BBA Report

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Haemolysis of washed human red cells by the combined action of *Naja naja* phospholipase A_2 and albumin

SAEED GUL and ANTHONY D. SMITH

The Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W1P 5PR (Great Britain)

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SUMMARY

1. Cobra venom phospholipase A_2 was found to hydrolyse phospholipids in washed human erythrocytes without causing significant haemolysis in an isotonic medium.

2. This indicates that phospholipids of the cell membrane are accessible to the enzyme action in isotonic buffer.

3. Addition of 2% albumin to the incubation medium either prior to or subsequent to the enzyme action causes haemolysis without any further increase in phospholipid splitting.

The action of whole cobra venom in haemolysing[1] washed red cells has been attributed to the synergistic action of direct lytic factor with phospholipase A_2 (ref. 2).

It has been reported that purified phospholipase A_2 does not haemolyse washed red cells unless the medium contains an external source of phospholipids[3], or contains sublytic concentrations of detergents which are themselves potentially lytic at higher concentrations[4,5], or is hypotonic[6].

Evidence is presented here that a purified phospholipase A_2 preparation from *Naja naja* venom will haemolyse washed human erythrocytes in the presence of albumin.

To determine haemolytic activity human blood was freshly collected in 3.8% sodium citrate (1 ml/10 ml) and centrifuged at 200 × *g* for 20 min. The supernatant plasma and buffy coat were removed and the cells washed three times with 10 vol. of 0.95% saline (pH 7.3), centrifuging each time at 1000 × *g* for 5 min with a fourth wash with 10 vol. of isotonic buffer, pH 7.3 or pH 5.8 (0.1 M glycylglycine in 0.6% NaCl).

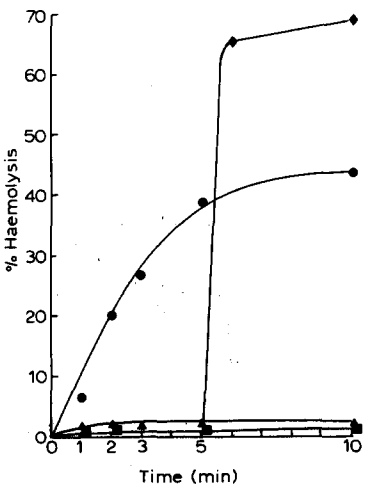
0.1-ml samples of the packed red cells were pipetted into glass stoppered tubes containing 0.8 ml buffer with or without bovine plasma albumin purified by the method of

Chen[7] and incubated for 10 min at 37 °C in a shaking water bath.

The reaction was started by adding 0.1 ml 100 mM $CaCl_2$ containing 20 μg of an enzyme preparation purified by the method of Braganca *et al.*[8] and free of direct lytic factor. Incubation was continued for various time intervals at the end of which 5 ml ice-cold saline (0.95% pH 7.3) containing 0.5% 200 mM EDTA (pH 7.3) were added, and the tube kept on ice until centrifuged. The degree of haemolysis was determined by measuring the absorbance at 540 nm and expressed as percent of total haemoglobin contained in 0.1 ml packed red cells when totally haemolysed with 5.9 ml water.

For estimation of free fatty acids, 4 vol. of chloroform–methanol (2:1, v/v) containing 0.5% 200 mM EDTA were added with rapid mixing. Blanks were included where solvent was added prior to enzyme. $C_{17:0}$ was added as an internal standard, the lower phase was pipetted out and the upper phase was washed once with 3 ml lower phase. After evaporation the residue was redissolved in 2 ml of a solvent system containing chloroform–hexane–diethyl ether (2:1:1, by vol.). Phospholipids were adsorbed on silicic acid and the supernatant taken to a small volume. The fatty acids were methylated with diazomethane and the methyl esters were run on a Perkin Elmer F11 gas chromatograph.

Fig. 1 shows the rate curves for haemolysis with and without 2% albumin and also with EDTA in the presence of albumin. It is evident that while in the absence of albumin phospholipase A_2 does not cause any significant haemolysis (< 2.0%), the addition of albumin brings about a marked degree of haemolysis which is abolished by EDTA, an inhibitor of this enzyme.



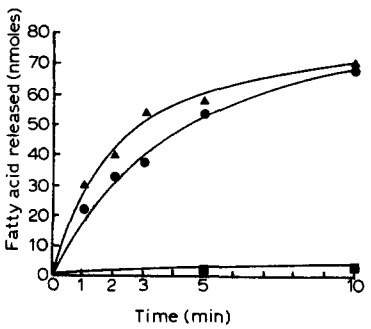


Fig. 1. Action of phospholipase A_2 on washed red cells in the presence and absence of albumin at pH 7.3. ▲—▲, no albumin; ●—●, *plus* 2% albumin; ◆—◆, *plus* 1 ml buffer containing 4% albumin after 5 min; ■—■, with 2% albumin *plus* 10 mM EDTA.

Fig. 2. Rate of release of free fatty acid by phospholipase A_2 in the presence and absence of albumin at pH 7.3. Symbols as in Fig. 1.

Haemolysis also occurs when albumin is added after the enzyme has acted upon the red cells for 5 min.

Fig. 2 shows the rate of release of free fatty acids as a result of the hydrolysis of the membrane phospholipids by this enzyme. No measurable amount of fatty acid is released from the blanks. It is clear that albumin did not increase the degree of hydrolysis (if anything, it somewhat decreased it) and here again EDTA was inhibitory. Addition of albumin after 5 min to the reaction system which had been proceeding without albumin caused no additional hydrolysis of phospholipid. The fatty acids ($C_{18:1}$, $C_{18:2}$ and $C_{20:4}$) which were cleaved were those to be expected from the β specificity of the enzyme, though a small proportion of $C_{16:0}$ was also found to be released. The pattern was similar with or without albumin, indicating that the same species of phospholipids are hydrolysed. Thin-layer chromatography showed that lecithin was preferentially attacked.

These results show that when human washed red cells are acted upon by phospholipase A_2 in an isotonic medium, albumin causes a pronounced degree of haemolysis, but has no effect on phospholipid splitting as judged by the release of fatty acids.

This indicates that albumin does not exert its effect by activating the enzyme and this is supported by the observation that on addition of albumin after the reaction has proceeded for 5 min, it will cause the cells to haemolyse without an additional release of fatty acid and this occurs much more rapidly than when the albumin is added with the enzyme. In the absence of albumin, despite appreciable phospholipid splitting, no significant haemolysis occurs.

The failure of purified phospholipase A_2 to haemolyse washed red cells in isotonic medium has been attributed to the failure of access of the enzyme to the phospholipids in the membrane. It has been proposed that in a hypotonic medium, the membrane configuration changes due to swelling of the cells, and thereby the phospholipids are exposed to the action of the enzyme and the cells are haemolysed[6]. These authors have also proposed that direct lytic factor and other agents exert their action by swelling and stretching the cell membrane. The results observed appear to depend on the source of the phospholipase A_2. In the case of the pancreatic enzyme, no haemolysis or hydrolysis of phospholipid occurs with intact erythrocytes[5]. However, there is evidence both from our results and from others[9] that appreciable amounts of fatty acid are cleaved as a result of the action of cobra phospholipase A_2 on intact erythrocytes in isotonic medium.

Since after washing with isotonic saline the cells become crenated, there is a possibility that in this crenated form some structural rearrangement occurs, so that in spite of membrane damage as evidenced by phospholipid splitting, the membrane is capable of retaining haemoglobin. If this structural abnormality were corrected and the membrane brought to its original shape, it is possible that the cells would haemolyse. The distortion in shape due to washing can be corrected by suspending the cells in isotonic buffer (pH 5.8) or 5% albumin solution[10] (though we have found that 2–4% albumin is sufficient). In order to test the hypothesis that haemoglobin release was connected with these changes in morphology, the reaction was carried out at pH 5.8. It can be seen from Table I that

TABLE I

EFFECT OF ALBUMIN AT pH 7.3 AND 5.8 ON HAEMOLYSIS BY PHOSPHOLIPASE A

pH	*μg enzyme*	*2% albumin*	*10 mM $CaCl_2$*	*Time*	*% haemolysis*
7.3	–	+	+	5 min	0.6
	20	–	+	5 min	1.30
	20	+	+	5 min	45.7
5.8	20	–	+	10 min	1.03
	20	+	+	5 min	49.0

negligible haemolysis was obtained in the absence of albumin but when 2% albumin was incorporated into the medium at this pH, the degree of haemolysis was almost identical with that at pH 7.3. These observations would rather suggest that haemolysis is not due to the return of the membrane to its normal shape in the presence of albumin although it cannot be stated with certainty that pH 5.8 buffer causes the same effect as 2% albumin.

One interesting finding is that in the absence of albumin less than 10% of the fatty acid is found in the supernatant after spinning down the cells, whilst 90% remains bound and recoverable when the sedimented cells are extracted with solvent. The opposite is the case when the medium contains albumin, when 90% of the released fatty acid is recoverable from the supernatant. The rate at which haemolysis occurs when albumin is added after the enzyme has acted for 5 min (with the haemolysis virtually complete after 1 min) seems consistent with a physical effect such as the binding of fatty acid. These observations would suggest that albumin may bind the fatty acids, removing them from the membrane, which can then no longer retain haemoglobin.

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