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Immunochemical studies on phospholipase A_2 from *Naja nigricollis* venom

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ABSTRACT

Four PLA_2 isoenzymes (*N. nigricollis* CM-PLA_2I-IV) were purified from *Naja nigricollis* (*N. nigricollis*) venom using Sephadex G-50 gel filtration, and ion exchange chromatography on CM-Sephadex C-25. The characterization of the isolated PLA_2 isoenzymes revealed similarities in molecular weights, and differences in the isoelectric points, the optimum temperature, optimum pH, optimum Ca^{+2} concentration and metal ion requirements. A good correlation (r> 0.7) for the *in vitro* neutralization of enzymatic PLA_2s activity and the ELISA titers was found for sera collected at one week from each boosting of the rabbits. The found correlations were particularly high (r>0.9) when the purified Seph-PLA_2 and CM-PLA_2II were used rather than the whole venom. The established correlations show the importance of the purified PLA_2 enzymes as immunogens for raising therapeutic antisera and as diagnostic reagents for *in vitro* determination of the potency of the therapeutic antisera.

Keywords: Antivenom, Correlation, *Naja nigricollis*, Phospholipase A_2, Snake venom

INTRODUCTION

Snakebite is a serious medical problem worldwide, especially in the tropics. The incidence of snakebite mortality is particularly high in Africa, Asia, Latin America and New Guinea (Gutierrez *et al.,* 2006). In Africa, snakebites cause more than one thousand deaths each year and thousands of cases of permanent physical disability (Theakston *et al.,* 2003). In Egypt, the Black-necked Spitting Cobra; *Naja nigricollis* (*N. nigricollis*) is one of the most venomous snakes distributed in the south part of Egypt (Saleh, 1997).

Biochemical characterization of the venom of a snake from a particular geographical location is of great importance because pathogenesis developing after a bite is dependent on the qualitative composition of the venom and on the quantitative distribution of different components in particular venom (Stocker, 1990; Warrell, 1997).

Venoms contain a variety of enzymes, non-enzymatic polypeptide toxins and non-toxic proteins. Toxicity of venom depends on the qualitative and quantitative distribution of different enzymes and toxins in the venom (Stocker, 1990). Phospholipase A_2 (PLA_2) is the single most toxic component of the venom and particularly the basic PLA_2 plays an important role in mortality and morbidity following snakebite (Bhat *et al.,* 1991; Mukherjee *et al.,* 1998; Kang *et al*., 2011).

The phospholipase A_2 (PLA_2) (EC. 3.1.1.4) superfamily consists of a broad range of enzymes defined by their ability to hydrolyze glycerophospholipids (water insoluble substrate phospholipids) at the *sn*-2 position of the glycerol backbone releasing equimolar amounts of lysophospholipids and free fatty acids (Kini, 2003).

The superfamily of PLA_2 enzymes currently consists of 15 groups and many subgroups (Schaloske & Dennis, 2006).

Venom $sPLA_2$ from Elapidae and Hydrophiidae have been classified into group IA with the exception of a few enzymes that belong to group IB. However, venom $sPLA_2$ from Crotalidae and Viperidae are found in group IIA, and group IIB contains some $sPLA_2$ from *Bitis species*. Finally, $sPLA_2$ from bee and lizard venoms belong to group III (Huang *et al.,* 1997; Six & Dennis, 2000).

Snake venoms are particularly rich in PLA_2s and contain multiple forms of PLA_2 enzymes (Kini & Evans, 1989; Singh *et al.,* 2000; Kini, 2003; Romero-Vargas *et al.,* 2010) that are similar in molecular size, isoelecteric points and even amino terminal sequences (Gao *et al.,* 2001; Kini, 2003). However, PLA_2 isoenzymes may exhibit a specific pharmacological effect, such as pre-synaptic or post-synaptic neurotoxicity, cardiotoxicity, myotoxicity, platelet aggregation, oedema formation, haemolysis, anticoagulation, convulsion, and hypotension (Kini & Evans, 1989; Singh *et al.,* 2000; Kini, 2003, Santos-Filho *et al.,* 2008; Zou *et al.*, 2012)

Whereas, many of the venom biological activities could be neutralized by the homologous and heterologous antivenins, the lethality of elapids could only be neutralized by the corresponding antivenins (Al-Asmari *et al.,* 1997; Mukherjee and Maity, 2002; Rodriguez *et al.,* 2006; Shashidharamurthy and Kemparaju, 2007).

Correlation of the *in vivo* neutralization of lethality and the *in vitro* PLA_2 neutralization to efficacy of the therapeutic antivenins for a number of elapids pointed to the importance of the elapid PLA_2 enzymes as immunogens for raising therapeutic antisera and as diagnostic reagents for *in vitro* determination of the potency of the therapeutic antisera. Also, Good correlations between the potency of antivenoms and the enzyme-linked immunosorbent assay (ELISA) titer against crude venoms and lethal toxin-containing fractions as antigens of viperid and elapid antivenoms were found (Theakston & Reid, 1979; Rungsiwongse & Ratanabanangkoon, 1991; Barbosa *et al.,* 1995; Heneine *et al.,* 1998; Maria *et al.,* 1998; Muniz *et al.,* 2000; Sells, 2003; Beghini *et al.,* 2004; Rial *et al.,* 2006; Halassy *et al.,* 2008).

The present study was designed to isolate and characterize PLA_2 enriched fractions from *N. nigricollis* venom. The isolated PLA_2 fractions were used as immunogens for raising therapeutic antisera and the correlation between the *in vitro* neutralization of the enzymatic PLA_2 activity and ELISA titer against the homologous venom was studied.

MATERIALS AND METHODS

Snake venoms

The *N. nigricollis* venom collected from cultivated lands in Luxor, Egypt was milked from several adult specimens. The venom was lyophilized, freeze-dried and stored at -20 °C until use. The venom samples were kept at -20 °C, thawed and centrifuged before use.

Purification of PLA_2 enzyme

The *N. nigricollis* (200 mg) venom dissolved in 1 ml equilibration buffer (0.05 M Tris-HCl, pH 6.8), was loaded on Sephadex G-50 column (2.6 × 50 cm). The sample-dissolving buffer was used for equilibration of the Sephadex column, and elution of the loaded samples. Fractions of 4 ml were collected at a flow rate of 48 ml/hr using fraction collector (Pharmacia LKB, Sweden). Fractions with phospholipase A_2 activity recovered from the previous step were pooled and directly applied to a CM-Sephadex C-25 column (1.6 × 25 cm) pre-equilibrated with the same buffer and eluted with a linear KCl gradient from 0 to 1.3 M in the same buffer. The enzymatic PLA_2 activity was detected by the indirect hemolytic assay according to Al-Abdulla *et al.* (1991). The active fractions were

pooled, dialyzed, freeze-dried, and stored as *N. nigricollis* CM-PLA_2I, II, III and IV.

Preparation of rabbit anti-PLA_2 antisera

Eight healthy rabbits (1.2-1.5 Kg body mass) were divided into two groups: Group A. Six Rabbits; were injected intramuscularly with doses containing 15 µg of the individual *N. nigricollis* Seph-PLA_2 and *N. nigricollis* CM-PLA_2 (I, II) dissolved in 0.5 ml isotonic saline and Group B**.** Two rabbits pre-immunized with the individual *N. nigricollis* Seph-PLA_2 were boosted with the homologous venom with doses containing 8 µg of whole venom dissolved in 0.5 ml isotonic saline. The primer dose was emulsified with 0.5 ml of complete Freund's adjuvant (CFA), whereas the first and second booster doses were emulsified with equal volume of incomplete Freund's adjuvant (IFA). Blood samples of the individual rabbits were collected at one week intervals and sera were separated, pooled and stored at -20 °C. Rabbit sera before immunization were used for control purposes.

Polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis analysis was performed in the Mini-Protean II Dual-Slab Cell (Bio Rad). Preparation of gels, samples, and electrophoresis were performed according to the conditions described by Laemmli (1970). Proteins were stained with 0.2% Coomassie Brilliant Blue R-250 in fixative solution [methanol/acetic acid/water (45/10/45) (v/v/v)] and destained by the same solution.

Analytical isoelectric focusing (IEF)

The analytical isoelectric focusing was performed in the MultiphorII unit (80-1106-20) connected to thermostatic circulator and the temperature was set in the range of (4-6) °C. Preparation of gels, samples and electrophoresis were performed according to Garfin (1990) with minor modification.

Phospholipase A_2 activity

Three methods were used to measure the PLA_2 activity of the crude venoms and the isolated fractions according to (Marinetti, 1965; Gutierrez *et al.,* 1988 and Moreno *et al.,* 1988) with slight modifications.

Method of Marinetti (1965)

This method was used to study the kinetics of the isolated *N. nigricollis* PLA_2 fractions. Briefly, a stock of egg yolk suspension was prepared by shaking one egg yolk in 0.9% NaCl to give a final volume of 100 ml. The working suspension used for the study of phospholipase A_2 was prepared by making 5-fold dilution of this stock suspension in 0.9% NaCl. The enzymatic activity was measured as follow: To 1 ml of the working egg yolk suspension, 4.9 ml of isotonic saline was added. The suspension was mixed, and stood for 5 min during this time the suspension equilibrated to a temperature of 41°C, then to the suspension, 0.1 ml of the test solution in saline was added. The contents were rapidly mixed and the absorbancy at 925 nm was recorded each 5 min for 15 min. The control was adjusted to give an absorbency of 0.6 (at 925 nm). The reaction velocity was determined as the absorbancy change at 925 nm per 5 min and was taken as a parameter for the relative enzyme activity.

Method of Gutierrez *et al.* (1988)

This method was used to quantify the specific PLA_2 activity in the enzyme isolation process. Briefly, Serial dilutions of the venom or the isolated fractions in 10 µl of physiological saline were added into 3 mm wells in agarose gels containing 4% washed human erythrocytes, 4% egg yolk suspension as a source of lecithin and 10 mM $CaCl_2$ and incubated for 20 hr at 37 °C. The square diameter of each hemolytic halo was measured then, the standard dose response curve for the PLA_2 activity was plotted (venom concentration versus the

square diameter of the hemolytic halos). One PLA_2 unit was defined as the venom concentration (μg) which induce a hemolytic halo of one cm^2 square diameter (r^2=1). The *in vitro* neutralization of the *N. nigricollis* venom PLA_2 activity was measured as follow; a constant amount of venom (one PLA_2 unit) was incubated with serial dilutions of anti- *N. nigricollis* venom and anti- *N. nigricollis* CM-PLA_2 antivenoms for 30 min at 37 °C. Then, 10 μl of each mixture was added to duplicate wells in agarose-erythrocyte-egg yolk gels; a non-immune rabbit serum was used as control. The square diameters of the hemolytic halos were measured and the neutralization of the enzymatic PLA_2 activity was calculated from the following formula: The % neutralization of enzymatic PLA_2 activity = 100% PLA_2-the % inhibition of PLA_2 activity. (The 100 % PLA_2 was the percentage of the haemolysis caused by the venom alone).

Method of Moreno *et al.* (1988)

After isoelectrofocusing (pH 3.5-10), or electrophoresis in 14% SDS-PAGE, the resolved proteins were eletrotransferred to nitrocellulose paper; the transfer was performed in the Semi-dry LKB - BROMMA - 2117- 250 NOVABLOT Electrophoresis Transfer Kit under constant current conditions of 100-150 mA for 1 hr according to Towbin *et al.,* (1979), the nitrocellulose paper was rinsed and incubated in incubation buffer (1% casein hydrolysate dissolve in 0.07 M barbiturate buffer, pH 8.6) for 2 hr at room temperature, the nitrocellulose membrane was then incubated with a substrate-fortified gel (1% agarose dissolved in PBS, pH 7.4, containing 4% of packed human erythrocytes, 4% of egg yolk as source of lecithin and 10 mM $CaCl_2$); the plate was kept at 37 °C in a humid chamber. After 3 hr incubation, the nitrocellulose paper was removed and the substrate gel was further incubated at 4°C for additional 21 hr, then the plate was photographed and the PLA_2 activity was indicated by the hemolytic bands (clear bands) in the substrate-fortified gel.

Effect of substrate concentration

Serial concentrations of the working egg yolk suspension (0.5-3 ml complete with isotonic saline) were added to constant concentration of the individual PLA_2 fraction then the reaction velocity was determined and plotted against substrate concentrations. The substrate Concentration (ml) that gives half-maximum velocity ($V_{max}/2$) was taken as the endpoint for calculation of relative K_m.

Effect of temperature

The optimum temperature was examined by incubating the reaction mixture at different temperatures ranging from 25 to 70 °C under standard assay conditions. The temperature value that gives the maximum reaction velocity was defined as the optimal temperature.

Effect of pH

The optimum pH was examined using Tris-HCl buffer over a broad pH range from 3 to 10. The pH value that gives the maximum reaction velocity was defined as the optimal pH.

Effect of Ca^{+2} concentration

Ca^{+2} concentrations between 0-100 mM of $CaCl_2$ were added to dialyzed substrate suspension (1 ml of dialyzed stock egg yolk suspension against 0.9% NaCl for 72 hr at 4 °C and 5 ml of isotonic saline) instead of substrate suspension as mentioned above. Reaction velocity of the individual PLA_2 fraction was measured and plotted against Ca^{+2} concentrations values. The Ca^{+2} concentration value that gives the maximum reaction velocity was defined as the optimal Ca^{+2} concentration.

Effect of metal ions and solvents on the relative PLA_2 activity

Metal ions (10 mM) of Na^{+}, K^{+}, Mg^{+2}, Ca^{+2}, Ni^{+2}, Co^{+2}, Zn^{+2}, Fe^{+2}, Mn^{+2}, $Al^{+3}$and citrate and solvents as 0.6% formaldehyde, 1% Ethanol, 1% and 5%

phenol, were dissolved in dialyzed substrate suspension. Then, the relative PLA_2 activity (%) was calculated and plotted against each metal ions and solvents.

Enzyme linked immunosorbent assay (ELISA)

ELISA was performed according to the method of Maria *et al.* (1998) using *N. nigricollis* venom (0.5 µg/well) as coating antigen, the conjugate (anti-rabbit IgG peroxidase) was used at 1:5000 dilution. A standard curve between log antisera dilution and log optical density (OD) at 490 nm was plotted. The dilution that gives 0.5 OD at 490 nm was taken as ELISA titer.

Protein determination

Protein concentrations were determined according to the method of Bradford (1976), using bovine serum albumin as standard protein.

RESULTS

Purification of *N. nigricollis* PLA_2s

The PLA_2 enzymes were isolated from *N. nigricollis* venom using gel filtration and ion exchange chromatography. In the First step, the *N. nigricollis* venom (200 mg protein) was applied on Sephadex G-50 gel filtration column (2.6 × 50 cm) and separated into three main protein peaks (Fig. 1), the PLA_2 activity fraction was detected under the first two peaks and designated as *N. nigricollis* Seph-PLA_2 with 75% recovery (Table 1). In the second step, the *N. nigricollis* Seph-PLA_2 fraction was applied on a CM-Sephadex C-25 column (1.6 × 25 cm), four major protein peaks were resolved as one unbound and three bound peaks. Four PLA_2 fractions were detected in the bound proteins and designated as CM-PLA_2 I, II, III and IV (Fig. 2). The *N. nigricollis* CM-PLA_2II fraction was found to possess the highest specific PLA_2 enzymatic activity and recovery (40 U/mg and 19%), respectively (Table 1).

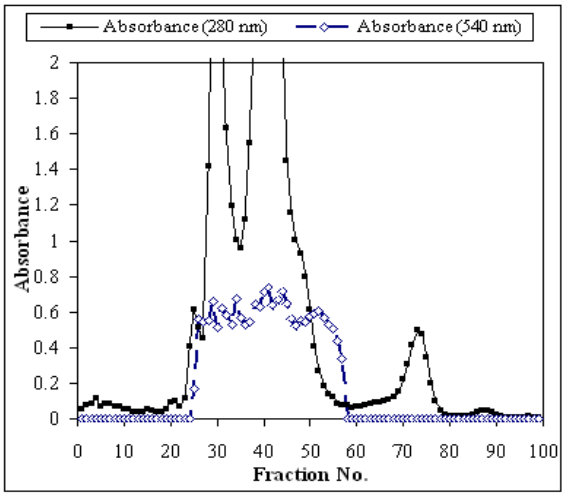

Fig. 1: Gel filtration of *N. nigricollis* venom on a Sephadex G-50. The venom (200 mg protein) dissolved in 0.05 M Tris-HCl, pH 6.8 buffer, was applied on the Sephadex G-50 column (2.6 × 50 cm) equilibrated with the same buffer. The column was eluted with the same buffer and 4 ml fractions were collected at a flow rate of 48 ml/hr, tubes from (25-58) show PLA_2 activity as assayed by the indirect hemolytic assay.

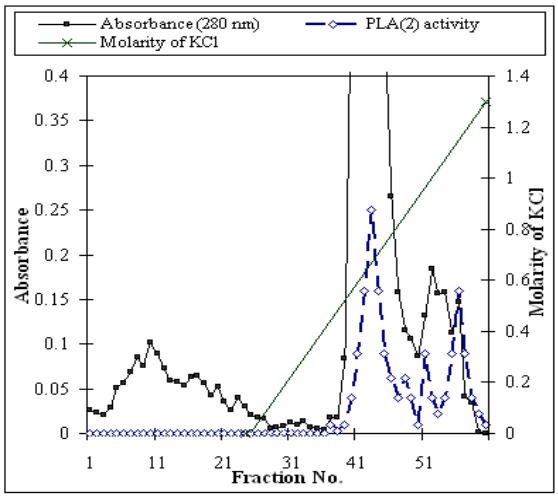

Fig. 2: Ion-exchange chromatography of *N. nigricollis* Seph-PLA_2 fraction on CM-Sephadex C-25. The fraction (120 mg protein) dissolved in 0.05M Tris-HCl, pH 6.8 buffer, was applied on the CM-Sephadex C-25 column (1.6 × 25) equilibrated by the same buffer. The unbound proteins were washed with the dissolving buffer and the bound proteins were eluted with a linear gradient from 0 to 1.3 M KCl in 0.05M Tris-HCl pH 6.8 buffer. Fractions of 4 ml were collected at a flow rate of 48 ml/hr. Four CM-PLA_2 fractions were resolved as indicated by the indirect hemolytic assay.

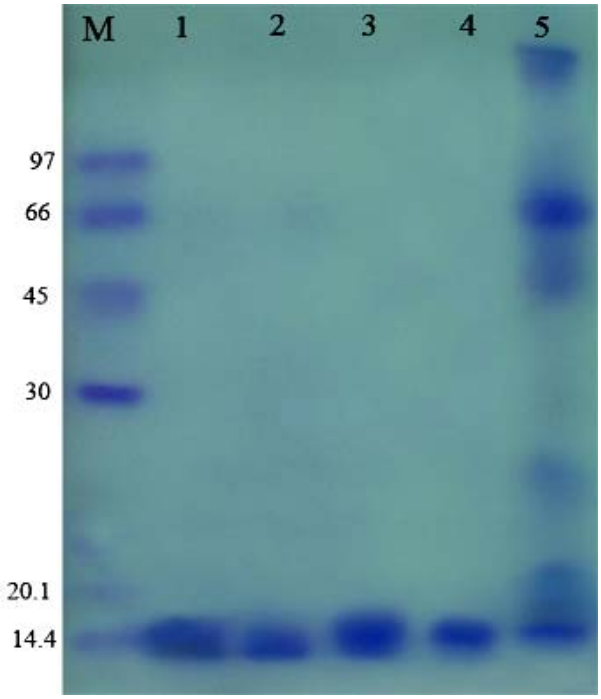

Fig. 3: 14% SDS-PAGE of *N. nigricollis* and their corresponding CM-PLA_2 variants (30-50µg protein/lane) under reducing conditions. The samples were: low molecular weight SDS- marker ranging from 14.4 to 97 kDa (M), *N. nigricollis* CM-PLA_2I (1), *N. nigricollis* CM-PLA_2II (2), *N. nigricollis* CM-PLA_2III (3), *N. nigricollis* CM-PLA_2IV (4) variants and *N. nigricollis* venom (5).

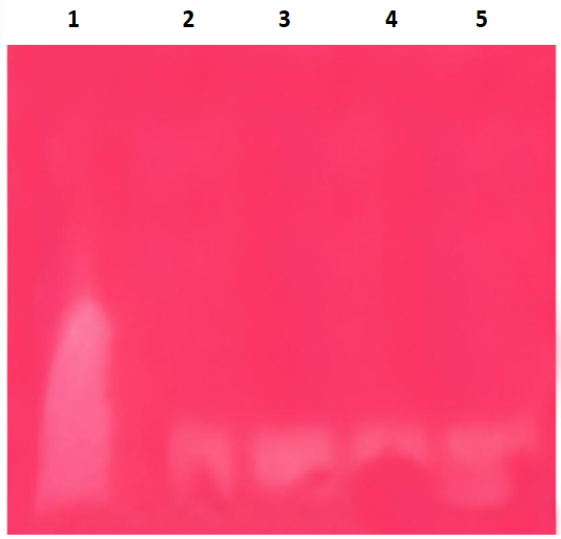

Fig. 4 : *N. nigricollis* venom proteins and the corresponding CM-PLA_2 variants (60 µg protein/lane) were separated in 14% SDS-PAGE, the resolved proteins were electrotransferred onto nitrocellulose paper then incubated with agaraose-RBCs (substrate) gel, The samples were: *N. nigricollis* venom (1), CM-PLA_2I (2), CM-PLA_2II (3), CM-PLA_2III (4) and CM-PLA_2IV (5) variants.

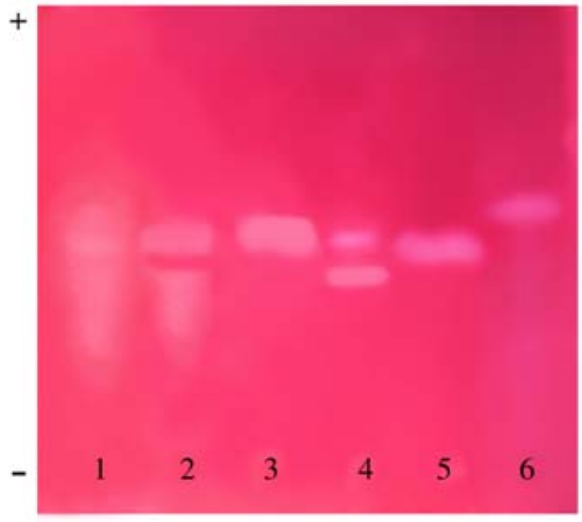

Fig. 5: Isoelectric points (*pIs*) of *N. nigricollis* venom and the corresponding CM-PLA_2 variants, (30 µg protein/lane) were focused at pH 3.5-10, the resolved proteins were electrotransferred onto nitrocellulose paper then incubated with agaraose-RBCs (substrate) gel. The samples were: *N. nigricollis* venom (1), Seph-PLA_2 (2), CM-PLA_2I (3), CM-PLA_2II (4), CM-PLA_2III (5) and CM-PLA_2IV (6) variants.

Table 1: The purification scheme of the PLA_2 fractions from *N. nigricollis* venom.

Sample	Total protein (mg)	*One unit PLA_2 (mg)	SpecificPLA$_2$ activity (U /mg)	Fold purification	PLA_2 activity	
					Total unit	*Recovery%*
N.nigricollis	200	100	10	1	2000	100
***N. nigricollis* Seph-PLA_2**	120	80	12.5	1.25	1500	75
***N. nigricollis* CM-PLA_2I**	33.8	150	6.67	0.67	225	11.3
***N. nigricollis* CM-PLA_2II**	9.5	25	40	4	380	19
***N. nigricollis* CM-PLA_2III**	10.8	100	10	1	108	5.4
N. nigricollis CM-PLA_2IV	6	65	15.39	1.54	92	4.6

*One PLA_2 unit was defined as the venom concentration (μg) which induce a hemolytic halo of one cm^2 (r^2=1).

Characterization of the *N. nigricollis* CM-PLA_2s

The electrophoretic separation of the The *N. nigricollis* CM-PLA_2 variants (I-IV) shows that the molecular weights of *N. nigricollis* CM-PLA_2 variants were between 14 and 15 kDa (Fig. 3), the activity patterns of the *N. nigricollis* CM-PLA_2 (I-IV) variants demonstrated that the molecular weights of the *N. nigricollis* CM-PLA_2 variants were of about 14 kDa (Fig. 4). The *N. nigricollis* CM-PLA_2 fractions (I-IV) were subjected to isoelectrofocusing technique using a wide range of ampholine (from 3-10), transferred to nitrocellulose paper, the PLA2 activity of the four fractions was then measured as mentioned above. The results indicated that these fractions have a range of isoelectric points (pIs) between 6 and 8 (Fig. 5).

Km values

The Km values of the *N. nigricollis* CM-PLA_2 fractions (I-IV) possessed close relative K_m values as shown in (Fig. 6 and Table 2). The Km values were 1, 1.3, 1.8 and 1.6 for *N. nigricollis* CM-PLA_2 I, II, III and IV, respectively (Table 2).

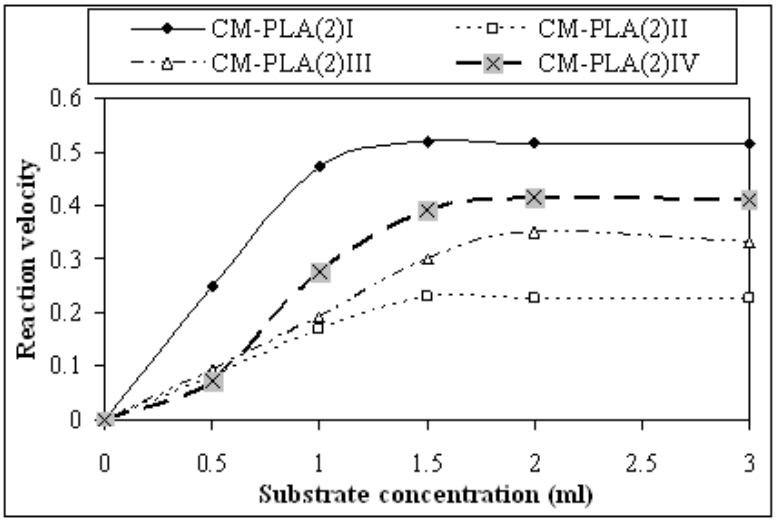

Fig. 6: Effect of substrate concentrations (egg yolk suspension) on the reaction velocity of four *N. nigricollis* CM-PLA_2 fractions.

Table 2: The relative K_m, the optimum temperature, pH and Ca^{+2} concentration values of the *N. nigricollis* CM-PLA_2 fractions.

CM-PLA_2 fractions	Relative K_m	*Optimum*		
		Temperature	pH	*Ca^{+2} Conc. (mM)*
***N. nigricollis* CM-PLA_2I**	1	41	7	10
***N. nigricollis* CM-PLA_2II**	1.3	44	7	10
***N. nigricollis* CM-PLA_2III**	1.8	52	8	5
N. nigricollis CM-PLA_2IV	1.6	52	8	5

Effect of temperature

The effect of temperature on the *N. nigricollis* CM-PLA_2 fractions was measured at incubation temperatures between (25-70° C), the results indicated that the *N. nigricollis* venom, *N. nigricollis* CM-PLA_2 (I, II) and *N. nigricollis* CM-PLA_2 (III, IV) fractions have significantly different optimal temperature values. The crude venom possessed its maximum PLA_2 activity at 65° C, the CM-PLA_2 (I, II) fractions exhibited their maximum activities at 41 and 44° C while the CM-PLA_2 (III, IV) fractions exhibited their maximum activity at 52°C (Fig. 7 A and Table 2).

pH optimum

The optimal pH values for *N. nigricollis* CM-PLA_2 fractions were measured at pH values between (3-10), the results revealed that the optimal pH values of *N. nigricollis* CM-PLA_2 (I, II) and *N. nigricollis* CM-PLA_2 (III, IV) fractions were different. the pH optimum of both *N. nigricollis* CM-PLA_2 (I, II) were 7, while that of *N. nigricollis* CM-PLA_2 (III, IV) were 8 (Fig. 7 B, Table 2).

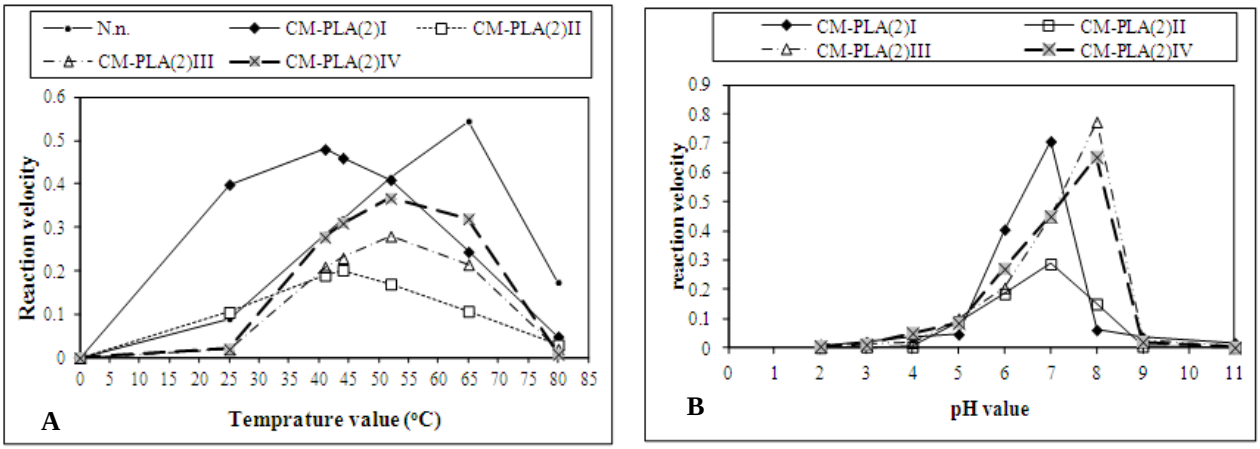

Fig. 7: Effect of temperature (A) and pH (B) on the reaction velocity of four *N. nigricollis* CM-PLA_2 fractions.

Effect of metal ions and solvents

The optimal Ca^{+2} concentration values for *N. nigricollis* CM-PLA_2 (I, II) and *N. nigricollis* CM-PLA_2 (III, IV) fractions were different (Fig. 8, 9 and Table 2). The Ca^{+2} at 10 mM increased the PLA_2 activity of the fractions in the order of (*N. nigricollis* CM-PLA_2 I, II> III, IV). K^+ ion activated all *N. nigricollis* CM-PLA_2 fractions except *N. nigricollis* CM-PLA_2I, whereas Mg^{+2} ion activated only *N. nigricollis* CM-PLA_2 II fraction (Fig. 9). Ethanol and phenol (1%) had no effect on the PLA_2 activity of the four *N. nigricollis* CM-PLA_2 fractions, whereas phenol (5%) and formaldehyde (0.6%) had inhibitory effect on the PLA_2 activity of the four *N. nigricollis* CM-PLA_2 fractions (Fig. 9).

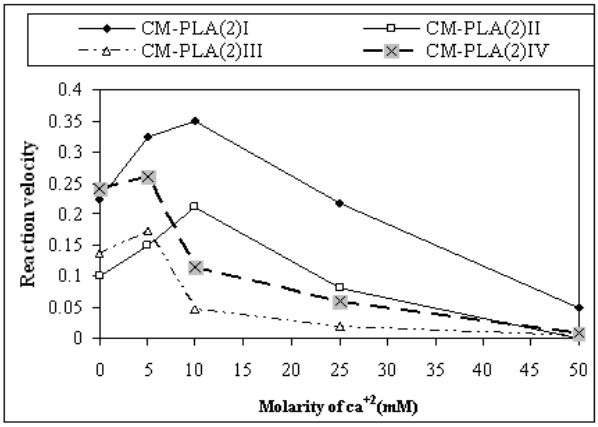

Fig. 8: Effect of Ca^{+2} concentrations on the reaction velocity of four *N. nigricollis* CM-PLA_2 fractions.

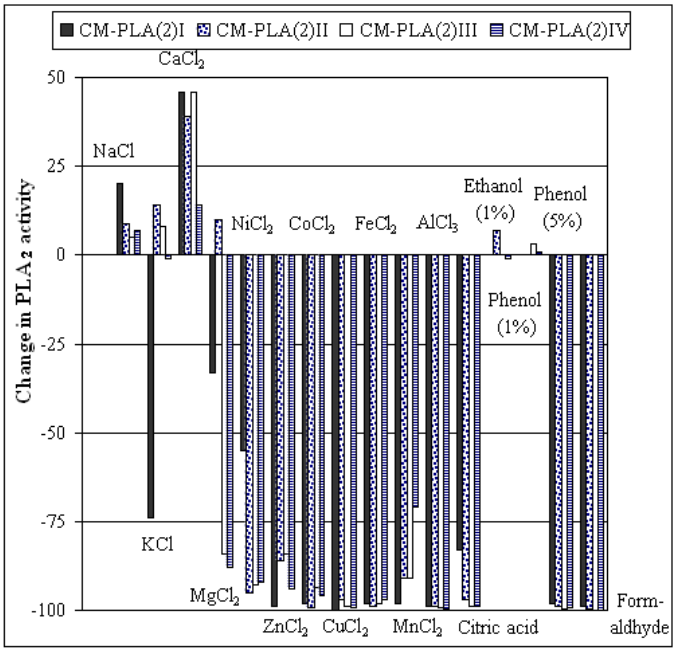

Fig. 9: Effect of metal ions and solvents on the enzymatic PLA_2 activity of four *N. nigricollis* CM-PLA_2 fraction.

Immunogenicity of the isolated PLA_2s:

Crude *N. nigricollis* venom and the corresponding PLA_2 isolated fractions were used as immunogens for production of the corresponding rabbit antisera. ELISA and neutralization of enzymatic PLA_2 activity confirmed the elicited rabbit antibodies (Abs) *in vitro* (Tables 3, 4).

Table 3: ELISA titer of different rabbit antisera

Weeks after first immunization	**ELISA titer of**			
	R. anti-N. nigricollis	***R. anti-N. nigricollis seph-PLA$_2$***	***R. anti-N. nigricollis CM-PLA$_2$I***	***R. anti- N. nigricollis CM-PLA$_2$II***
0	0	0	0	0
1	13022	41	8	13
4	10108	53214	196	156861
7	28443	48595	12775	184438

Table 4: Potency of the R. anti-*N. nigricollis* and R. anti- *N. nigricollis* PLA_2s fractions against the homologous venom.

Weeks after first immunization	% Neutralization of N. nigricollis P LA_2 venom with			
	R. anti-*N. nigricollis*	**R. anti-*N. nigricollis* seph-PLA_2**	**R. anti-*N. nigricollis* CM-PLA_2I**	***R. anti- N. nigricollis CM-PLA_2II***
0	43.8	0	0	0
1	43.8	0	23.4	23.4
4	60.9	43.8	60.9	60.9
7	75	60.9	75	86

Potency of the rabbit anti-*N. nigricollis* and its corresponding isolated PLA_2s

The ELISA titer of the rabbit antisera to the venom antigens (presumably the venom PLA_2) were detected after three weeks from the first injection and progressively increased over the immunization schedule. The ELISA titers of the rabbit anti-CM-PLA_2I reached the highest peak after one week from the third injection (12775 EU), while the ELISA titers of the rabbit anti-Seph-PLA_2 and rabbit anti-CM-PLA_2II showed the highest ELISA titers in the eighth (60191 EU) and the sixth (727307 EU) weeks, respectively (Table 3). The results revealed that the CM-PLA_2II was the most immunogenic PLA_2 fractions, whereas CM-PLA_2I was the least immunogenic.

***In vitro* neutralization of the enzymatic PLA_2 activity**

Antisera to the elapid venoms and the isolated venom PLA_2 isoenzymes were raised and investigated for its efficacy to neutralize the enzymatic PLA_2 activity of crude venom. The neutralization of the venom PLA_2 activity by the various harvests of the rabbit antisera revealed a progressive increase in the PLA_2 neutralization potency over the immunization period. Also, Neutralization by antisera to the isolated PLA_2s was found to be more efficient than neutralization by rabbit antisera to the whole venoms. Furthermore, compared to the crude venom, the isolated PLA_2s were found to be more immunogenic (Table 4).

Correlation between ELISA antibody titers and *in vitro* neutralization of enzymatic PLA_2 activity

The neutralization of the venom enzymatic PLA_2 activity and the levels of antibodies measured by ELISA were correlated, a good correlation was found for the rabbit anti-*N. nigricollis* ($r > 0.7$). However, attempts to correlate the same traits for all the samples from an individual immunized animal or for timed samples from different animals were not successful ($r < 0.6$). The found correlations were particularly high ($r > 0.95$) when the isolated Seph-PLA_2 fraction were used rather than the whole venom for establishing the correlations. Most pronouncedly the correlation for neutralization of the enzymatic PLA_2 activity of the *N. nigricollis* with the rabbit anti-*N. nigricollis* CM-PLA_2II was the best ($r > 0.98$) (Table 5).

Table 5: The correlation between ELISA antibodies titers and the *in vitro* neutralization of *N. nigricollis* PLA_2 enzymatic activity.

Rabbit antisera against homologous venom	Correlation coefficient value (r)
Anti-*N. nigricollis*	0.747
Anti- *N. nigricollis* Seph- PLA_2	0.938
Anti- *N. nigricollis* CM- PLA_2I	0.853
Anti- N. nigricollis CM - PLA_2II	0.989

DISCUSSION

The occurrence of PLA_2 from snake venom as multiple isoenzymes is a common observation (Singh *et al.,* 2000; Kini, 2003; Ramirez-Avila *et al.,* 2004; Cogo *et al.,* 2006; El Hakim *et al.,* 2008; Romero-Vargas *et al.,* 2010). These isoenzymes have been reported to share high identity in their amino acid sequence and possess similar three-dimensional structure (Arni & Ward, 1996; Scott, 1997; Tan *et al.,* 2003). However, the exact number of the isoenzymes in each venom is not certain. The uncertainty could be explained in terms of the fact that the specific substrate for each of the variants is not yet known.

Three different assays were used in the present study to assess the PLA_2 activity of one of the most medically important Egyptian snake venom and its isolated PLA_2 fractions. A sensitive assay described by Gutierrez *et al.* (1988), was used in the present study to quantities the specific PLA_2 activity of *N. nigricollis* venom and the coressponding isolated PLA_2 fractions, this assay based on the measurement of hemolytic haloes induced by venom on the agarose-erythrocyte-egg yolk (fortified-substrate) gel. A standard curve for PLA_2 activity of snake venom or isolated fractions was established by plotting protein concentration versus the square diameter of hemolytic halo using selected reference venom (data not shown). The enzymatic PLA_2 specific activity was calculated as U/mg proteins. Another assay of Moreno *et al.* (1988) was used to measure the enzymatic PLA_2 activity based on the transfer of the resolved venom to a substrate-fortified gel. This assay was highly useful in identifying the molecular weights and the *pIs* of the PLA_2 isoenzymes present in biological fluids in general and snake venoms in particular.

Based on the measured characteristics for the venom PLA_2, it was first resolved as one peak by gel filtration, followed by separation by cation exchange chromatography according to the *pI* differences (Soares *et al.,* 1998; Ramirez-Avila *et al.,* 2004; Zouari-Kessentini *et al.,* 2009; Romero-Vargas *et al.,* 2010; Landucci *et al.,* 2012; Zou *et al.,* 2012). Recently, other methods which include reverse-phase high-performance liquid chromatography (RP-HPLC) (Sim, 1998; Higuchi *et al.,* 2007; Rodrigues *et al.,* 2006; Calgarotto *et al.,* 2008; Pereanez *et al.,*2009; Romero-Vargas *et al.,* 2010), capillary zone electrophoresis and mass spectrum were used for purification and identification of the venom PLA_2s (Zhang and Gopalakrishnakone, 1999; Singh *et al.,* 2000; Weinberger, 2001).

At the molecular exclusion step, almost all of the *N. nigricollis* venom enzymatic PLA_2 activities were recovered in the pool designated as *N. nigricollis* Seph-PLA_2 fraction representing 75 % of the venom. At the cationic-exchange chromatography step the *N. nigricollis* Seph-PLA_2 was separated into four *N. nigricollis*-PLA_2 (named *N. nigricollis* CM-PLA_2I, II, III and IV) with recovery of (11.3, 19, 5.4 and 4.6 %), respectively.

The specific activity of *N. nigricollis* CM- PLA_2I, II, III, IV fractions were 6.7, 40, 10 and 15.4 U/mg protein, respectively, demonstrating that enzymatic PLA_2 specific activity of the isoenzymes are quite different. In addition, the results showed that the highest PLA_2 specific activities were 40 U/mg for *N. nigricollis* CM-PLA_2II indicating four-fold increase in the *N. nigricollis* venom PLA_2 specific activity.

The molecular weights of the *N. nigricollis* CM-PLA_2 isoenzymes resolved from *N. nigricollis* venom were close to 14 kDa which agree with the range generally reported for snake venom PLA_2 (Kemparaju *et al.,* 1999; Bonfim *et al.,* 2008; Landucci *et al.,* 2012; Zou *et al.,* 2012). The kinetics of the PLA_2

isoenzymes revealed that, the relative K_m values for *N. nigricollis* CM-PLA_2 isoenzymes were close (1, 1.3, 1.6 and 1.8) for *N. nigricollis* CM-PLA_2 (I, II, IV and III), respectively.

The optimal temperature values of the *N. nigricollis* venom, *N. nigricollis* CM-PLA_2 (I, II) and *N. nigricollis* CM-PLA_2 (III, IV) fractions were different. Very weak PLA_2 activities were observed between 10 and 22° C for all *N. nigricollis* CM-PLA_2 isoenzymes except *N. nigricollis* CM-PLA_2I, while the PLA_2 activity of these isoenzymes were completely inhibited at 80° C. The crude *N. nigricollis* venom possessed maximum activity at 65° C, which agrees with Nair *et al.* (1976) who found that the venom of several *Naja* species (Elapidae) showed their maximum activity at 65°C.

The optimal pH values at 41° C for *N. nigricollis* CM-PLA_2 (I, II) and *N. nigricollis* CM-PLA_2 (III, IV) were different. The catalytic activity of the *N. nigricollis* CM-PLA_2I was completely inhibited at pH 8. Whereas, the catalytic activity of all the other fractions was completely inhibited at pH 9. These differences may be related to the enzyme conformational change by pH (Viljoen *et al.,* 1975; Arni & Ward, 1996).

Effect of metal ions on enzymatic PLA_2 activity of the isolated fractions of *N. nigricollis* is markedly different. As most of the PLA_2s (Murakami *et al.,* 2006), the Ca^{+2} ion was found to be essential for the PLA_2 mediated hydrolysis of phospholipids (Ca^{+2}-dependent). The optimal Ca^{+2} concentration values were different, 10 mM for all fractions except 5 mM for the *N. nigricollis* CM-PLA_2 III and IV. These differences in the concentrations of Ca^{+2} are probably related to the enzyme conformational change produced by this divalent ion. The catalytic activity was slightly activated by the Na^+ and variably inhibited by the AL^{+3}, Co^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2} and Ni^{+2} ions. The *N. nigricollis* CM-PLA_2 (II, III) were activated by K^+, whereas, the *N. nigricollis* CM-PLA_2II was activated by Mg^{+2} ion. However, all the other fractions were completely inhibited by the K^+, Mg^{+2} and Zn^{+2}. This diversity in metal requirements might reflect different requirements for the different PLA_2s functions. The data obtained from the present study provide biochemical information about the *N. nigricollis* phospholipase A_2 that help in studying their pharmacological effects.

In attempt to investigate whether or not the potency of antisera correlates with the inhibition of PLA_2 enzymatic activity of the homologues venoms. Antisera to *N. nigricollis* venom and the corresponding PLA_2 fractions were raised in rabbits using the same immunization schedule.

In the present study, the elicited antibodies to the PLA_2 isoforms contribute differently but significantly to the *in vitro* neutralization of enzymatic PLA_2 activity of the corresponding venom. A highly significant correlation between the *in vitro* neutralization of enzymatic PLA_2 activity and the ELISA antibody titers elicited to selected partially isolated PLA_2 was established.

The *in vitro* neutralization of PLA_2 activity and ELISA titers have been exploited to evaluate the neutralizing ability of antivenoms, therefore, avoiding large-scale use of mice, antigen and antiserum and to simplify and cut down the expenses for potency evaluation (Alape-Giron *et al.,* 1997; Maria *et al.,* 1998; Rial *et al.,* 2006), in addition to adherence to ethical concepts.

There is a strong body of evidence indicating that the functional activity of some PLA_2 is independent of its *in vivo* activity (Kini & Evans, 1989; Kini, 2003). In spite of these observations, Successful correlates for testing the potency of horse antivenins were established (Alape-Giron *et al.,* 1997; Maria *et al.,* 1998; Rial *et al.,* 2006). These correlates include the in *vivo*

neutralization of lethality, neutralization of the hemolytic activity (PLA_2) and levels of antibodies measured by ELISA. In the present study, a good correlation for neutralization of the enzymatic PLA_2 activity and the ELISA titers was found for sera collected at one week from each boosting of the rabbits ($r>0.9$). The found correlations were particularly high when the isolated Seph-PLA_2 fraction were used rather than the whole venom for establishing the correlations. However, attempts to correlate the same traits for all the samples from an individual immunized animal or from timed samples from different animals were not successful.

In the present study, the indirect hemolytic assay developed by Gutierrez *et al.* (1988) for *in vitro* neutralization of PLA_2 activity was applied. The assay has several advantages, which include sensitivity and detection of very low levels of antibodies (Abs), easy, fast, and many samples can be studied simultaneously. This allows testing of the immune response of individual animals during extended immunization schedule in order to monitor antibodies production (Alape-Giron *et al.*, 1997).

The results in the present study revealed that the antisera to the isolated PLA_2 were more potent than the anti-whole venom. This could be attributed to the complexity of the venom antigens compared to the isolated PLA_2s that are antigenically less diverse (Freitas *et al.*, 1990; Beghini *et al.* 2005) suggested that commercial antivenoms enriched in anti-PLA_2 antibodies could be useful for treating neurotoxicity in envenomed persons. In addition, the production of antivenoms against the main toxic component(s) of the venom of interest would reduce the amount of non-essential antibodies and proteins injected during antivenom therapy and could therefore result in safer treatment (less risk of side effects such as anaphylactic shock).

The data obtained from the present study give potential application in evaluation of the antivenom production. As the therapeutic antivenoms are traditionally produced in large animals, the established correlations helps in cutting down the production costs and overcome the ethical arguments concerning the extensive use of experimental animals.

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