°. After 1 hr, buffer and then hemolysin solution were added to give a final volume of 4 ml. The mixtures were incubated for another hour at 37° (the standard time of incubation for hemolytic tests [1]), and then centrifuged at 4000 rev/min for 5 min. The supernatant was separated and its optical density determined at 540 nm. To determine the effect of the time of preincubation with inhibitor on the extent of inhibition, saponin was added after various preincubation periods.

(b) Erythrocyte suspension (2 ml) was incubated with buffer (0·1 ml) for 1 hr at 37°. Then buffer and

hemolysin were added and the procedure described under (a) was repeated. Percentage of inhibition was calculated as:

$$\left(1 - \frac{\text{O.D. with inhibitor}}{\text{O.D. without inhibitor}}\right) \times 100$$

Percentage of hemolysis was determined by comparison with a sample for which 100% hemolysis was obtained.

The effect of aldonolactones on tomatidin and digitogenin hemolysis was tested as described for the saponins. In this case however, 1 ml of a 5×10^{-4} M and 2×10^{-3} M solution of tomatidin and digitogenin, respectively, in dimethylsulfoxide–water 5:1 (1 ml) were used. These concentrations of tomatidin gave 65% hemolysis, and of digitogenin 70% hemolysis.

Inhibition of erythrocyte glycosidase. Two sets of experiments were run in parallel.

(a) Erythrocyte suspension (2 ml) was incubated with 0·1 ml of aldonolactone solution (8×10^{-1} M) at 37°. After 1 hr buffer (1·8 ml) and substrate (*p*-nitrophenyl-glucoside or *o*-nitrophenyl-galactoside) solution (0·1 ml) were added. The mixture was incubated at 37° for a further hour, then centrifuged at 4000 rev/min. To 3 ml of the resulting supernatant, 1 M Na_2CO_3 solution (0·5 ml) was added thus bringing the pH to 3. After 1 hr the optical density was read at 420 nm.

(b) Erythrocyte suspension (2 ml) was incubated with buffer (0·1 ml) for 1 hour at 37°; then buffer (1·8 ml) and substrate solution (0·1 ml) were added and the procedure described under (a) repeated. Percentage of inhibition was calculated as described above.

RESULTS AND DISCUSSION

The inhibitory effect of two aldonolactones, D-gluconolactone and D-galactonolactone, on the hemolytic activity of various saponins was tested.

The constitution of the saponins used for these investigations is outlined in Table 1. Three types of glycosides were tested, those having both a glucosidic

Table 1. The constitution of saponins tested

Saponin	Structure	Reference
Digitonin	β-D-Ga (1→2) β-D-Gl; β-D-Gl (1→4) β-D-Ga; β-D-Xy (1→3) β-D-Gl; β-D-Ga (1→3) digitogenin	[8]
Tomatin	β-D-Xy (1→3) β-D-Gl; β-D-Gl (1→2) β-D-Gl; β-D-Gl (1→4) β-D-Ga; β-D-Ga (1→3) tomatidin	[9]
Aescin	β-D-Gl (1→4) β-D-Glr; β-D-Gl (1→2) β-D-Gl; β-D-Glr (1→3) aescigenin	[8]
Pariphyllin	α-L-Rh (1→4) β-D-Gl; α-L-Ar (1→3) β-D-Gl; β-D-Gl (1→3) diosgenin	[10]
α-Hederin	α-L-Rh (1→2) α-L-Ar; α-L-Ar (1→3) hederagenin	[8]
Glycyrrhizin	β-D-Glr (1→2) β-D-Glr; β-D-Glr (1→3) glychyrrhetinic acid	[11]

Symbols: Gl, glucose; Ga, galactose; Xy, xylose; Glr, glucuronic acid; Rh, rhamnose; Ar, arabinose.

Table 2. The inhibitory and augmentory effects of gluconolactone on the hemolytic activity of various saponins

Saponin	Concentration of saponin (M)	% Hemolysis	Percentage inhibition or augmentation of hemolysis: 2×10^{-2} M Lactone % Inhib.	2×10^{-2} M Lactone % Aug.	1×10^{-2} M Lactone % Inhib.	1×10^{-2} M Lactone % Aug.	5×10^{-3} M Lactone % Inhib.	5×10^{-3} M Lactone % Aug.	2×10^{-3} M Lactone % Inhib.	2×10^{-3} M Lactone % Aug.	1×10^{-3} M Lactone % Inhib.	1×10^{-3} M Lactone % Aug.	5×10^{-4} M Lactone % Inhib.
Digitonin	5×10^{-6}	94	5		3		15		16		0		
	$4{\cdot}5 \times 10^{-6}$	76	5		5		26		26		0		
	$3{\cdot}5 \times 10^{-6}$	49	6		7		30		29		0		
	$2{\cdot}7 \times 10^{-6}$	21	5		5		32		27		0		
Tomatin	$1{\cdot}5 \times 10^{-5}$	85	100		88		53		20		12		0
	$1{\cdot}25 \times 10^{-5}$	59	100		88		58		33		24		0
	1×10^{-5}	25	100		76		64		34		20		0
Aescin	$6{\cdot}6 \times 10^{-6}$	95	4		5		4		0				
	$5{\cdot}4 \times 10^{-6}$	86	2		5		6		0				
	$4{\cdot}4 \times 10^{-6}$	72	0		7		3		0				
	$3{\cdot}3 \times 10^{-6}$	51	0		4		11		0				
Pariphyllin*		90			30		30		0				
		45	53		40		32		0				
		28	49		39		40		0				
α-Hederin*		36		100% hemolysis		100% hemolysis		100% hemolysis					
		14											
Glycyrrhizin	3	36		70		45		36		15	0		32
	1·2	23		30		10		10		0	23		40

* Aliquots were taken from a saturated solution.

Table 3. The inhibitory and augmentory effects of galactonolactone on the hemolytic activity of various saponins

Saponin	Concentration of saponin (M)	% Hemolysis	2×10^{-2}M Lactone % Inhib.	2×10^{-2}M Lactone % Aug.	1×10^{-2}M Lactone % Inhib.	1×10^{-2}M Lactone % Aug.	5×10^{-3}M Lactone % Inhib.	5×10^{-3}M Lactone % Aug.	2×10^{-3}M Lactone % Inhib.
Digitonin	$4{\cdot}5 \times 10^{-6}$	75	30		0		0		
	$3{\cdot}3 \times 10^{-6}$	50	22		0		0		
	$2{\cdot}9 \times 10^{-6}$	36	35		0		0		
Tomatin	$1{\cdot}5 \times 10^{-5}$	85	81		63		31		6
	$1{\cdot}25 \times 10^{-5}$	60	78		67		36		4
	$1{\cdot}1 \times 10^{-5}$	30	68		64		57		5
Aescin	$5{\cdot}4 \times 10^{-6}$	86	0		0				
	$3{\cdot}3 \times 10^{-6}$	51	0		0				
Pariphyllin*		90	30		24		20		
		45	20		0		0		
α-Hederin*		36		100% hem.		100% hem.			
		14		100% hem.		100% hem.			
		10		100% hem.		100% hem.			
Glycyrrhizin	3	36		40		15	8		28
	2·4	23		40		0	7		20

* Aliquots were taken from a saturated solution.

and a galactosidic bond (digitonin and tomatin), those possessing a glucosidic but no galactosidic bond (aescin and pariphyllin), and those saponins which have neither a glucosidic nor a galactosidic bond in the molecule (α-hederin and glycyrrhizin). The inhibitory effects of the aldonolactones were tested at various saponin and inhibitor concentrations. The inhibitor concentration could not be raised above 2×10^{-2} M without the aldonolactones causing hemolysis by themselves.

The results, which are summarized in Tables 2 and 3, show unequivocally that aldonolactones do inhibit saponin-induced hemolysis. The extent of inhibition of hemolysis by aldonolactones is dependent on the time of preincubation of erythrocytes with the aldonolactones (Fig. 1); therefore a standard preincubation time of 1 hr was chosen for all inhibitory experiments. Determinations of percentage hemolysis both in the presence and in the absence of inhibitors were reproducible to within 3 per cent hemolysis. Consequently, the results in the case of strongly inhibitable saponins at concentrations normally giving between 30 and 90 per cent hemolysis can be considered quantitatively reliable, whereas the other results should be considered qualitative. In all cases the average of at least three measurements was taken.

Of the two aldonolactones tested, gluconolactone is undoubtedly the more potent. It inhibits the activity of all saponins tested to a much higher extent than does galactonolactone. It was necessary to conduct the experiments over a narrow range of saponin concentrations because of the steep increase in hemolysis. Thus with the slightly different saponin concentrations used, the percentage of aldonolactone inhibition measured did not show large variations. The inhibition is, however, highly dependent on the nature of the saponin and the concentration of the inhibitor. Tomatin is the most inhibitable saponin. Its hemolytic effect can be totally abolished with high concentrations of

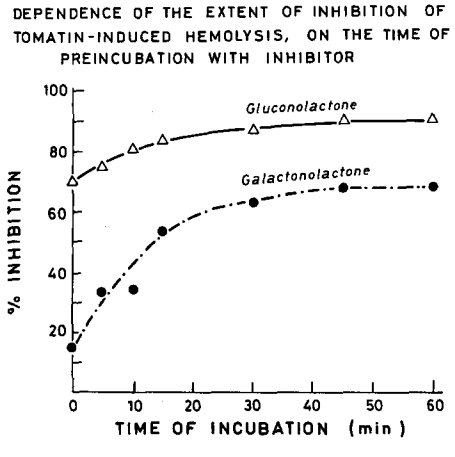

Fig. 1. Tomatin concentration $1{\cdot}5 \times 10^{-5}$ M. Inhibitor concentration 1×10^{-2} M. For details see Experimental.

gluconolactone and to an extent of 80 per cent with galactonoactone. Tomatin is also the only saponin in which inhibition decreases markedly with the reduction of the aldonolactone concentration. For all other saponins, optimal inhibition is obtained either over a wide range of inhibitor concentration (between 2×10^{-2} M and a lower concentration) or at a definite peak below 2×10^{-2} M. The existence of such an optimal concentration can be explained by assuming that the aldonolactones, although by themselves being non-hemolytic, may have more or less pronounced accelerator effects on saponin-induced hemolysis [12]. That such augmentation may occur with glucono- and galactonolactones is exemplified by the cases of glycyrrhizin and α-hederin. With the latter 100% hemolysis is achieved with saponin concentrations giving 10% hemolysis under normal conditions.

Since it is a well established fact that saponin-induced hemolysis may be non-specifically inhibited by various types of compounds [11], it was necessary to confirm that the observed inhibition by the aldonolactones is specific for the glycosidase enzymes and not an inhibition of the hemolytic process itself. To verify this assumption two sets of experiments were carried out.

Firstly the effect of the aldonolactones on the extent of hydrolysis of glycosides catalyzed by erythrocytes was investigated. Gluconolactone (2×10^{-2} M) inhibited the hydrolysis of *p*-nitrophenyl-glucoside by 90 per cent and galactonolactone (2×10^{-2} M) inhibited the hydrolysis of *o*-nitrophenyl-galactoside by 60 per cent. Moreover the dependence of the inhibition on the time of preincubation with inhibitor was similar to that observed for hemolysis [3].

Secondly it was found that the aldonolactones had no inhibitory effect on the hemolysis induced by the aglycones tomatidine and digitogenin, which correspond to the glycosides tomatin and diginonin. We may therefore conclude that the inhibition of saponin-induced hemolysis by aldonolactones is due to the inhibition of hydrolysis of the glycosidic bond only.

The aldonolactones, although being specific glycosidase inhibitors, reduce the hemolytic activity of saponins without the corresponding glycosidic bond. Both lactones inhibit glycyrrhizin-induced hemolysis (at low inhibitor concentrations for which no augmentation is observed), although glycyrrhizin is a glucoronido-glucoronide. Likewise, pariphyllin is inhibited by galactonolactone, although it possesses no galactosidic bond. These observations may indicate that a single enzyme is responsible for the hydrolysis of various glycosidic bonds, as has been proved for several glycosidases from various other sources [5].

When different saponins are tested, great variations are observed in the inhibitory activities of the aldonolactones, even if the saponins possess appropriate glycosidic bonds. These variations are most striking in the case of gluconolactone action on the hemolytic activity of tomatin, digitonin, pariphyllin and aescin, all of which bear β-glucosidic bonds. Digitonin is the only saponin in which the relatively low activity may be

explained by assuming an augmentation, thus appearing to show a slight inhibition. However, no enhancing effect can explain the almost complete lack of inhibition in aescin-induced hemolysis. It seems probable that the nature of the aglycone or of the other sugar units making up the glycosidic part markedly influence the kinetics of glycosidase hydrolysis. Aldonolactones were shown to be competitive inhibitors [13]. The extent of their inhibition is consequently markedly affected by the K_m values, even when fixed concentrations of both substrate and inhibitor are used [14]. The great differences between the inhibition of tomatin- and aescin-induced hemolysis may thus be accounted for by assuming great variations in the K_m values of the glucosidase for the two substrates. The nature of the erythrocyte glycosidases is now under investigation.

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