Journal of Enzyme Inhibition and Medicinal Chemistry, 2002 VOL. 17 (1), pp. 55–59

Inhibition of *Naja Nigricolis* Venom Acidic Phospholipase A_2 Catalysed Hydrolysis of Ghost Red Blood Cells by Columbin

A.J. NOK[a,*], E. BALOGUN[a], J.A. LORI[b] and M.S. ABUBAKAR[c]

[a]*Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria;* [b]*Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria;* [c]*Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria*

(Received 5 December 2001)

The inhibitory effects of a naturally occurring diterpenoid furanolactone, columbin, on partially purified acidic phospholipase A_2 (PLA_2) from *Naja nigricolis* was investigated. Columbin inhibited the *N. nigricolis* PLA_2 in a dose related pattern with an IC_{50} value of 2.5 μM. Double reciprocal plots of initial velocity data of inhibition by columbin revealed a non-competitive pattern. The K_M remained constant at 19 μM, while the V_{max} changed from 54 μmoles/min/mg to 32 μmoles/-min/mg and 20 μmoles/min/mg in the presence of 2 and 10 μM of columbin, respectively. Extrapolated K_i values were 3 and 6.28 μM at 2 and 10 μM inhibitor, respectively. Columbin also inhibited PLA_2 hydrolysis of ghost RBC in a dose-dependent fashion. At least 70% suppression of PLA_2-catalysed haemolysis of RBC was observed in the presence of 2 μM columbin.

Keywords: *Naja nigricolis*; PLA_2; Columbin; Inhibition

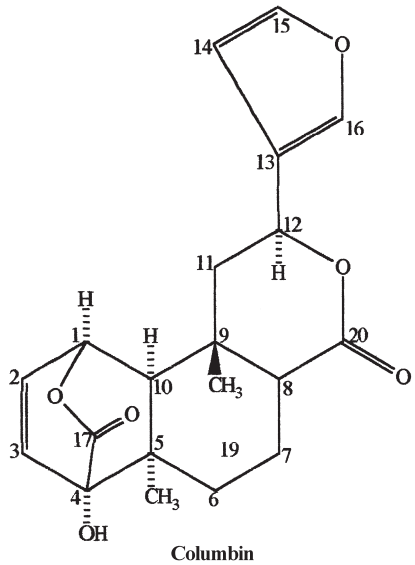
INTRODUCTION

The actions of different snake venom are broad and the understanding of the multiple poisoning process is desirable in the formulation of a satisfactory antidote. Phospholipase A_2 (PLA_2) is a common enzyme found in most snake venom.[1–4] Its central pathological role in the venom has heightened interest in the search for potential inhibitors of the enzyme for drug development. Medicinal plants are commonly used in the treatment of snakebites,[5] however, their mode of action and active ingredients are usually largely unknown. In a previous work, we observed that *Arostolichia albida* a medicinal plant used in the treatment of snake poisoning[6] has no effect on the non-hemorrhagic proteolytic activity of the venom from *Bitis arietans*.[7] Here we investigated the effect of columbin (Figure 1), a component of the plant, on partially purified acidic PLA_2 from *Naja nigricolis* and report for the first time its inhibition of the enzyme's catalysed hydrolysis of lecithin and ghost red blood cells.

MATERIALS AND METHODS

Materials

Naja nigricolis was caught from the wild at Zaria in the Northern part of Nigeria. It was identified in the



Columbin

FIGURE 1 Chemical structure of columbin.

*Corresponding author. Tel.: +234-69-550510. E-mail: jandrew@skannet.com

ISSN 1475-6366 print/ISSN 1475-6374 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/14756360290011762

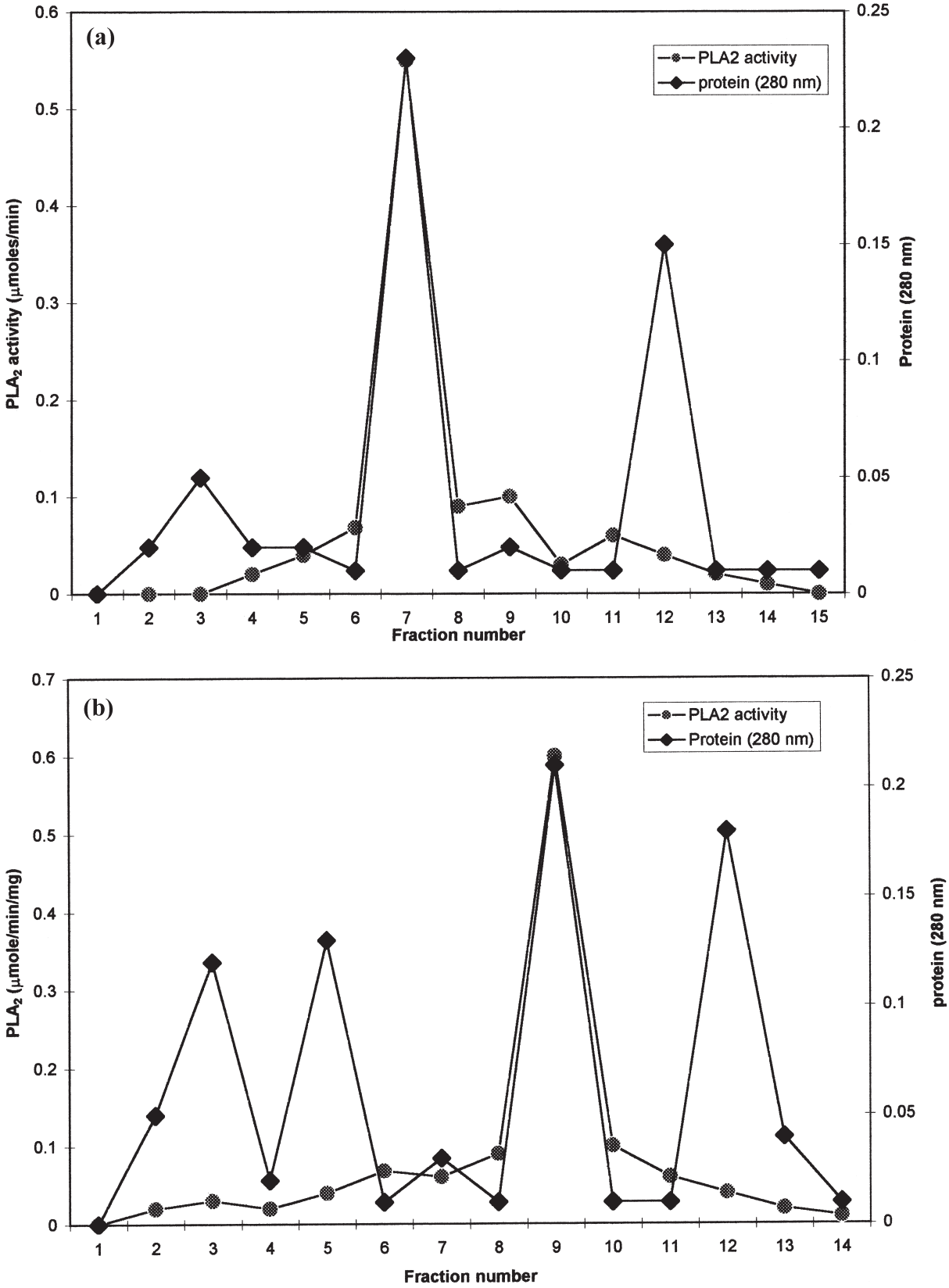


FIGURE 2 a. Elution profile of *Naja nigricolis* acid PLA_2 from Sephadex G-75 column. The column (2.6 × 80 cm) was pre-equilibrated with 0.1 M acetate buffer pH 5.5 and run at a flow rate of 1.0 ml/min. b. Elution profile of *Naja nigricolis* acid PLA_2 from CM-Sephadex (2 × 15 cm) column pre-equilibrated with 0.1 M acetate buffer pH 5.5 and run at a flow rate of 1.0 ml/min. The enzyme was eluted by linear NaCl gradient (0.05–0.2 M NaCl).

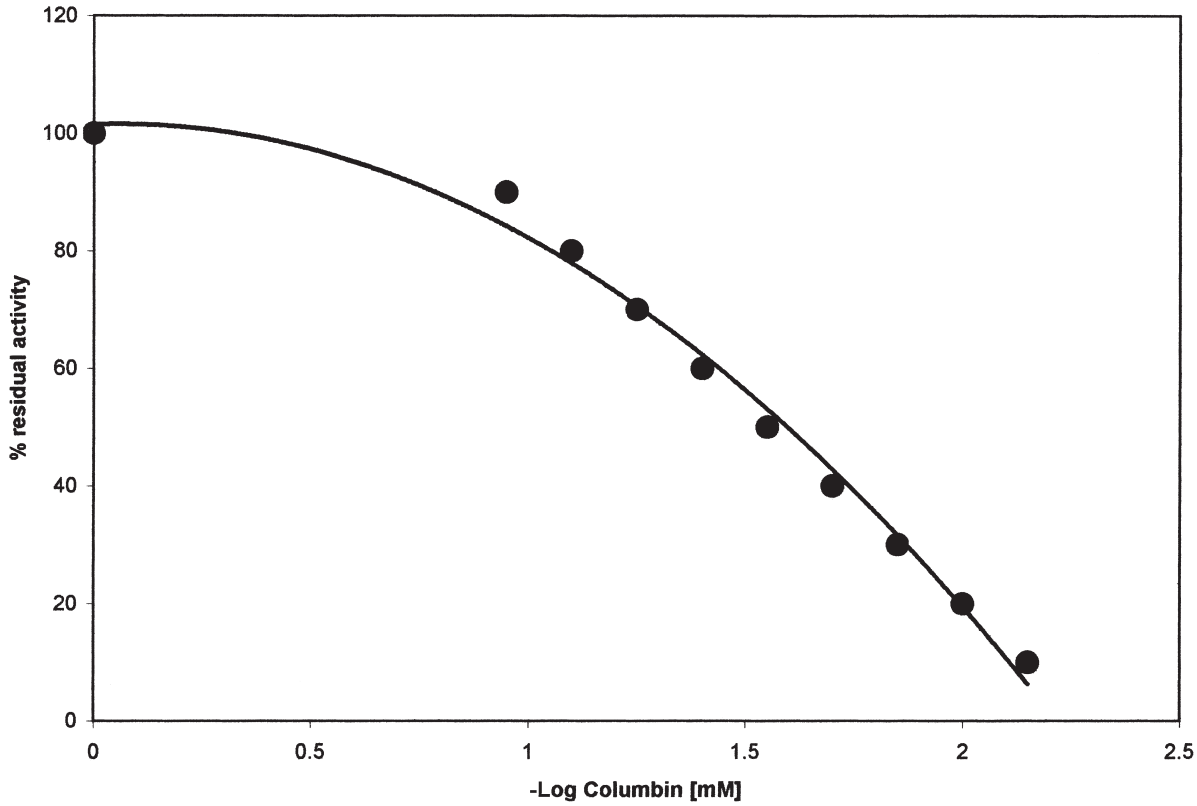


FIGURE 3 Percentage residual activity of *Naja nigricolis* PLA_2 activity after incubation at different levels of columbin for the determination of IC_{50}. The enzyme at 50 μl was initially incubated with 50 μl of 2–10 μM columbin at 37°C. At the end of 1 h, the mixture was incubated with 500 ml of NBD-phosphotidyl choline and incubated at 37°C for 1 h. The reaction was stopped by chilling under ice and the released NBD-aminocaproic acid was quantified at 465 nm using a Beckman Spectrophotometer.

department of pharmacognosy and drug development, Ahmadu Bello University, Zaria, Nigeria. The venom was collected fresh as described,[8] freeze dried and stored at 5°C.

Columbin was a generous gift from Dr M.K. Choudhury of the Department of Pharmaceutical Chemistry, Ahmadu Bello University, Zaria, Nigeria.

General laboratory and inorganic chemicals were obtained from Aldrich and were of analytical grade. Organic chemicals were from Sigma (St. Louis, USA).

Purification of PLA_2

The venom (200 mg) was dissolved in 5 ml of 50 mM acetate buffer pH 5.5 and applied onto a Sephadex G-75 column (2.6 × 80 cm) pre-equilibrated with 0.1 M acetate buffer pH 5.5. Twenty five fractions of 8 ml each were collected at a flow rate of 1.0 ml/min. The fractions were assayed for protein and PLA_2 activity. Active fractions were pooled and reduced to 5 ml by freeze drying and loaded onto a CM sephadex column (2 × 15 cm) equilibrated with the same buffer. Twenty fractions of 5-ml each were collected by NaCl gradient (0.05–0.2 M) and assayed for protein and PLA_2 activity. The active fractions were concentrated as previously described.

PLA_2 Assay

A routine assay for PLA_2 was conducted as described.[9] Briefly, 50 μl of the enzyme was incubated with 500 μl of 10 mM NBD-phosphatidyl choline at 37°C for 30 min. The released NBD-aminocaproic acid was determined using a Beckman Spectrophotometer at 465 nm.

Kinetic Analysis

Lecithin [0.01–0.1 M] was prepared in ethanol. The enzyme (50 μl) in 50 mM acetate buffer pH 5.5 was incubated with 5 ml of the substrate in 95% ethanol at 37°C. At the end of 1 h incubation, the reaction was stopped by cooling under ice for 5 min. The reaction mixture was titrated using 0.02 M ethanolic NaOH to phenol red end point. The activity of the enzyme was defined as the amount of enzyme that catalysed the formation of 1 μmole free fatty acid [FFA]/min under the defined assay condition.

Inhibition using columbin was done in the presence of 2–10 μM of the compound dissolved in 50 mM acetate buffer pH 5.5.

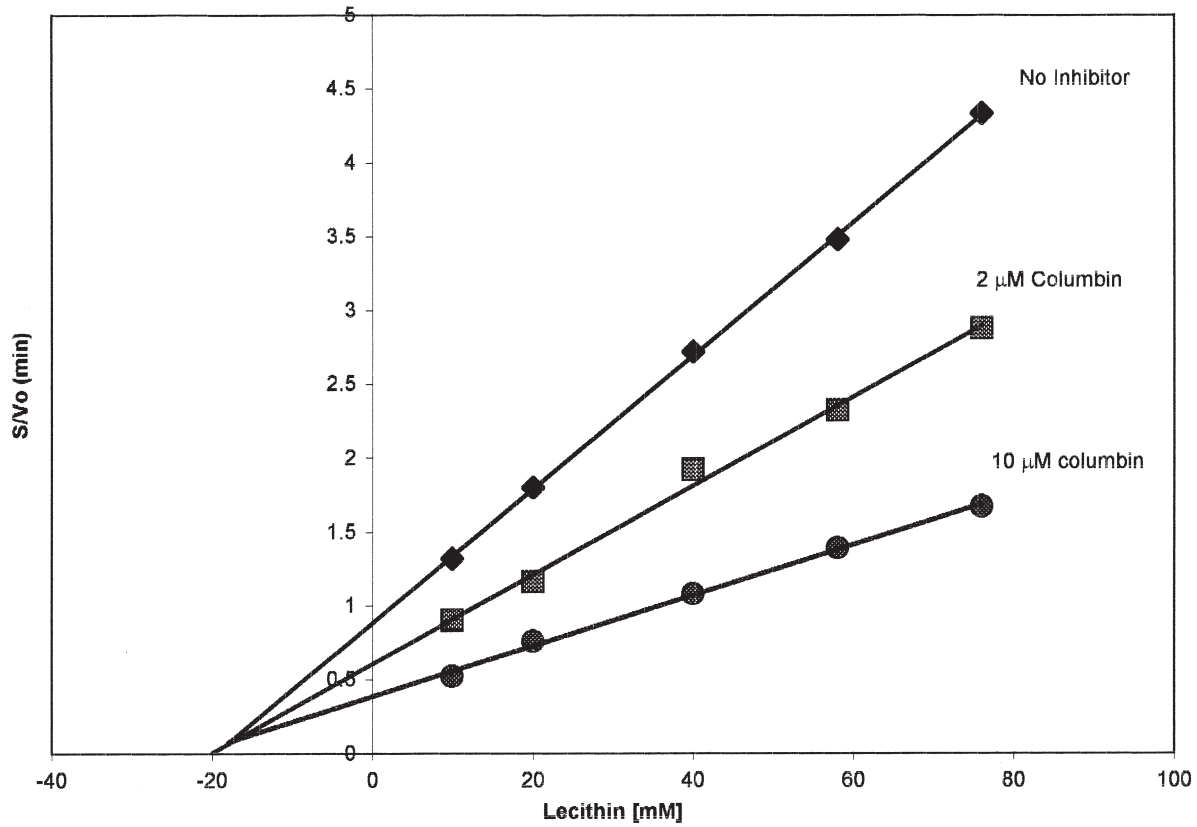


FIGURE 4 Hanes–Wolf Plot of Initial velocity data for the elucidation of the kinetic parameters: K_M and V_{max} of *Naja nigricolis* acid PLA_2 and K_i values for columbin at 2 and 10 μM. The experiment was conducted in the presence of 5 ml aliquots of: 0.01–0.1 M lecithin prepared in 95% ethanol. The reaction was commenced by dispensing 50 μl of the enzyme into the substrate solution and then incubated at 37°C. At least 3 experiments were conducted for each situation and the means used for the plots.

Inhibition of PLA_2 Hydrolysis of Washed Erythrocytes from Rats

Ghost RBCs were prepared as described.[10] Exactly 5 ml of the cells was incubated with 50 μl of the partially purified PLA_2 in the presence and absence of 2 and 10 μM columbin. Heat inactivated PLA_2 was used as control. The activity was followed as a function of the release of FFA as described in the preceding section.

RESULTS AND DISCUSSION

The PLA_2 activity of *N. nigricolis* venom was eluted in the void volume from the Sephadex G-75 column (Figure 2a). After ion exchange chromatography on CM sephadex column, a single active peak eluted at 0.1 M NaCl (Figure 2b). The enzyme was apparently homogenous on polyacrylamide gels [not shown]. When the partially purified PLA_2 from *Naja nigricolis* was incubated with columbin and assayed for residual activity, the enzyme lost activity in a dose-dependent pattern with IC_{50} of 2.5 μM (Figure 3). The micromolar level required for inhibition clearly shows the high efficacy of the compound against the venom's PLA_2. A Hanes–Wolf plot for the evaluation of inhibition type by columbin revealed non-competitive patterns (Figure 4) with K_i values of 3 and 6.28 μM at 2 and 10 μM, respectively. At these concentrations, the physiological index of efficiency of the enzyme [V_{max}/K_M] decreased from 17.18 h^{-1} to 10.21 and 7.65 h^{-1}, respectively. PLA_2 is a highly pathological enzyme found in several species of snake venom world wide.[11] It is known to be toxic to cell membrane, causing local cell and tissue damage as well as systemic effects in snake bitten victims.[12] Beside enzymatic activity, venoms induce various pharmacological effects including myotoxic, anti-coagulant effects and insulin stimulating effects.[13] The inhibition of PLA_2 can therefore be significant in ameliorating the myotoxic and cell damaging effect by the venom on red blood cells which leads to anaemia.

The non-competitive inhibition pattern suggests that sites other than the active site could be involved in the inhibition. Since pre-incubation of the enzyme with columbin followed by dialysis failed to reverse the inhibition, it strongly suggests that the columbin–PLA_2 interaction involved strong covalent forces affecting the structure. These events does not support the degeneration of the tertiary

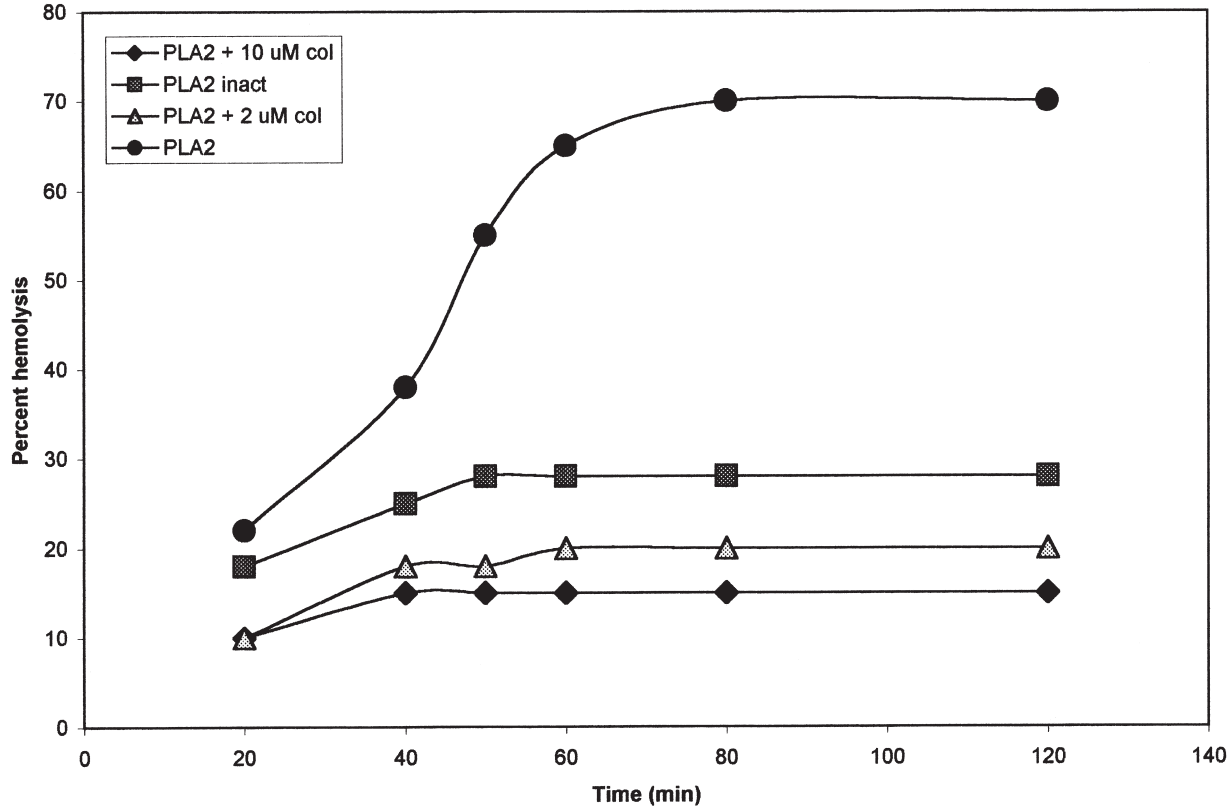


FIGURE 5 Haemolysis of washed Rat Red blood cells (RBC) with *Naja nigricolis* acid PLA_2 in the presence and absence of 2 and 10 μM columbin. The reaction was commenced by dispensing 50 μl of the enzyme into a total of 5 ml solution of the RBC and then incubated at 37°C. Three experiments were conducted for each situation and the means used for the plots.

complex: [I.E.S] i.e. columbin–PLA_2–lecithin to yield products. The K_i values (3–6.28 μM) which is far less than the K_M 19 μM is further proof of the high affinity of the inhibitor for the enzyme, which is independent of substrate requirement. The *N. nigricolis* PLA_2 also catalysed the extensive hydrolysis of ghost RBCs (Figure 5). About 50% haemolysis was achieved after 50 min of incubation whilst maximal haemolysis was reached after 80 min. However, in the presence of 2 μM of columbin, there was a sharp fall in the hydrolysis of the ghost RBCs to about 18%. Since the hydrolysis was followed as a function of released FFA, it can be construed that the hemolytic activity is linked to hydrolysis of membrane lecithin. Moreso the hydrolysis of lecithin generates FFA and lysolecithin, while the former leads to acidosis, the latter is a powerful hemolyzing agent with detergent-like effects.[14] The suppression of haemolysis by columbin suggests that the columbin–PLA_2 interaction protects the cells from haemolytic anaemia. This is particularly significant in the potential application of columbin in the management of anaemia in a snake bitten victim. The present report thus reveals for the first time the target of a component of the plant *A. albida* used in the treatment of snake poison. Further work in this area is desirable since it concerns, columbin-associated control of insulin stimulation in anti-snake therapy.

References

[1] Diaz, C., Leon, G., Rucavado, A., Rojas, N., Schroit, A.J. and Gutierrez, J.M. (2001), *Arch. Biochem. Biophys.* **391**, 56–64.
[2] Yamaguchi, Y., Shimohigashi, Y., Chijiwa, T., Nakai, M., Ogawa, T., Hattori, S. and Ohno, M. (2001), *Toxicon* **39**, 1069–1076.
[3] de Olivera, A.H., Giglio, J.R., Andriao-Escario, S.H., Ito, A.S. and Ward, R.J. (2001), *Biochemistry* **40**, 6912–6920.
[4] Nuchprayoon, I., Sai-Ngam, A., Suntrara chum, S., Norphrom, J., Pakmenche, N., Chanhome, L., Nuchprayoon, S. and Sitprija, V. (2001), *J. Med. Ass. Thai.* **84**, 99–105.
[5] Abubakar, M.S., Sule, M.J., Pateh, U.U., Abdulrahman, E.M., Haruna, A.K. and Jahun, B.M. (2000), *J. Ethnopharmacol.* **69**, 253–257.
[6] Haruna, A.K. and Choudhury, M.K. (1995), *Indian J. Pharm. Sci.* **27**, 222–224.
[7] Nok, A.J. (2001), *J. Biochem. Mol. Toxicol.* **15**, 215–220.
[8] Markfarlane, R.G. (1967), *Br. J. Haematol.* **13**, 437–451.
[9] Simuzu, S., Tan, I.Y., Yamada, H., Tabata, M. and Murachi, T. (1980), *Anal. Biochem.* **107**, 193–198.
[10] Gull, S. and Smith, A.D. (1972), *Biochim. Biophys. Acta* **288**, 237–240.
[11] Komori, Y., Nikai, T. and Sugihara, H. (1990), *Comp. Biochem. Physiol.* **97**, 507–514.
[12] Lizano, S., Lambeau, G. and Lazduski, M. (2001), *Int. J. Biochem. Cell Biol.* **33**, 127–132.
[13] Gao, R., Kini, R.M., Li, G., Luo, R., Sclavanayagan, Z.E. and Gopalakrishnakone, P. (2001), *Biochim. Biophys. Acta* **154**, 30–40.
[14] Nok, A.J., Esievo, K.A.N., Ibrahim, S., Ukoha, A.I. and Ikediob, I.C.O. (1993), *Cell. Biochem. Func.* **11**, 125–130.