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Phosphodiesitrase activity of some Egyptian snake venoms: biochemical and immunological characteristics and effect on blood coagulation of phosphodiesterase enzyme from *Naja nigricollis* venom

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ABSTRACT

Phosphodiesterase (PDE) enzyme activity was screened in four Egyptian snake venoms belonging to families Elapidae and Viperadae and a highly active enzyme (Nn-PDEII), from the richest snake venom species Naja nigricollis, was purified and characterized in a set of biochemical, immunological and biological assays. Purification was followed in two successive steps including gel filtration and ion exchange chromatography. The enzyme exists as a dimer with a disulphide bridge between the two subunits. The enzyme showed its optimum activity at 60 ^{o}C and pH 8 and was best stable in alkaline medium (pH 8-9) at 40 ^{o}C up to 1 h, however, it loses ~50% of its activity at 60 ^{o}C. PDE activity was markedly increased by Mn^{+2}, Co^{+2} and Mg^{2+} , increased to a lesser extent by Ca^{2+}, Ba^{2+}, Ni^{2+}, K^{+} and Na^{+} but not significantly affected by Zn^{2+} ions and iodoacetic acid. On the other hand, Al^{3+} and Cu^{2+} ions, PMSF, DTT, EDTA, O-phenanthroline and L-cysteine exhibited obvious inhibitory effects. Nn-PDEII was highly immunogenic to rabbits and could induce antibodies specific to N. nigricollis venom antigens. In addition, anti-PDEII antibodies were able to partially neutralize, in vitro, PDE activity of the enzyme in a dose-dependent manner. Nn-PDEII exhibited potent anticoagulant effect most probably via inhibiting one or more of the coagulation factors within the intrinsic pathway in the coagulation cascade. Characterization of a component of N. nigricollis venom that affect blood coagulation at specific stage could have potential in identifying new drug leads in the treatment of hemostatic disorders.

Key words: Phosphodiesrerase, *Naja nigricollis*, purification, Biochemical, Immunological, blood coagulation

INTRODUCTION

Phosphodiestrases (PDEs; EC 3.1.4.1) are a superfamily of enzymes that catalyze the hydrolysis of phosphodiesters to phosphomonoesters. They exist both intracellularly and extracellularly in a wide variety of organisms [1]. Intracellular PDEs play a key role in signal transduction by regulating the cellular concentration of cyclic nucleotides; cyclic adenosine monophosphate (c-AMP) and cyclic guanosine monophosphate (c-GMP) [2-3]. For their special role as specific target enzymes, PDEs have been particularly attractive for researchers in the field of pharmacology [4-5], and [6] described the ongoing time exciting for the field of PDE research advances for the development of new drugs.

Snake venom PDEs are present extracellularly and are referred as exonucleases as they also catalyze DNA and RNA in a characteristic progressive fashion beginning at the 3′ end of polynucleotides, liberating 5′-mononucleotides at basic pH [7]. Subsequently, they have long been used to elucidate the structure and sequence of nucleic acids [8,9]. Venom PDEs represent one of the more minor, though pervasive, enzyme group of snake venoms [10] and are found more abundant in the venoms of Viperidae and Crotalidae [11-12] than Elapidae and Hydrophidae families [13-15], however, few studies dealt with the immunological and/or biological activities of the enzyme. This may be due to a previous assumption that these enzymes are non-toxic and are only involved in the digestion of the prey. The renewed interest evolved after [16-17] suggested the central role of PDE in envenomation strategies where they

endogenously liberate purines and pyrimidines derivatives which act as multitoxins. Generation of adenosine, in particular, is pharmacologically important as it seems be involved in smooth muscle relaxation and vasodilation as well as other effects on cardiovascular function [18-19] which facilitate prey immobilization via paralysis and hypotension [20]. Being unaffected by intracellular PDEs suggested that venom PDEs are particularly distinct at their functional sites. Such high functional specificity makes venom PDEs good targets for designing specific drugs that would have minimal side effects [21].

As snake venom PDEs are very interesting biochemically, medically and pharmacologically and as they do not have enough description in Egyptian snake venoms, we aimed in this report to investigate the presence of these enzymes in the venoms of some Egyptian snake species and study some biochemical, immunological and biological properties of the major PDE enzyme isolated from the snake species, *Naja nigricollis*, that exhibited the highest PDE enzymatic levels among venoms from other tested snake species.

EXPERIMENTAL SECTION

Snake venoms and commercial antivenom

Pooled venoms from snakes of two Elapids; *Naja haje* (Nh) and *Naja nigricollis* (Nn), and two vipers; *Cerastes cerastes* (Cc) and *Cerastes vipera* (Cv), were milked from several members collected from their natural habitat. The venoms were lyophilized and stored at - 20 °C until used.

Purification of PDE enzyme From N. nigricollis venom

Pooled *Nn* venom (100 mg) dissolved in 1 ml of 50mM Tris-HCl, pH 8 buffer was loaded on Sephadex 100 column (1.6 × 90 cm). The buffer was used for equilibration of the column and elution of the loaded samples. Fractions of 4 ml were collected at a flow rate of 24ml/h using a fraction collector (Pharmacia LKB, Sweden). The active fractions showing PDE activity were pooled and directly applied on DEAE-Sepharose column (1.6 × 15 cm) equilibrated with the same buffer. The unbound proteins were washed with the equilibration buffer, while the bound proteins were eluted by step-wise gradient of NaCl (0 - 1 M) at a flow rate of 36 ml/h. 3 ml fractions were collected and the PDE active fractions were pooled and stored at 4 °C. Absorbance of elutes was monitored at 280 nm and the enzyme fractions were stored at -20 ^{o}C.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis under non-denaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of [22] using Tris-glycine, pH 8.3 buffer. Protein bands were located by staining with silver nitrate according to [23].

Molecular weight determination

Molecular weight (MW) was determined by: (***i***) Gel filtration technique using Sephacryl S-200 according to [24]. Sephacryl S-200 column (1.6 × 90 cm) was calibrated with the standard protein markers; cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and b-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume. (*ii*) SDS-PAGE; Electrophoretic analysis was performed in the Mini- Protean II Dual-Slab Cell (BioRad, USA). Preparation of gels, samples, and electrophoresis was performed according to [25]. Prestained protein markers were used as a standard.

Phosphodiesterase (PDE) activity

The assay was conducted in a 96-well microplate in duplicate according to the method of [26] with slight modifications. Briefly, 10 µL of crude venom/enzyme was incubated with 90 µl of 5 mM of bis (p-nitrophenyl) phosphate as a substrate dissolved in 50 mM of Tris–HCl, pH 8.0 buffer at 37 ^{0}C for 1 h. The reaction was stopped by the addition of 50 µl of 50 mM NaOH, and the absorbance was read at 405 nm in a microplate reader and corrected to a path length of 1 cm. The specific activity of phosphodiesterase was expressed in nmol of p-nitrophenol/min/mg lyophilized venom. One unit of PDE is defined as the amount of the enzyme that hydrolyzes 1 nmole of bis (p-nitrophenyl) phosphate per min under the specified assay conditions.

Protein determination

Protein content in venom samples was determined according to [27] method using bovine serum albumin (BSA) as a standard.

Enzymatic properties

Effect of temperature and pH on Nn-PDE activity

Nn-PDE activity was measured individually either at different temperatures in the range between 4^{o}C and 100^{o}C using 50 mM Tris-buffer pH 8, or at different pHs in the range between 3-9 using 50 mM of citrate buffer (pH 3–4),

acetate buffer (pH 4–5.5), sodium phosphate buffer (pH 5.5-7.5), and Tris–HCl buffer (pH 7.5–9) at 37^{o}C. The final enzymatic activity was determined according to Sales and Santoro (2008).

Effect of temperature and pH on Nn-PDE stability

Thermal stability was determined by pre-incubating a constant volume (10 µl) of PDE enzyme individually for 1 h at different temperatures (4-100 °C), followed by cooling in ice bath prior to substrate addition. While the pH stability was determined by pre-incubating separately 10 µl of the enzyme (s) with different buffers as mentioned above in the range between pHs 3 and 9 pHs at 37 ^{o}C for 1 h, before adding the substrate. The final enzymatic activity was determined as mentioned above.

Effect of metal ions and inhibitors

The influence of different metals and inhibitors on Nn-PDE activity was investigated by co-incubating the enzyme with the metal ions (10 mM) or the inhibitors (5 mM) individually at a final volume of 10 µl in Tris-HCl buffer, pH 8 at 37 °C prior to addition of the substrate. The residual PDE activity was then measured as mentioned above.

Effect of different concentrations of bis (p-nitrophenyl) phosphate

The assay was performed using different concentrations of bis (p-nitrophenyl) phosphate substrate (0 - 7.5 mM) and PDE enzyme at a final volume of 10 µl containing 0.05 µg protein. The V_{max} and k_m were calculated from a Lineweaver-Burke plot according to [28], where V_{max} represents the maximum rate achieved by the system, at maximum saturation of the substrate concentration while the K_m, Michaelis constant, is the substrate concentration at which the reaction rate is half that of V_{max}.

Immunological studies

Immunization of rabbits and preparation of anti-PDE antisera

Polyclonal antibodies against Crude *N. nigricollis*, PDEI, PDEII and PDEIII were raised in New Zealand rabbits (2-3 kg). All rabbits received a constant dose of 5 µg in 0.5 ml physiological saline at ~three week intervals all over the immunization period (12 weeks). The primer dose was emulsified in 0.5 ml of complete Freund's adjuvant, whereas subsequent booster doses were emulsified in 0.5 ml of incomplete Freund's adjuvant. The rabbits were bled every week from the eye vein. The blood was incubated at 37^{o}C for 2 h then placed in the refrigerator overnight at 4 ^{0}C. The serum was separated from blood cells by low speed centrifugation (2000 rpm) for 10 min at room temp and kept at -20 ^{0}C till used. Serum samples from a rabbit receiving only saline was used as a reference control.

Indirect ELISA

96 wells microtiter plates (Dynatech) were coated overnight at 4^{0}C with 100µl of 5µg/ml solution of the crude *N. nigricollis* in the coating buffer (0.05 M carbonate buffer pH 9.6). The plates were washed 3 times with PBS-T buffer (100 mM PBS pH 7.5 containing 0.05% Tween 20) and incubated for l h at 37^{0}C with 150µl/well of the blocking buffer (100 mM PBS pH 7.5containing 2% gelatin). After wash, serial two fold dilutions of the tested rabbit antisera in the PBST were dispensed into duplicate wells previously coated with the above mentioned antigens and incubated for 1 h at 37^{0}C. After wash, rabbit anti-horse IgG-peroxidase conjugate diluted in PBS (1/3000) was added (100ml/well) and incubated for 1 h at 37^{0}C. The plates were thoroughly washed for 3–5 times with PBST buffer before allowing them to react with orthophenelene diamine (OPD) in (100ml/well). The reaction was allowed to proceed for 15 min at room temperature in the dark before the addition of 50 ml 2 N Sulfuric acid. The developed absorbance at 490 nm was measured in a Micro ELISA Reader Photometer. The dilutions at which the absorbance of the test antisera was 0.5 were graphically interpreted, their reciprocals were considered as the ELISA titers against each of the tested antigen.

Immunoblotting

SDS-PAGE using 12% separating gel was first performed as mentioned above. Transfer of venom proteins from gels to nitrocellulose sheets was performed according to the method of [29]**,** using the Semi-dry LKBBROMMA-21117-250 NOVABLOT Electrophoresis Transfer Cell, at 100 mA for 60 min. Immobilized venom proteins were probed with antivenoms (dilution 1:1000) and the patterns were developed using anti-rabbit IgG peroxidase conjugate (Sigma, USA, dilution 1:3000) and 4-Chloro-I-Naphthol substrate.

Neutralization of PDE activity by anti Nn-PDEII antisera

Neutralization experiments were performed by incubating a constant amount of PDE enzyme (10 units) with different dilutions of rabbit anti-Nn-PDEII, commercial anti-snake antiserum (obtained from the Egyptian organization for biological products and vaccines (VACSERA), or a mixture (1:1) of both antisera for 30 min at 37°C. After then, 10 µl of each mixture was incubated with the substrate in Tris buffer, pH 8 (90 µl) and the residual PDE activity was measured under standard assay conditions as mentioned above. A non-immune rabbit serum was used as a control.

Effect on blood coagulation

Human normal citrated plasma

Human normal citrated plasma was prepared from blood of healthy human donors who do not receive any medication in the last three weeks. A blood sample (5ml) was drawn into a test tube containing 3.2% sodium citrate (400 µl). The tube is inverted upside down gently to ensure full citration of the blood and then centrifuged at 2000g for 15 min at 25^{o}C. The plasma obtained was used to perform the following assays.

Prothrombin time (PT) assay

Various concentrations of Nn-PDEII enzyme (1.25–20 µg) in 50mM sodium phosphate buffer, pH 7.2 (100 µl) were incubated with the prepared human plasma (100 µl) for 3 min at 37°C. PT reagent (thromboplastin with calcium chloride, 200 µl) obtained from biomed – Liquiplastin - S diagnostics kit and pre-warmed at 37°C, was added. The time taken for clot initiation was optically measured and recorded in seconds.

Activated partial thromboplastin time (APTT) assay

Various concentrations of Nn-PDEII enzyme (1.25–20 µg) in 50mM sodium phosphate buffer, pH 7.2 (100 µl) were incubated with the prepared human plasma (100 µl) for 3 min at 37°C. APTT reagent from biomed – Liquicelin - E diagnostics kit (100 µl) was added and the mixture was incubated for another 3 min at 37°C. Finally, 100 µl of 0.025 M $CaCl_2$ (pre-warmed at 37°C) was added and the clotting time was recorded in seconds.

RESULTS AND DISCUSSION

Screening of phosphodiesterase (PDE) activity in some selected Egyptian snake venoms

Although venom PDEs are known to hydrolyze a wide variety of biologically important nucleotides; ATP, GDP, NAD+, NADP+, DNA and RNA, and despite numerous PDEs that have been isolated from different snake venoms, very little is known about their immunological properties, and may be fewer about their biological and pharmacological potential [20]. In the present study, we found it interesting to search for the presence of PDE activity in some common Egyptian snake species. We described the purification and some major characteristics of a highly active PDE enzyme from *N. nigricollis* snake species.

Uzawa [30] had reported as early as 1932 that PDE activity is nearly found in all snake venoms and higher activity was associated with Viperidae family. Our results showed the presence of PDE in all the Egyptian venoms tested but higher activity was observed in *N. nigricollius* venom belonging to family Elapidae. This could be attributed to the geographical and age variations among snake venom species [31-33]. Further studies had to be done on individual snake venoms to study inter- and intra-species variability.

The results revealed that the venoms of elapids, *N. haje & N. nigricollis*, displayed higher PDE activity than the tested viperids, *C. cerastes* & *C. vipera. N. nigricollis* venom possessed the highest PDE activity among the tested venoms with 4400 U/mg protein, whereas *C. cerastes* venom showed the lowest PDE activity with 500 U/mg protein (Table 1).

Table 1: PDE Specific activities of some Egyptian snake venoms

Venom	specific activity (Unit/mg protein*)
N. haje	2500
N. nigricollis	4400
C. vipera	1200
C. cerastes	500

One unit of PDE is defined as the amount of the enzyme that hydrolyzes 1 nmole of bis (p-nitrophenyl) phosphate per min under the specified conditions. Data represent the mean of two independent experiments.

Purification and molecular mass determination of PDE enzyme from N. nigricollis venom

Two step purification protocol was followed for isolation of PDE from *N. nigricollis* venom as summarized in Table (2). In the first step, *N. nigricollis* venom was applied on Sephadex G-100 where the venom was resolved into two main protein peaks and the PDE activity was detected in the second peak (Fig.1A). The enzymatically active fractions, designated as Seph-PDE, were pooled and applied directly on DEAE-Sepharose ion exchange chromatography where one major and several minor enzyme isoforms eluted by step-wise NaCl increasing concentrations, 0 - 1 M (Fig. 1B). The major PDE enzyme, designated as Nn-PDEII, eluted late by 0.1M NaCl and migrated as a single prominent band on native PAGE. Minor peaks, showing promiscuous less prominent bands on native SD-PAGE, got no further analysis**.** The molecular mass of Nn-PDEII was estimated as 125 kDa by gel

filtration on Sephacryl S-200 (Fig. 1C). On SDS_PAGE, Nn-PDEII split into two subunits in presence of 2-mercaptoethanol corresponding to apparent molecular masses of 65 & 58 kDa (Fig. 1D).

Table 2.Purification scheme of PDE from *N. nigricollis* venom

Sample	Total protein (mg)	Total activity (U)	Specific activity (U /mg)	% Recovery		Purification fold
				Protein	Activity	
Nn venom	50	200000	4 000	100	100	1
Nn Seph-PDE	30	180 000	6 000	60	90	1.5
Non bound PDE	0.188	1 312.5	7 000	0.375	0.656	1.75
Nn-PDE I	0.3	3 150	10 500	0.6	1.575	2.62
Nn-PDE II	9.375	157 500	16 800	18.75	78.75	4.2
Nn-PDE III	1.5	15 000	10 000	3	7.5	2.5
Nn-PDE IV	0.75	2 500	3 333	1.5	1.25	0.83

Snake venom PDE had been isolated from several species by different methods [34,35, 36]. Here we adopted a combination of gel filtration and ion exchange chromatography for the production of apparently homogenous PDE. We obtained different isoforms with different elution properties from anion exchange chromatography. One major enzyme, PDEII that constituted ~ 18% of the total *N. nigricollis* venom protein and ~80% recovery of the enzymatic activity was evident. No further attempt was made for characterization of the isoforms due to their low abundance.

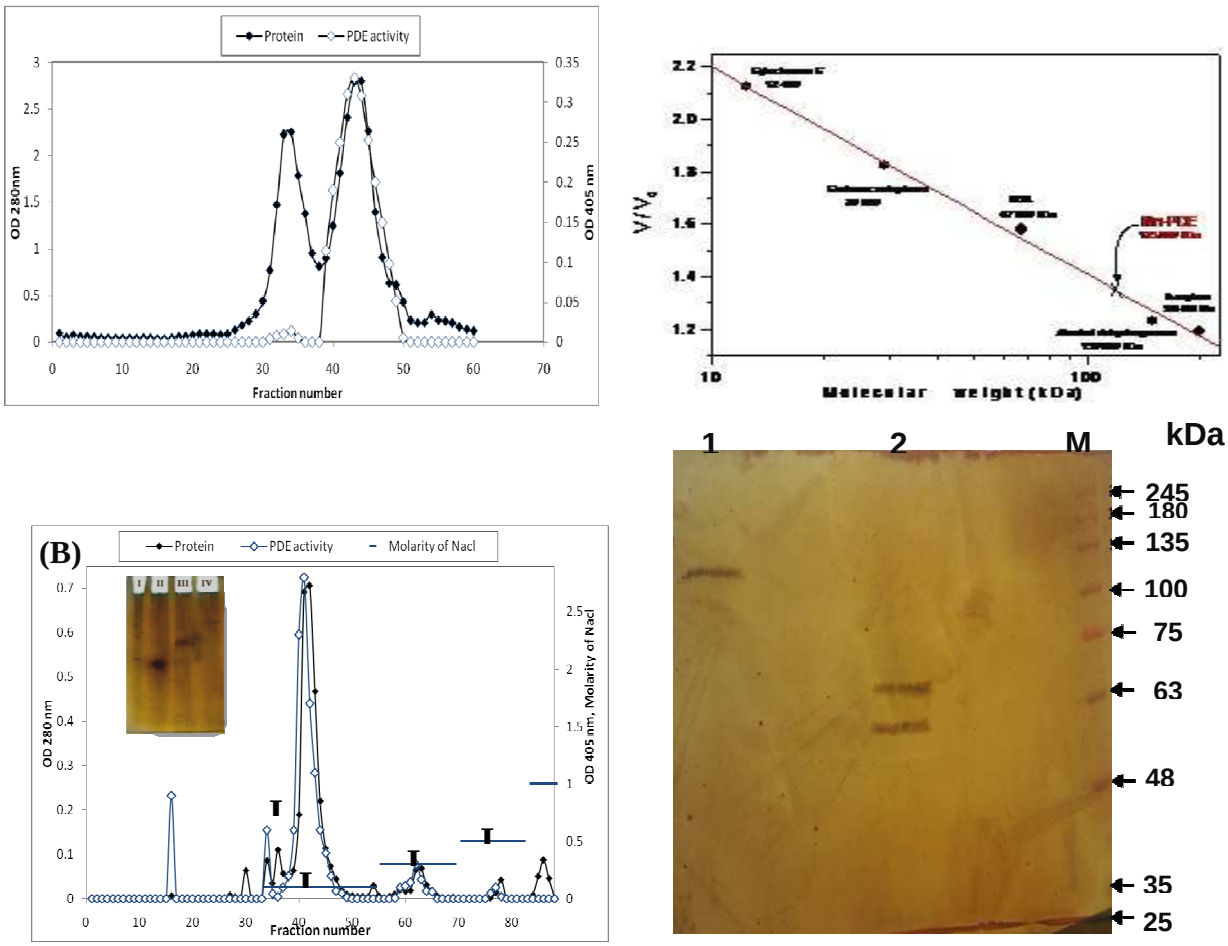

Fig. 1. Purification of PDEs from N. nigricollis venom

A) Gel filtration of venom (50 mg), collected from N. nigricollis snakes inhabiting Aswan (inserts), and loaded on Sephadex G-100 column. The enzymatically active fractions were pooled and subjected to ion-exchange chromatography. (B) DEAE- Sepharose column of Seph-PDEII pool eluted by step-wise NaCl gradient (0-1M). The active pool (Nn-PDEII), migrated as single prominent band on 7.5% native-PAGE (insert), was collected for further characterization. (C) Molecular mass determination of Nn-PDEII by gel filtration chromatography on Sephacryl S-200 using Dextran blue (2,000,000 kDa) to determine the void volume and the standard protein markers to calculate the molecular mass. (D) 10% SDS-PAGE of Nn-PDEII (20 µg) in absence (1) and presence (2) of 2-mercaptoethanol, and the standard molecular weight markers (M).

Most venom PDEs are high molecular mass (> 90 kDa) single polypeptide chains [20] with some exceptions where PDE exist as a homodimers with disulfide bridge between the subunits [7,37,38]. Similarly, the native form of Nn-PDEII enzyme showed a single band of 125kDa as determined by gel filtration on calibrated Sephacryl S-200 column. In presence of 2-mercaptoethanol, however, it split into two obvious subunits of 66 and 59 kDa on SDS-PAGE suggesting that the enzyme exists is a heterodimeric form with a disulphide bridge between the two subunits.

Biochemical characterization of Nn-PDEII

Effect of temperature and pH

Nn-PDE enzyme activity was determined at different temperatures and pHs; the maximum enzyme activity was recorded at 60^{o}C (Fig. 2 AI) using Tris-HCl buffer, pH 8 (Fig. 2 BI). The stability of the enzyme was also studied at different temperatures and pHs. The enzyme could be considered thermally stable, since no loss of enzymatic activity was recorded after 1 hour incubation at 40°C (Fig. 2 AII). However, incubation at 60 ^{o}C for I hour resulted in 50 % loss of enzymatic activity and complete loss of activity was recorded above 70 ^{o}C . Moreover, the enzyme maintained its full activity in alkaline medium pH 8-9 (Fig. 2 BII).

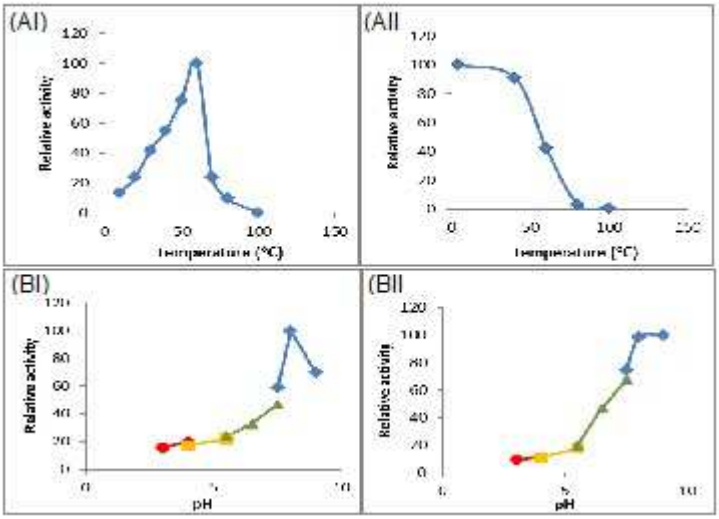

Fig. 2. Effect of temperature (A) and pH (B) on activity (AI, BI) and stability (AII, BII) of Nn-PDEII

Values are the mean of two independent experiments with duplicates. For determination of pH effect, a set of eour buffers were used; citrate phosphate buffer (pH 3-4), sodium acetate buffer (4-5.5), sodium phosphate buffer (5.5-7.5), and Tris-HCl buffer (7.5-9)

The results in the present study revealed that the optimal temperature and pH values of the purified Nn-PDE II enzyme were 60 ^{o}C and 8, respectively. The enzyme maintained its full activity at alkaline pH (pH 8-9) up to 40 °C, however, it lost ~50% of its activity at 60 ^{o}C and a complete loss of activity was detected above 70 °C. These results fully agree with that of PDE from *C. cerastes* [34], *Bothrops alternates* [35], and *Walterinnesia aegyptia* [36]

Effect of metal ions and inhibitors

The effect of metal ions and inhibitors are illustrated in Fig. 3. The results revealed that Nn-PDEII enzymatic activity was markedly increased by Mn^{+2}, Co^{+2} and Mg^{2+} , increased to a lesser extent by Ca^{2+}, Ba^{2+}, Ni^{2+}, K^{+} and Na^{+} but not significantly affected by Zn^{2+} ions and iodoacetic acid. On the other hand, Al^{3+} and Cu^{2+} ions, PMSF, DTT, EDTA, O-phenanthroline and L-cysteine exhibited obvious inhibitory effects.

Nn-PDE II enzyme was active in absence of addition of any divalent ions. The necessary cation is suggested to be present on the enzyme [37]. Several transition metal ions were shown to activate PDEs from different sources [19, 38-39].

Zn^{2+} ions have shown to be critical for several venom PDEs [20]. However, the catalytic activity of Nn-PDEII in the present study seems to be unaffected by Zn^{2+} ions but greatly enhanced by Mn^{2+} and Co^{2+} ions, moderately increased by Ca^{2+}, Ba^{2+}, Ni^{2+} ions, insignificant increased by Na^{+}, K^{+} ions while AL^{3+} and Cu^{2+} ions exhibited obvious inhibitory effect. Whether metal ions act as stabilizer or as essential cofactor is not yet clear and has to be investigated.

Cysteine, DTT, EDTA, and O-phenanthroline inhibited the Nn-PDE II activity while iodoacitic acid had no effect on the enzymatic activity. These findings are in agreement with the observations that metal chelators (EDTA and o-phenanthroline) generally inhibit PDE activity [7,20,31] and suggest that Nn-PDEII is a metalloenzyme. Inhibition by cysteine and DTT but not by iodoacetic acid suggests that the S - S bond, in particular, is essential for activity as with other venom PDEs [21,34,35,40]. PMSF also inhibited Nn-PDEII activity indicating the importance of serine residues for activity as with PDE from *Doboia russelli russelli* [21].

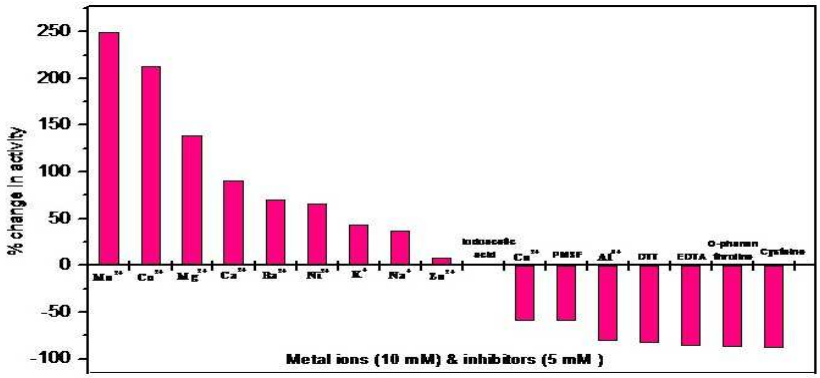

Fig. 3. Effect of metal ions (10mM) and inhibitors (5 mM) on the enzymatic activity of Nn-PDEII
Data represent the mean of two independent experiments

Enzyme kinetics
The enzymatic activity of PDE was determined at different concentrations of bis (p-nitrophenyl) phosphate substrate. The K_m and V_{max} of the enzyme, calculated for bis (p-nitrophenyl) phosphate substrate from Lineweaver-Burk plot (Fig. 4), were found to be 3.92 mM and 0.218 µmole/min, respectively.

The Km value of Nn-PDEII enzyme is in the same order as reported by others e. g. 5.6×10^{-3} M and 8.3×10^{-3} M [40,41] by using same substrate but recently Santoro et al. [9] reported 8.5×10^{-4} M for the same substrate which is lower than the values reported to date.

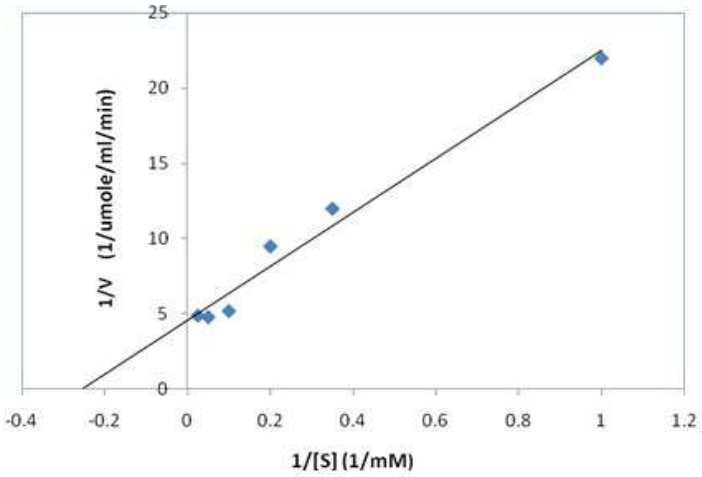

Figure 4. Line weaver-Burk plot of Nn-PDE II using different concentrations of the substrate "bis (p-nitrophenyl) phosphate" under the standard assay conditions
Each point represents the average of two experiments with duplicate at each concentration

Immunological properties of Nn-PDEII enzyme
Preparation of rabbit antisera
The crude *N. nigricollis* and the purified Nn-PDEs were used as immunogens for preparation of the corresponding rabbit antisera. The response of the rabbits immunized over a period of ~three months was monitored by ELISA **(**Fig. 5). The results revealed that Nn-PDE II was much more immunogenic than all other isomers tested but less immunogenic than the crude *N. nigricollis* venom. A characteristic zigzag behavior is evident for Nn-PDE II enzyme isomer, the antibody levels seem to increase one week after each booster and rapidly fall at the next week until the further booster is injected. The greatest antibody level was recorded at the 9th week with an ELISA titer of

~ 2962. Although numerous PDE have been isolated from snake venoms, only few studies have dealt with their immunological properties [35, 42, 43]. Here we used rabbits to raise antibodies to the purified Nn-PDEII enzyme over ~ ten week immunization program. Our results showed that Nn-PDEII was a good immunogen as a 5 µg enzyme per injection could induce the production of highly reactive antibodies of reasonable ELISA titre (~3000) as shown in ELISA and immunoblotting assays.

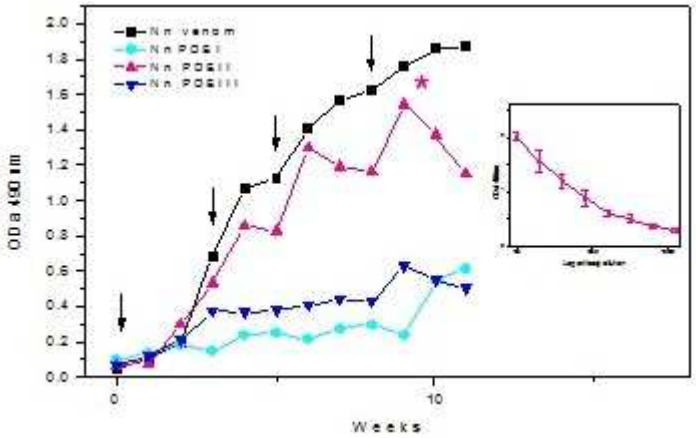

Fig.5. ELISA antibody levels of rabbit antisera to *N. nigricollis* venom and the corresponding PDE fractions using crude *N. nigricollis* venom as the coating antigen

The solid arrows indicate the time of injection and booster doses. The star indicates the highest titer obtained at the 9th week, estimated as 2961.8 ELISA titer (insert).

Immuno-reactivity of anti Nn-PDEII with different snake venoms

The immune-reactivity of the prepared rabbit anti Nn-PDEII towards the corresponding Nn-PDEII and the crude venoms derived from the parent and other snake species was studied using immunolotting technique. It is evident that the rabbit antibodies react specifically with the analogous Nn-PDE enzyme and cross-reacted with other protein components of the parent venom, but not with those from other snake venom species (Fig. 6).

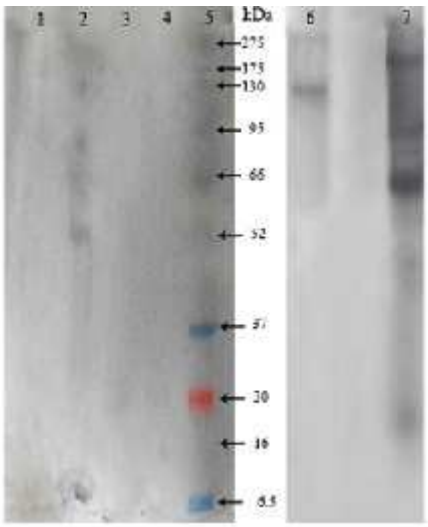

Fig.6. Immunoreactivity of rabbit anti-PDEII antisera towards Nn-PDEII enzyme and venoms of different snake species clarified by immunoblotting analysis. (1) *N. haje* [20µg], (2) *N. nigricollis* [20µg], (3) *C. vipera* [20µg], (4) *C. cerastes* [20µg], (5) pre-stained molecular weight markers, (6) Nn-PDEII [50µg], (7) *C. cerastes* [50µg]

The prepared anti - Nn-PDEII antisera could react specifically with the corresponding Nn-PDE antigens and cross-react with other components of the parent venom but not with those from other species. These results support the idea that major the factor underlying immunological cross-reactivity of snake venoms seems to be the structural similarities among analogous venom proteins [44]. However, the ability to cross-react with structurally &/or functionally unrelated proteins of the parent species implies the presence of common epitopes shared and conserved among proteins of the corresponding source species; an observation that was previously reported by Stábeli et al.[45].

3.4.3 Neutralization of PDE activity

The neutralization power of the prepared rabbit anti Nn-PDEII towards the purified enzyme was studied and compared to that of commercial polyvalent antiserum (Fig. 7). The results revealed that both antisera could neutralize, dose, dependently, the PDE activity of the purified enzyme, however, the commercial polyvalent antiserum was more efficient at each of the concentrations used. On the other hand, the mixture (1:1) of both antisera has higher neutralizing power than rabbit anti Nn- at higher concentrations (lower dilutions, ~ 1:10 - 1:100), indicating higher antibody content of the mixture antisera, but had lower neutralization power at lower concentrations (higher dilutions, ~1:300 - 1:1200) than the rabbit anti Nn-PDEII, indicating higher affinity of the later than that of the mixture antisera.

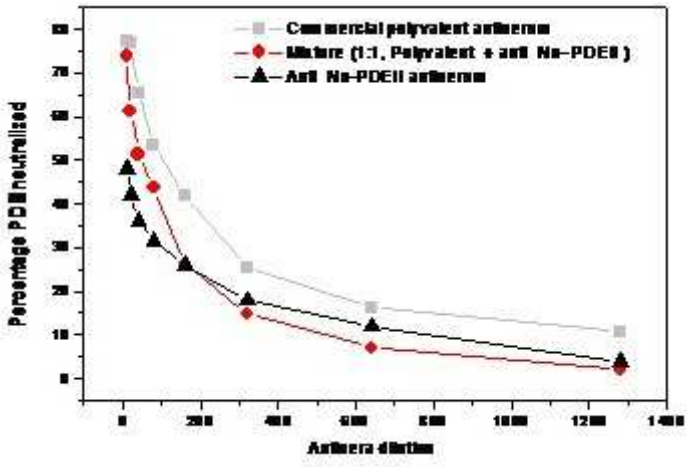

Fig.7. Neutralization of PDEII enzyme activity (10 Units) by different concentrations of commercial polyvalent, specific anti-Nn PDEII and a combination (1:1) of both antisera

PDE was neutralized by rabbit antisera and also by commercial polyvalent anti-snake antiserum. However, the neutralization power of the anti Nn-PDE antibodies was much weaker compared to that of commercial polyvalent antisera which can be explained in terms of dilution and purification factors where the later antisera have been subjected to several purification and quality control steps prior to being commercially available that were not applied to native anti-NnPDE antisera. Despite the lower neutralization ability of anti – Nn-PDEII, it seems to possess high affinity to Nn-PDEII enzyme that could be advantageous to raise efficiency of protective anti-snake antisera.

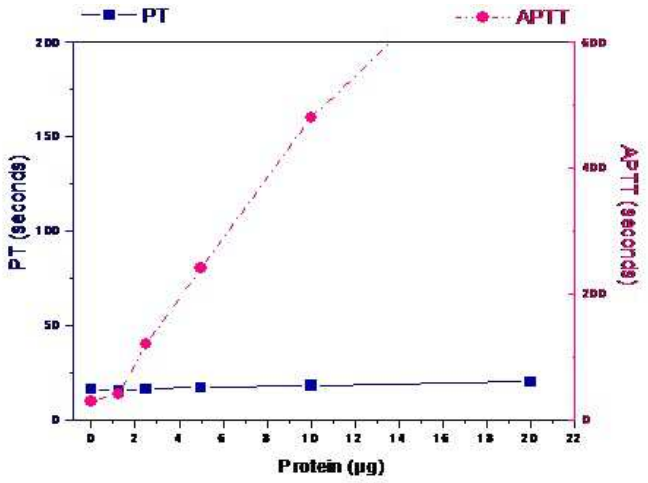

Fig. 8. Effect of Nn-PDEII on humanblood coagulation tested by prothrombin time (PT) and activated partial thromboplastin time (APTT) assays

Bioactivity of Nn-PDE enzyme: effect on blood coagulation time

The effect of Nn-PDEII on the coagulation time of normal blood plasma was assessed by prothrombin time (PT) and the activated partial thromboplastin time (APTT) assays as shown in Fig. (8). It is evident that purified Nn-PDEII had very weak anticoagulant effect, if any, on the extrinsic pathway and the common coagulation pathways, whereas

it seems to inhibit one or more of the coagulation factors within the intrinsic pathway that resulted in a potent anticoagulant effect manifested by the apparent prolongation of the partial thromboplastin time especially at higher Nn-PDE concentrations.

Nn-PDEII, like PDEs isolated from other snake species [36] exhibited potent anticoagulant effect. It seems to inhibit one or more of the coagulation factors within the intrinsic pathway, in particular, as manifested by the apparent prolongation of the partial thromboplastin time especially at higher Nn-PDE concentrations. Characterization of a component of *N. nigricollis* venom that affect blood coagulation at specific stage could have potential in identifying new drug leads in the treatment of hemostatic disorders.

CONCLUSION

With the increasing risk of atherosclerosis which is the primary cause of life-threatening diseases such as, stroke and myocardial infarction, particularly among increasingly aged population throughout the world [46], search for new anticoagulants that target specific active sites are becoming essential. The biodiversity of snake venom components that affect blood coagulation have been attractive sources for new lead compounds for treatment of thromboembolic disorders. Here we demonstrate a component of *N. nigricollis* venom, Nn-PDEII, which specifically interferes with the intrinsic hemostastic pathway resulting in a potent anti-coagulation effect. The structural, biochemical and immunological characteristics have been investigated. Such studies could have medicinal or pharmacological impact and could open doors for further studies for identifying new drug leads from deadly snake venoms.

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