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Partial Purification and Characterization of Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum

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Authors' contributions

This work was carried out in collaboration between all authors. Author FAA designed the study, wrote the protocol and author AJN supervised the work. Authors FAA and NA carried out all laboratories work and performed the statistical analysis. Author FAA managed the analyses of the study. Author NA wrote the first draft of the manuscript. Author EO managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The most effective and acceptable therapy for snakebite victims is the immediate administration of antivenin following envenomation which is limited by problems of hypersensitivity reactions in sensitive individuals and its inability to resolve the local effects of the venom. Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum (PIES) was isolated, partially purified and characterized. The neutralizing protein from *E. ocellatus* serum inhibited the *E. ocellatus* (carpet viper) venom phospholipase A_2 (PLA_2) enzyme in a dose dependent manner. A two step purification process on sephadex G-200 column chromatography and DEAE- cellulose chromatography gave an active fraction that inhibited the venom PLA_2 by 78%. The result from SDS-PAGE showed the inhibitor to

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be a 24.98kDa protein and its kinetic study revealed a mixed pattern of inhibition on the carpet viper venom PLA_2 with an estimated K_i values of 3.8%(v/v) to 7.3%(v/v). The study was carried out at the Department of Biochemistry, Faculty of Science, Ahmadu Bello University Zaria, Nigeria from June 2011 to August 2012. The relevance of these findings towards understanding the biochemistry of carpet viper envenomation and the development of a novel antivenin drug in future targeting the activity of PIES are discussed.

Keywords: Envenomation; antivenin; carpet viper; phospholipase A_2; neutralising protein.

1. INTRODUCTION

Snakebite is a global medical health problem especially in the rural areas of the tropics with about 40,000 deaths and 375,000 cases of permanent physical disabilities worldwide annually [1,2]. The annual snakebite incidence in northern Nigeria has been estimated to be 497 per 100,000 populations with 12.2% mainly due to the carpet viper, *Echis ocellatus* [3]. Based on hospital records, the four most deadly northern Nigerian snakes are *Echis ocellatus*, *Naja nigricollis, Bitis arietans* and *Naja katiensis* [4]. The incidence of snakebite is often associated with agricultural activities with the highest at the beginning of the rainy season. The other important factor responsible for the increase in snakebite cases is flood which drives out snakes from their burrows [5].

Snake venom, the most complex of all poisons is a mixture of enzymatic and non enzymatic toxic compounds as well as other nontoxic proteins, non proteins including carbohydrates and metals all stored in the poison gland. The pathophysiologic base for morbidity and mortality is the disruption of normal cellular functions by these enzymes and toxins. Some of these proteins include enzymes like phospholipase A_2, B, C, D, hydrolases, hyaluronidases, phosphatases, esterases, phosphodiesterase, neuclosidases and metalloproteases [6].

Phospholipase A_2 (PLA_2) is a lipolytic enzyme that hydrolyses the fatty acyl ester bonds at the 2-sn position of membrane phospholipids producing equimolar amounts of free fatty acid (FFA) and lysophospholipid, mainly arachidonic acid (AA); these products then become available for conversion to potent pro inflammatory mediators, such as platelet-activating factor and eicosanoids [7,8] respectively. The enzyme from snake venom is primarily used for trophic and defence functions in most species but show wide range of pharmacological activities such as neurotoxicity, myotoxicity, cardiotoxicity, with a greater impact on platelet aggregation and blood coagulation [9-11].

Venomous snakes are resistant to their own venom and several natural neutralizing proteins have been identified in their plasma [12]. In particular, PLA_2 inhibitors have been isolated from various snake sera and their primary structures have been determined [13]. However, information as regards PLA_2 inhibitor from *E. ocellatus* serum is still scanty and yet to be fully elucidated.

The main concern about the empirical use of antivenin is the limited efficacy against the local tissue damaging activities of venoms, its relative scarcity and cost factor [14,15]. Thus, specific inhibitors may alleviate the local effects of the venom, which is difficult to achieve with conventional antivenins. Therefore, in this study, we report on the purification and characterization of PLA_2 inhibitor from *E. ocellatus* serum with the view to obtain the kinetic data and establish other characteristics of the protein that would be important in achieving the said objective.

2. MATERIALS AND METHODS

2.1 Materials

Reagents were purchased from Sigma Chemical Company, St. Louis, U. S. A. Desiccator, Refrigerator, Micro pipette, Burette, Hot air oven, Water bath, SDS chamber. Twelve (12) adult *Echis ocellatus* snakes were obtained from Kaltungo, Gombe State, Nigeria and identified at the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria, Nigeria.

2.2 Collection and Preparation of Venom

Echis ocellatus identified were kept in wooden boxes. They were fed raw meat fortnightly. Venom was collected by modified milking method as described by Markfarlane. Briefly silica gel was activated at 80℃ for 2 hours in an oven, placed in a dessicator and kept in a refrigerator. The snakes were restrained at the position of the joint between the last cervical vertebrae and the skull, using the thumb and the fore-finger, care

was taken to avoid strangulation. A cellophane piece was used to cover a beaker and held in position using rubber band. The second hand was used to hold the beaker and brought to make contact with the mouth of the snake, such that as the snake bites through the cellophane using its upper fangs, venom was ejected into the beaker. The venom from all the snakes were pooled and immediately placed in a desiccator with activated silica and allowed to crystallize at 4℃. The crystallized venom was then transferred into eppendorf tubes, labelled properly and kept in a deep freezer at – 18℃. These were referred to as crude venom [16].

2.3 Collection and Preparation of Serum

1ml per day of blood for 3 days was obtained from each snake through the caudal vein on alternate days. The blood collection was carried out under the supervision of Dr. Ofemile Peter, Department of Veterinary Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. After blood clotting at room temperature, the serum was separated by centrifugation at 3000xg for 5mins and kept in the refrigerator until further use [17].

2.4 Partial Purification of Phospholipase A_2 from Crude Venom

Briefly, 2 ml of 10 mg/ml of crude *E. ocellatus* venom was loaded onto DEAE cellulose column (1.5 x 50 cm) pre-equilibrated with 50 mM phosphate buffer pH 6.8. The column was eluted stepwise with NaCl gradient (0.01 – 0.1 M) at a flow rate of 0.2 ml/min. 2 ml each of 20 fractions were collected and assayed for phospholipase A_2 activity and total protein. The PLA_2 active fractions were pooled together and loaded on sephadex G-75 column equilibrated with 50 mM phosphate buffer (pH 6.5). The column was eluted with the same buffer, maintaining a flow rate of 1ml/min. 2ml each of 20 fractions were collected and assayed for PLA_2 [18] and total protein concentration by taking absorbance at 280 nm [19].

2.5 PLA_2 Assay

Briefly, 25 µl of 1 mg/ml L-α-lecithin substrate was incubated with 10 µl of the partially purified enzyme from *E. ocellatus* venom for 10 min at 37℃. The reaction was then terminated by immersing the tube in a boiling water bath for 2 min and the amount of released free fatty acid measured titrimetrically at pH 8.0 using 20 mM sodium hydroxide and phenolphthalein indicator. The activity of phospholipase A_2 was defined as the amount of enzyme that hydrolyses 1 µmole of fatty acids from L-α -Lecithin per minute under standard conditions.

$$\text{(L-α-LECITHIN} \xrightarrow{PLA_2} \text{FREE FATTY ACID (FFA) +LYSOLECITHIN)}$$

The phospholipase A_2 activity was given as thus,

Volume of 20 mM NaOH that neutralized free fatty acid = ymL

$0.02 \times y \times 10^{-3}$ = k moles/10 min

Enzyme activity = k moles/10 min = Z moles/min.

2.6 Determination of Protein Concentration

The protein concentration of the partially purified PLA_2 from *Echis ocellatus* venom was determined spectrophotometrically by taking absorbance at 280 nm wavelength.

2.7 Purification of PLA_2 Inhibitor from *Echis ocellatus* Serum

PLA_2 Inhibitor from *Echis* Serum (PIES) was partially purified by a modified method. Briefly, 2 ml of serum collected from the snakes was directly fractionated at 4℃ on a sephadex G-200 column, pre equilibrated with 0.05 M Tris HCl buffer, pH 8.1 containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 3ml/hr. 2ml of twenty fractions were collected and inhibition studies (assay for residual PLA_2 activity) was carried out and fractions exhibiting inhibitory activity against the partially purified PLA_2 from *Echis ocellatus* venom were pooled together and directly loaded on a DEAE cellulose column pre equilibrated with 0.05 M phosphate buffer, pH 6.8. The column was eluted with a stepwise linear gradient of sodium chloride (0.01- 0.1M NaCl) at a flow rate of 5min/ml. 2ml of fifty fractions collected were assayed for residual PLA_2 activity and total protein and fractions exhibiting inhibitory activity against *Echis ocellatus* PLA_2 were pooled together, dialysed and stored at -4℃ until further use.

2.8 Inhibition Studies

To analyze the effect of PIES on PLA_2 activity, briefly, 10 µl of partially purified PLA_2 from *Echis ocellatus* venom, 25 µl of 1 mg/ml L-α-lecithin substrate was incubated for 10 min at 37℃ with

25 µl of various concentrations of PIES (10-50%v/v). The reaction was then terminated by immersing the tube in a boiling water bath for 2 min and the amount of released free fatty acid measured titrimetrically at pH 8.0 using 20 mM sodium hydroxide and phenolphthalein indicator. The inhibition of phospholipase A_2 activity by PIES otherwise referred to as residual PLA_2 activity was defined as the amount of residual enzyme that hydrolyzes 1 µmole of free fatty acids from L-α -Lecithin per minute in the presence of PIES under standard conditions.

2.9 Kinetic Studies

The effect of partially purified PIES on partially purified PLA_2 activity was carried out with varying concentrations of the substrate (2-5 mg/ml) at 0%, 5%, 10% and 20% of PIES. Initial velocity values obtained were used to draw Double Reciprocal plot to determine the inhibition binding constatant (K_i) and to ascertain the kind of inhibition.

2.9.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of the partially purified PIES under denaturing conditions was performed with 12% Acrylamide gel using a tris-glycine buffer, pH 8.3. The protein band was located by staining with Coomassie brilliant blue [20].

3. RESULTS

3.1 Purification of PLA_2 from *E. ocellatus* Venom

The results of the purification of PLA_2 from *E. ocellatus* venom are summarised on Table 1. The crude extract contained about 2.28 mg of total protein with a total activity and specific activity of 7.40 µmol/min and 3.25 µmol/min/mg respectively. Fractionation of the crude venom on DEAE- cellulose chromatography gave a specific activity of 21.5 µmol/min/mg. The subsequent gel filtration on sephadex G-75 chromatography gave an active peak (Fig. 2) with a specific activity of 53.17 µmol/min/mg, 16.36 purification fold and 43.11% recovery.

Fig. 1 shows the elution profile of *Echis* PLA_2 after ion exchange chromatography on DEAE cellulose column. An active peak eluted in fraction 13 of the void volume, had a protein concentration of 0.2 mg/ml and a corresponding enzyme activity of 4.3 µmol/min eluted by 80 mM Nacl.

From the elution profile of *Echis* PLA_2 after gel filtration on Sephadex G-75 column (Fig. 2), an active peak (fraction 12) was found to contain about 0.06 mg/ml protein and a corresponding PLA_2 activity of 3.19 µmol/min.

3.2 Purification of Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum

The results of partial purification of phospholipase A_2 Inhibitor from *Echis ocellatus* serum (PIES) are shown in Figs. 3 and 4. From the results (Fig. 3), the elution of PIES on sephadex G-200 column chromatography produced an active peak (fraction 15) with a protein concentration of about 0.022 mg/ml and a relative inhibition of about 70% against the partially purified *E. ocellatus* PLA_2. Further purification on DEAE cellulose column (Fig. 4) gave an active peak (fraction 37) with a relative inhibition of 0.778 (77.8%) and about 0.26 mg/ml of protein.

3.3 Molecular Weight of Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum (PIES)

The purity and molecular weight of the partially purified inhibitory protein were determined by Sodium Dodecyl Sulphate Polyacrilamide Gel electrophoresis (SDS-PAGE). Fig. 5 shows the electrophoretic pattern of the sample under denaturing conditions. A distinct band of the protein sample was visualised against the standard marker proteins on the gel and the molecular weight of the partially purified PIES (Band A) was estimated to be 24,986.19Da. From the plot of log of molecular weight of the marker proteins against their respective relative mobility (R_f), the molecular weight of the partially purified PIES was found to be 24,986.19Da (Fig. 6).

3.4 Characterisation of Partially Purified Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum (PIES)

A study of the inhibition kinetics of partially purified PIES on partially purified PLA_2 revealed that PIES exhibited a mixed pattern of inhibition (Fig. 7) with K_M decreasing from 4.54 mg/ml without inhibitor to 3.27 mg/ml, 3.27 mg/ml and 3.12 mg/ml at 0%, 5%, 10% and 20% concentration of PIES respectively. The K_i and K_{ii} values were estimated to be 3.89% (v/v) and 7.28% (v/v) (Figs. 8 and 9), respectively.

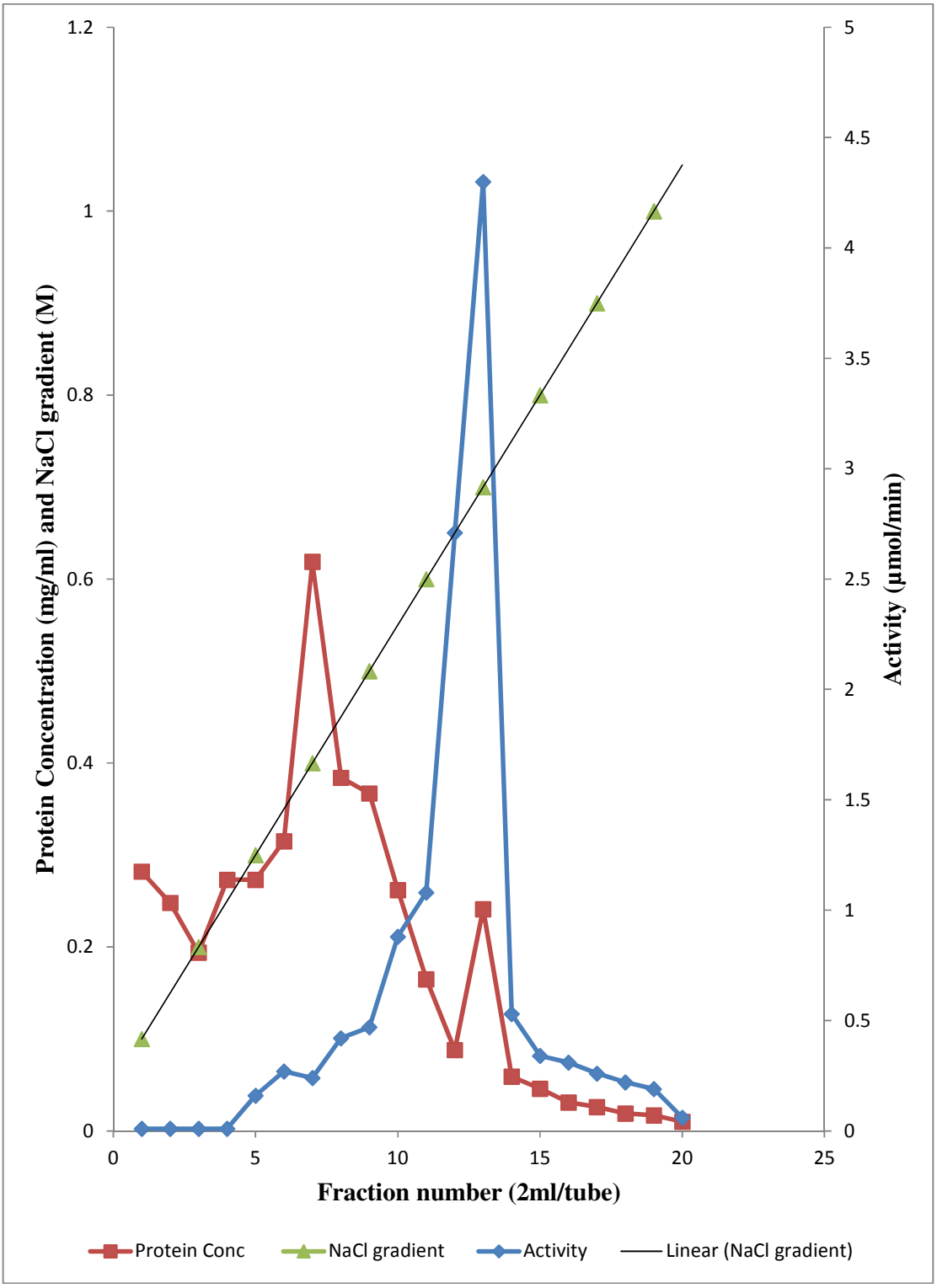

Fig. 1. Elution profile of phospholipase A_2 from *Echis ocellatus* venom on DEAE-cellulose column chromatography (1.6 x 50 cm)

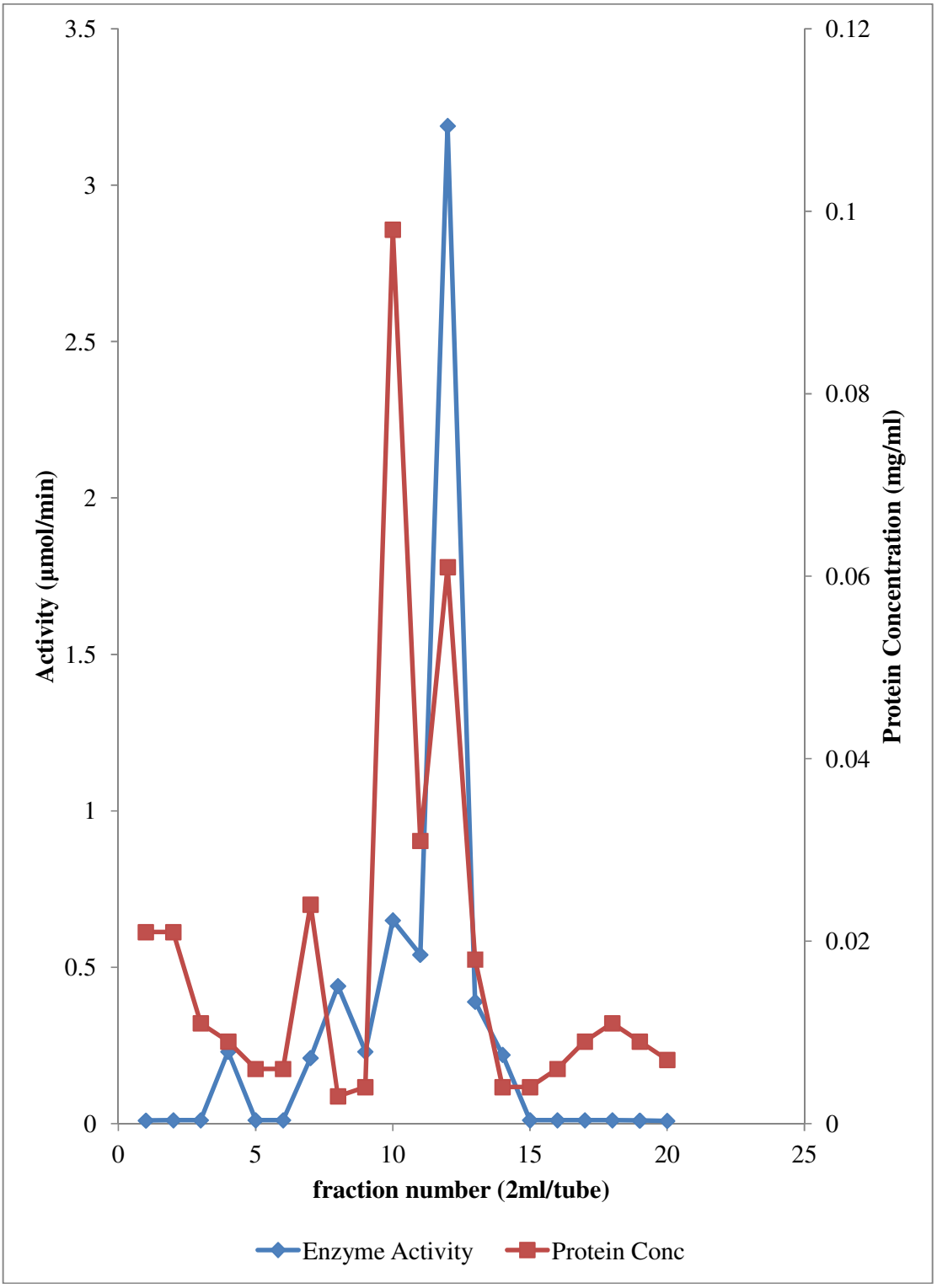

Fig. 2. Elution profile for Phospholipase A_2 from *Echis ocellatus* venom on Sephadex G-75 column chromatography (1.6 x 90 cm)

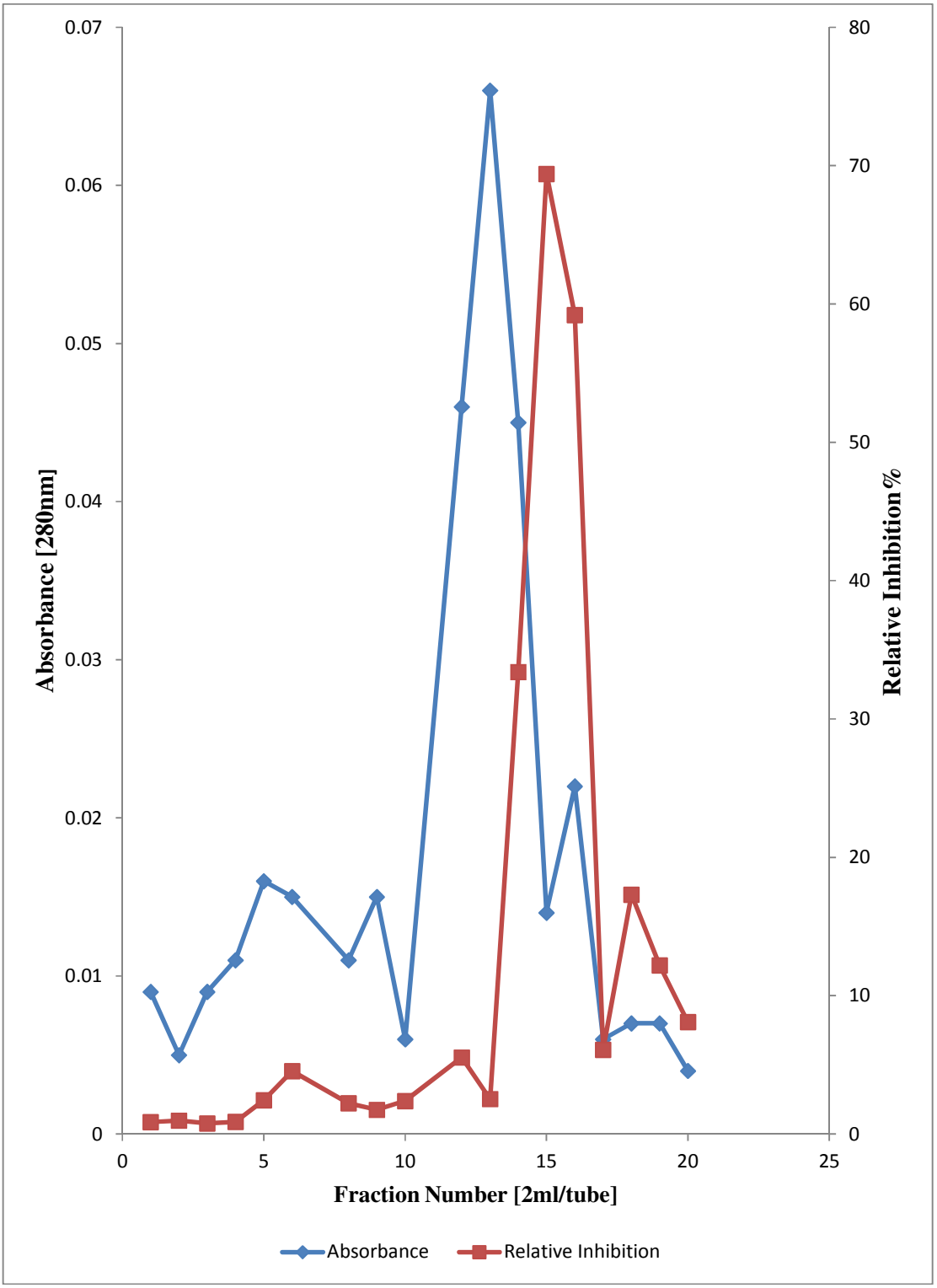

Fig. 3. Elution Profile of phospholipase A_2 inhibitor from *Echis ocellatus* serum (PIES) on Sephadex G- 200 column showing relative inhibition (%) and total protein at 280 nm

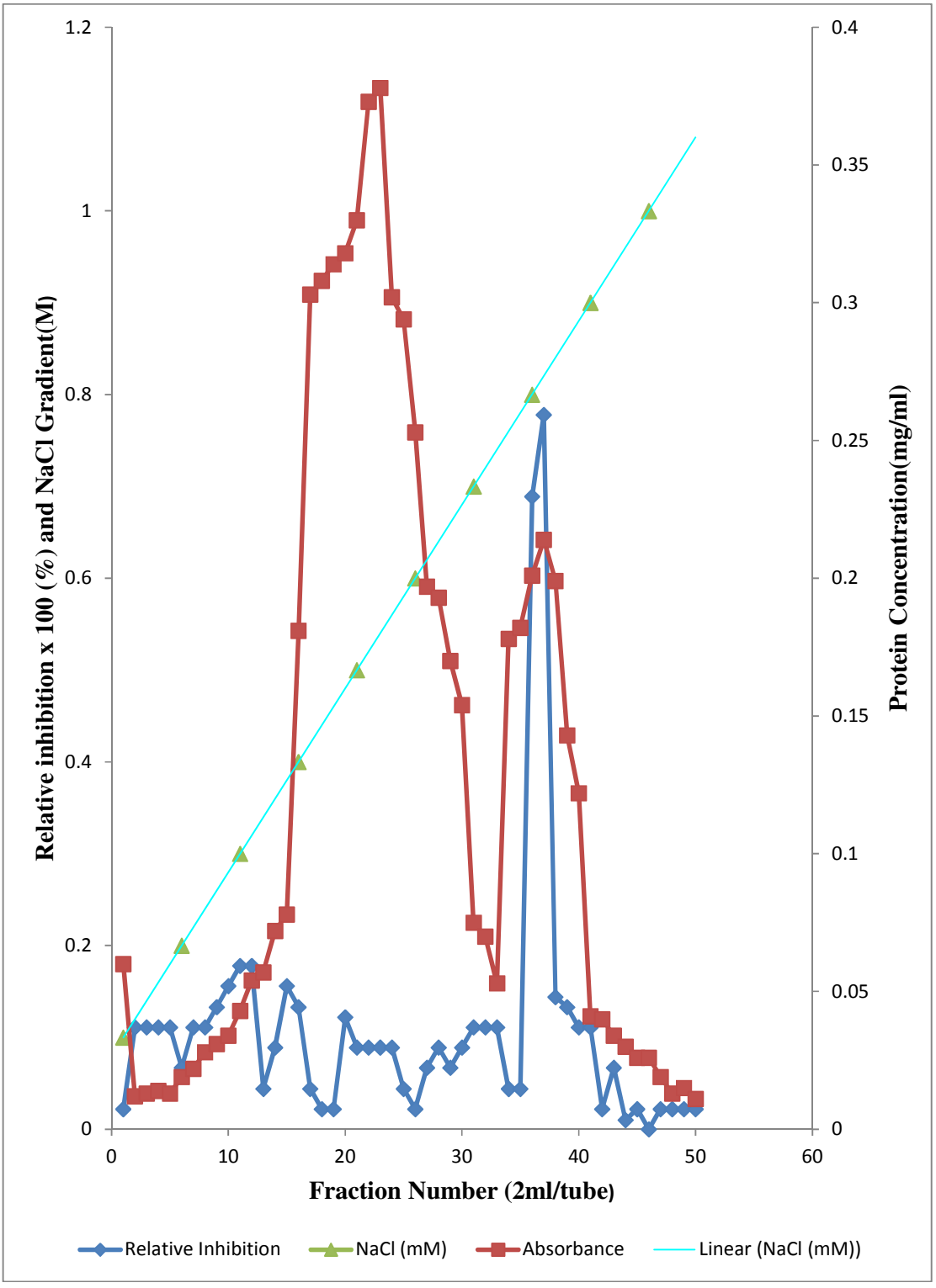

Fig. 4. Elution Profile for Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum on DEAE-cellulose showing Relative Inhibition (%), total protein at 280 nm and NaCl gradient (M)

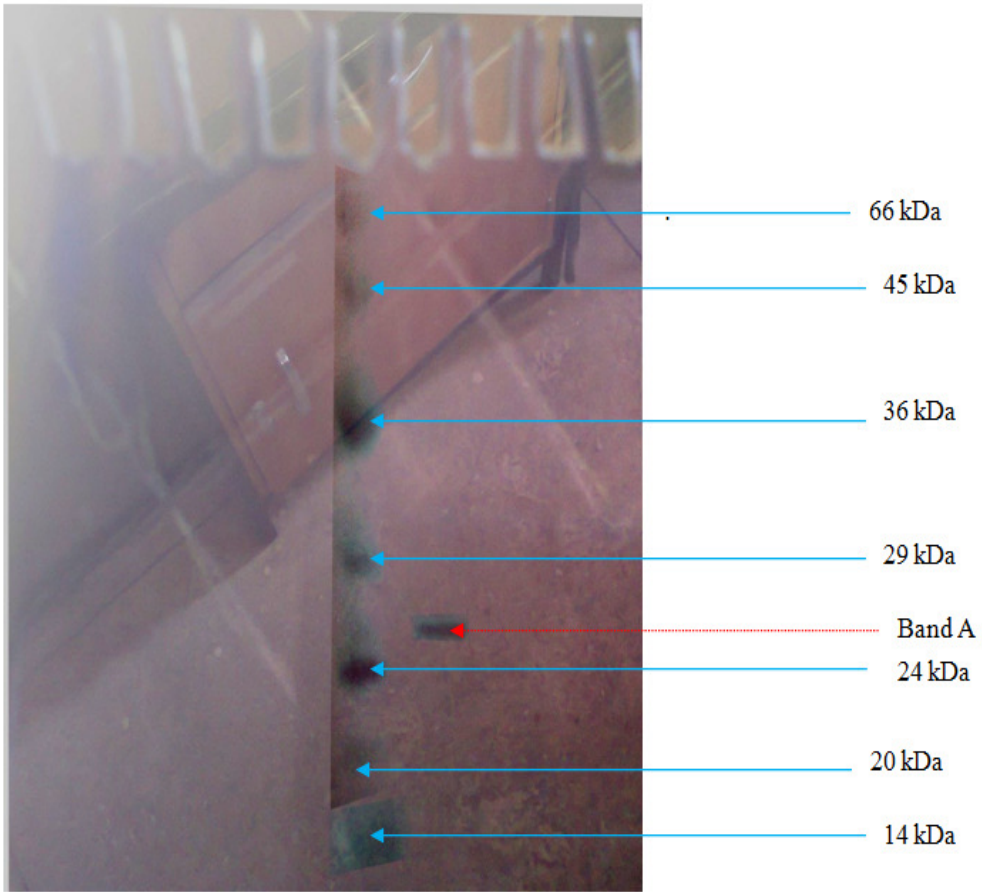

Fig. 5. Electrophoregram of Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum (PIES) on Polyacrilamide Gel using Coomasie brilliant blue staining

Band A represent PIES with an estimated molecular weight of 24.986kDa

Table 1. Purification profile of phospholipase A_2 from *Echis ocellatus* venom

Purification step	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)	Purification fold	Percentage yield
Crude	2.28	7.40	3.25	1.00	100
Ion exchange chromatography on DEAE cellulose	0.20	4.30	21.5	6.62	58.11
Gel filtration on Sephadex G-75	0.06	3.19	53.17	16.36	43.11

**One unit of Phospholipase A_2 was defined as the amount of enzyme that hydrolyses 1 µmole of fatty acids from L-α -Lecithin per minute under standard conditions*

4. DISCUSSION

Toxicological properties of snake bite are thought to be associated with enzymes especially Phospholipase A_2 (PLA_2), which is found to be its most toxic component [21]. Considering the role of PLA_2 in envenomation, understanding the characteristics of the enzyme from snake venom has raised concern for venom researchers, as it would help in the production of effective therapeutic antivenins [22]. In this study, PLA_2 from *E. ocellatus* venom which belongs to the Class II PLA_2 predominantly found in *Viperidae* snakes was isolated and partially purified .The PLA_2 active fraction was eluted in the void volume from ion exchange chromatography on DEAE-cellulose column (Fig. 1). After gel filteration on sephadex G-75 column, an active peak eluted (Fig. 2). From the results, there was an increase in purification fold

from 6.62 to 16.36 (Table 1); while the specific activity also increased from 21.5 µmol/min/mg to 53.17 µmol/min/mg. An increase in purification fold and specific activity of the crude venom PLA_2 after the two purification steps could be attributed to the removal of other synergistically interacting components of the venom.

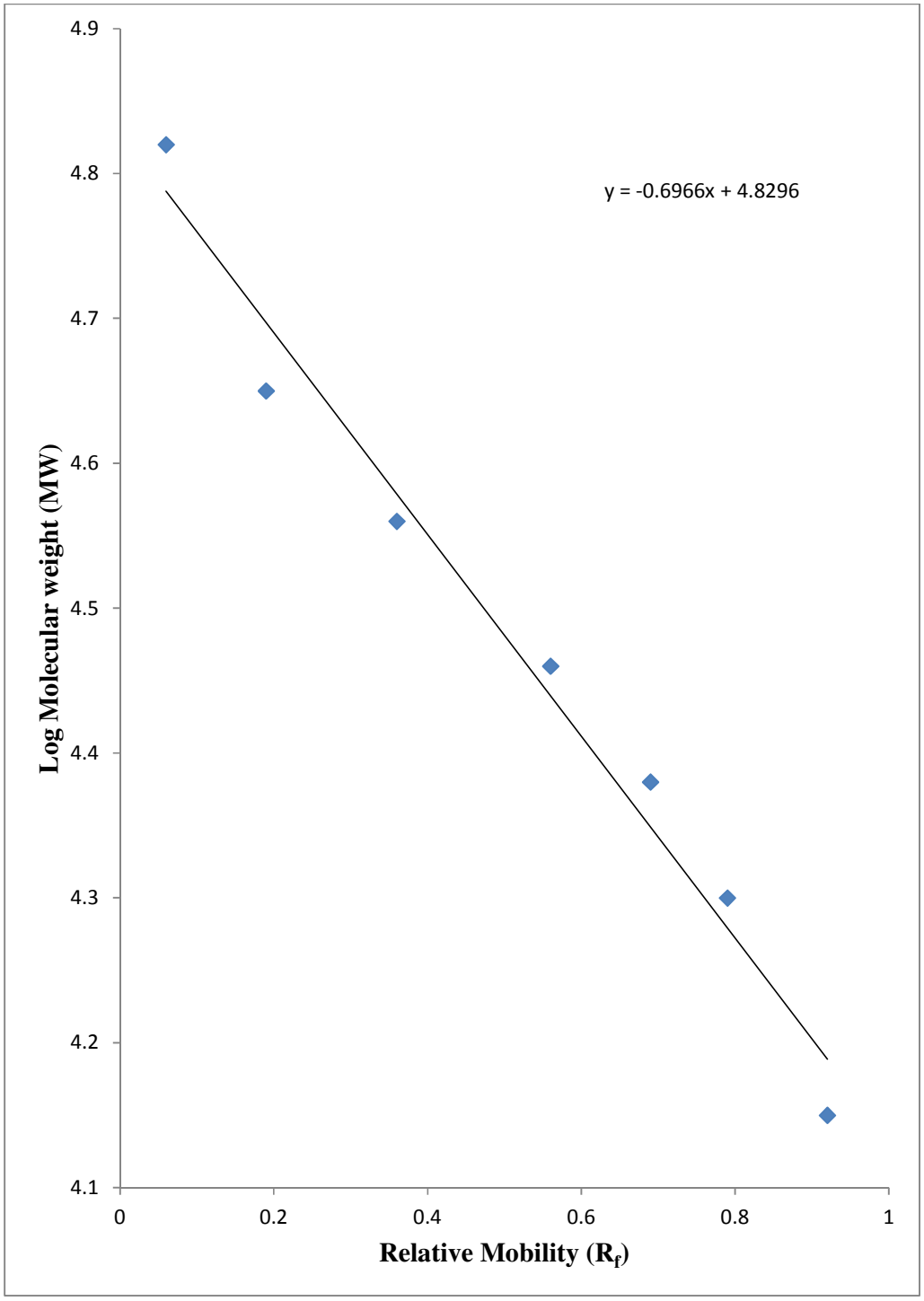

Fig. 6. A plot showing the Log of molecular weight against the R_f of the marker protein bands used to estimate the molecular weight of partially purified PIES

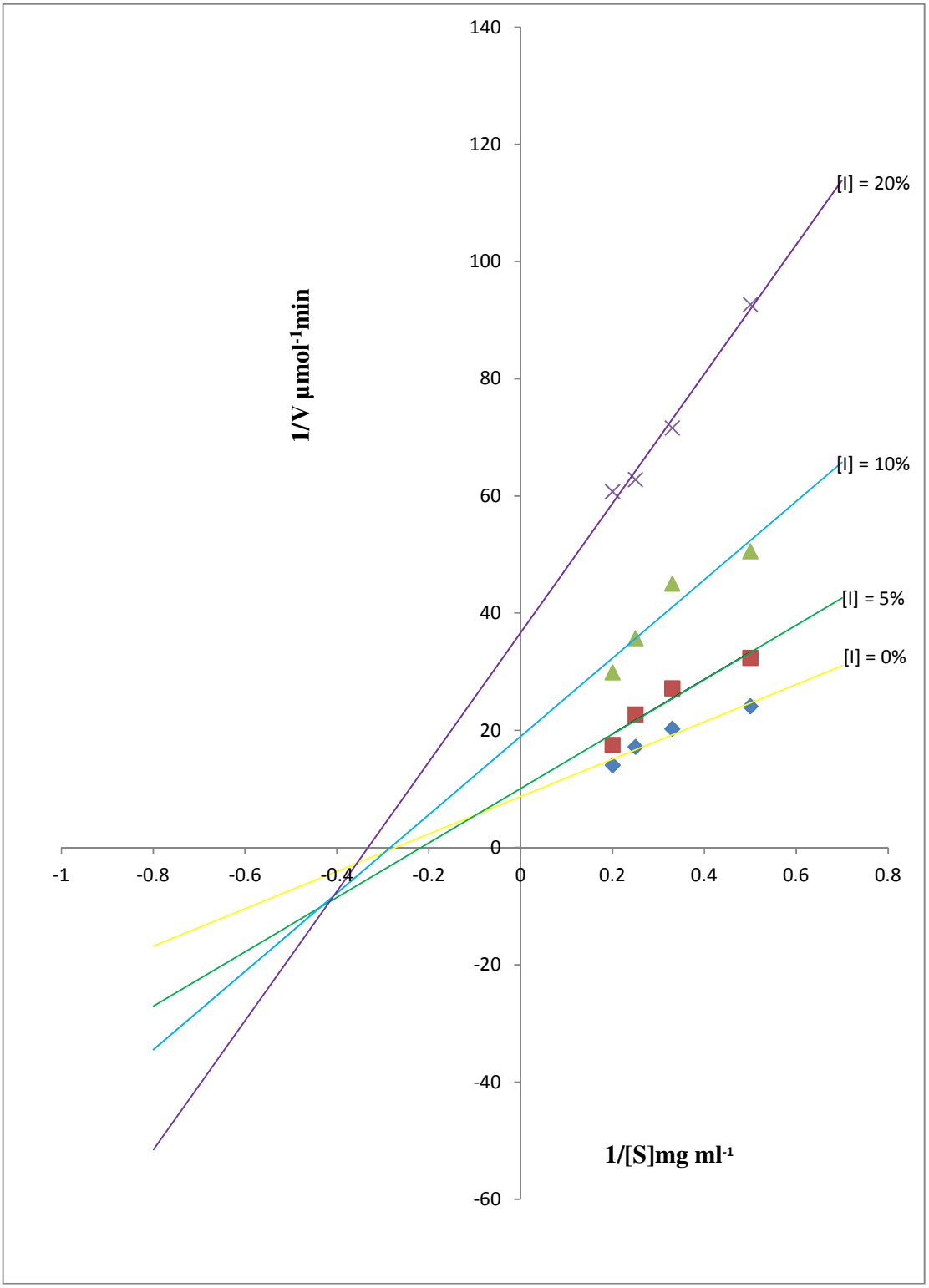

Fig. 7. Double Reciprocal plot showing the effect of partially purified PIES on partially purified *Echis* PLA_2 activity

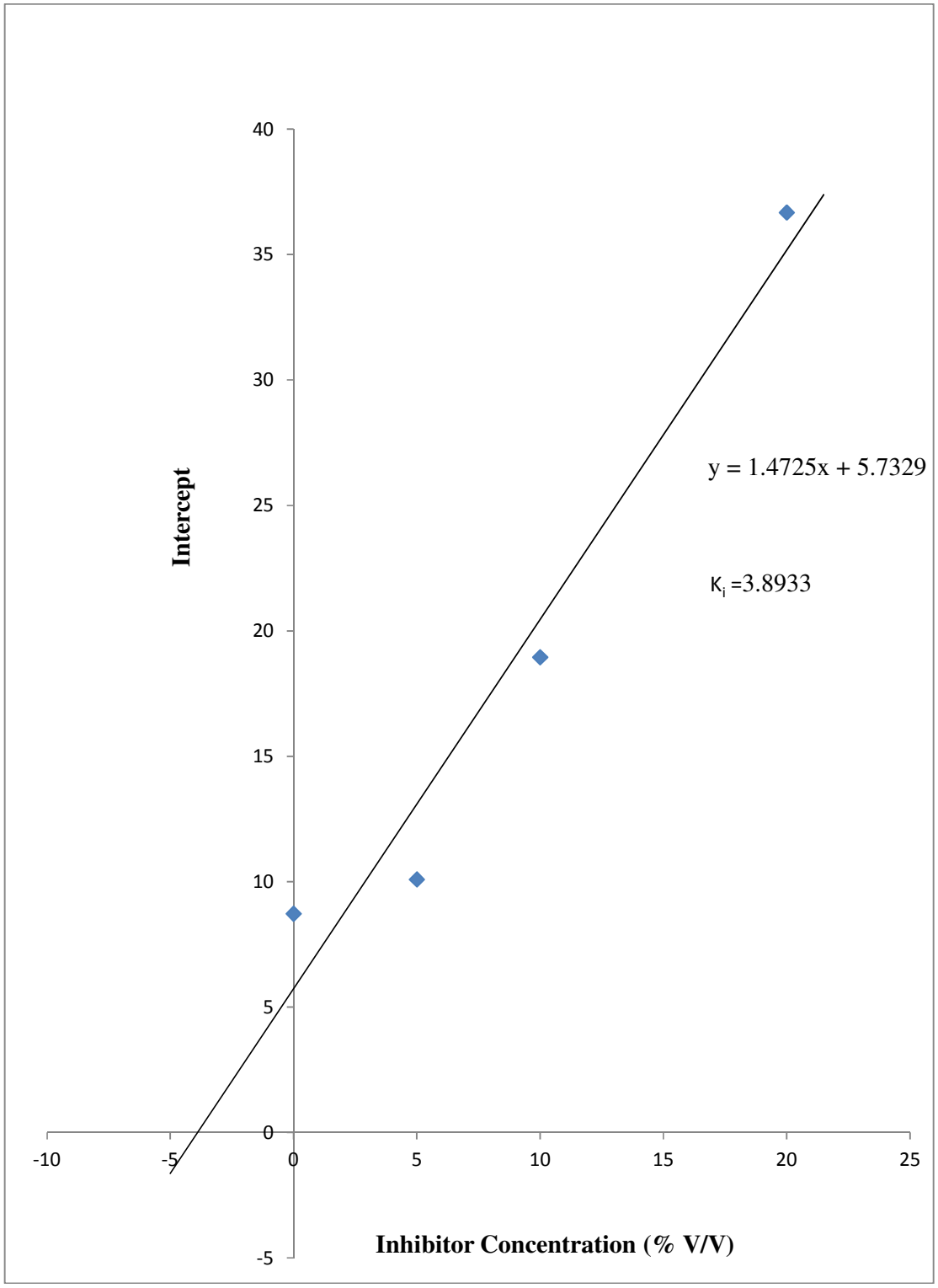

Fig. 8. Secondary plot of intercept against inhibitor concentration showing K_i

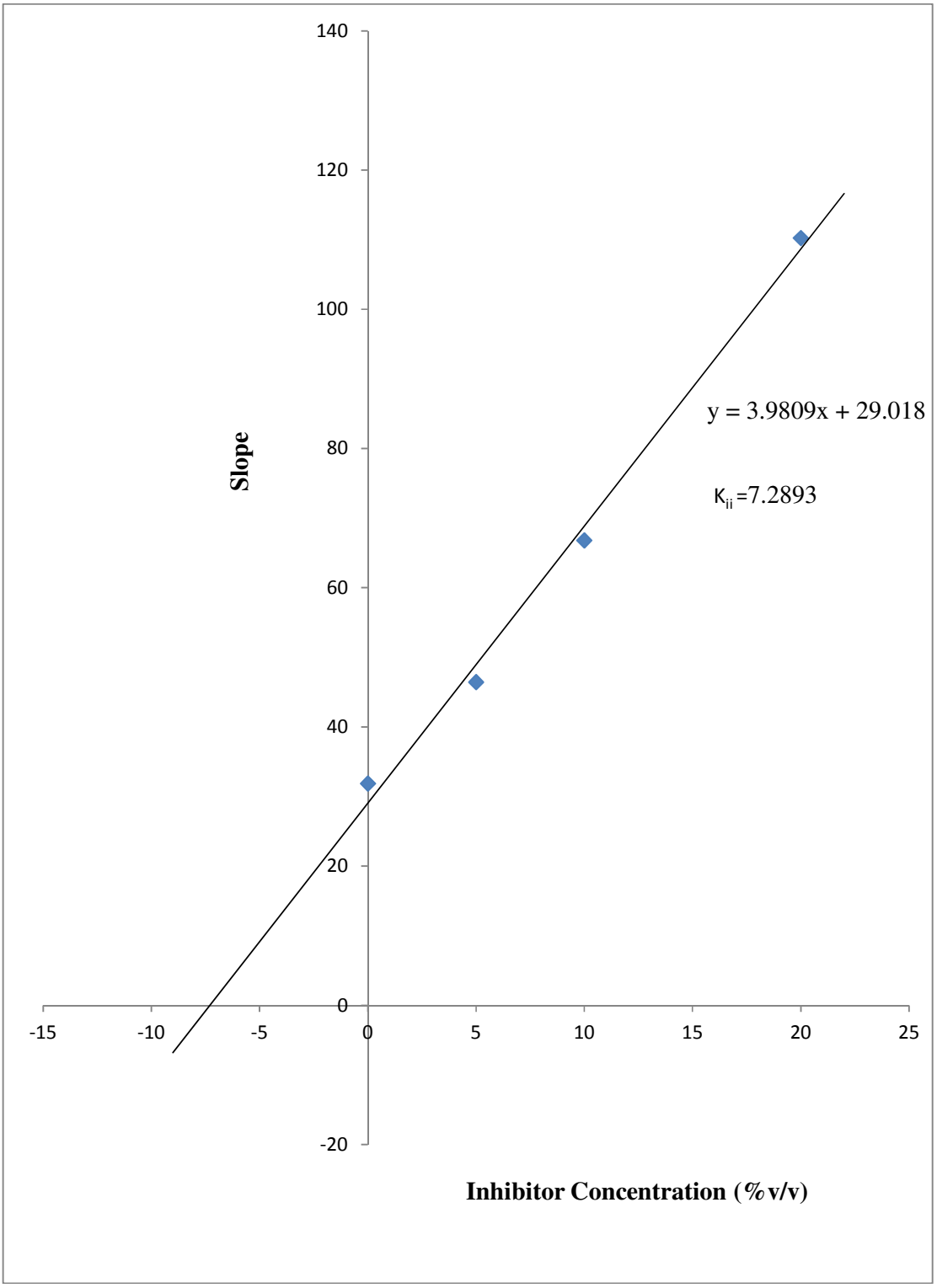

Fig. 9. Secondary plot of slope against Inhibitor concentration showing K_{ii}

Venomous snakes are resistant to their own venom and certain molecules like endogenous antitoxic serum proteins with neutralising capacity against snake venom have been previously reported [23]. In particular, PLA_2 inhibitors have been isolated from various snake sera and their primary structures have been determined [24]. In the present study, a protein PLA_2 Inhibitor from *E. ocellatus* Serum (PIES) was purified in a two-step purification process on sephadex G-200, (Fig. 3) and ion-exchange chromatography on DEAE-cellulose (Fig. 4). An active peak eluted at 80 mM NaCl inhibited the partially purified PLA_2 from the snake venom by 77.8%. The inhibitory effect of PIES may be attributed to its ability to chelate Ca^{2+}, an important co-factor of PLA_2 [25]. The formation of enzyme-inhibitor or toxin-inhibitor complexes has been previously shown to be responsible for the inhibition of the PLA_2 enzyme from *T. flavoviridis* and for neutralization of the major toxins from *Naja naja atra* venom [26]. An anti-neurotoxic factor isolated from the serum of *Vipera palaestinae* forms a complex with an acidic component of the venom which participates synergistically in the neurotoxic action of this venom [27].

The purity of partially purified PIES was checked by SDS-PAGE as shown in Fig. 5. The inhibitor appeared as a single sharp band with an estimated molecular mass of about 25 kDa. This is similar to the molecular weight of PLA_2 inhibitor isolated from the serum of *Crotalus d. terrificus* snake. The isolation of a 23.6-kDa antivenin factor from *Crotalus d. terrifcus* plasma has also been reported [28]. However, proteins with higher molecular weight possessing antivenin activity have also been previously reported, for example, PLA_2 inhibitors isolated from the plasma of *T. flavoviridis* [29] and *Agkistrodon b. siniticus* [30] were found to b 100 kDa and 75 kDa proteins, respectively.

Kinetic study of this research reveals that PIES exerts a mixed pattern (non competitive and uncompetitive) of inhibition on *Echis* PLA_2 (Fig. 7). This is suggestive that a site other than the active site could be involved in the inhibition since incubation of the *Echis* PLA_2 with PIES in ice for several hours did not reverse the inhibition. The result of this study is consistent with the findings that the leaves extract of *Ceiba pentandra* exerts a mixed pattern of inhibition on *Echis* PLA_2 [31]. This may also likely suggest that the PIES-PLA_2 interaction involves strong covalent forces affecting the structure [32]. The K_i values (3.8933-7.2893%) from this study are indicative of a relatively high affinity of the inhibitor for the enzyme (Figs. 8 and 9).

5. CONCLUSIONS

From this study, it is concluded that *E. ocellatus* serum contains phospholipase A_2 (PLA_2) inhibitor with biochemical characters similar to other *Viperidea* PLA_2 inhibitors. It is also envisaged that PLA_2 inhibitor from *E ocellatus* serum (PIES) might prove to be useful in the treatment of the various diseases in which PLA_2 enzymes have been implicated.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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