Biochemical and Biological Properties of the Phosphodiesterase-1 Purified from King Cobra Venom

[1]SAAD S.M. AL-SALEH*, [1]SAMI ULLAH KHAN AND [2]MOHAMMAD ASHRAF
[1]Department of Clinical Laboratory Sciences, College of Applied Medical Science, King Saud University P.O. Box 10219. Riyadh 11433, Saudi Arabia.
[2]Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Bahawalpur. Pakistan.

(Received 3rd June 2008 accepted in revised form 21st October 2008)

Summary: A phosphodiesterase-1 (PDE-1, EC 3.1.4.1) have been purified from King cobra; *Ophiophagus hannah (O. hannah)* venom by preparative native PAGE. A single protein band was observed in analytical native PAGE and SDS-PAGE. The molecular mass was found to be 148 kDa. The enzyme was free from 5'-nucleotidase and alkaline phosphatase activities. The enzyme showed an optimum pH 10.0 in Tris-HCl buffer. The optimum temperature was found to be 50 °C. Energy of activation (Ea) was calculated to be 164. The PDE-1 is a glycoprotein and exhibited basic pI. The V_{max} and K_m of PDE-1 calculated were 1.53 μM/min/mg and 2.6×10^{-3} M, respectively. The K_{cat} and K_{sp} values are 9.2^{s-1} and 58.8 M^{-1} Min^{-1} respectively. Cysteine caused a non-competitive inhibition, with a K_i 8.2×10^{-3}M. The IC_{50} was 3.9 mM. The adenosine diphosphate (ADP) caused a competitive inhibition, having K_i 1.0×10^{-3} M. The IC_{50} was 12.0 mM. Glutathione, o-phenanthroline, Zinc and EDTA inhibited the PDE-1 activity, whereas magnesium slightly potentiated the activity. The enzyme hydrolyzed thymidine 5'-mono phosphate p- nitro-phenyl ester most readily (10 fold) while cyclic 3'-5'-AMP was least readily hydrolyzed substrate. The PDE-1 up to 4.0 mg/Kg i.p was not lethal in mice. The PDE-1 exhibited an anticoagulant effect whereas the crude venom showed strong coagulant effect. The above mentioned studies show that the *O. hannah* PDE-1 is very similar to that isolated from other snake venoms.

Introduction

Snake venom is a cocktail of hundreds to thousands of biologically active proteins and enzymes used in both attack and defense. The snake venom proteins are enzymes, toxins or nerve growth factors. The biological activities are predominantly found in the protein fractions rather than the non-protein fractions [1]. The diversity of the symptoms arising after a snake bite such as bleeding, shock, hemorrhage, necrosis and muscular paralysis are caused by particular venom component possessing enzymatic and/or toxic properties [2].

Enzymes are important and most noticeable components of snake venom. More than 26-39 different enzyme activities have been detected in snake venoms [3, 4]. Because these proteins are capable of modulating the physiological response of envenomed animals, they show promise as potential pharmacological tools and as drug leads. A number of snake venom enzymes are extensively used as tools in biochemical research or as diagnostic or therapeutic agents. Purified snake venom enzymes with a known mode of action and narrow substrate specificity, resistant to inhibitor systems present in blood and tissues, have found multiple uses in therapeutics, diagnostics or preparative procedures in hemostaseology, while only a few practical applications of snake venom enzymes are known from other biochemical or biomedical disciplines [5-10]. Tsai *et al.,* reported for the members of each toxin family, protein sequences were aligned and subjected to molecular phylogenetic analyses. The results indicated that the PLAs and a Kunitz inhibitor of W. aegyptia are most similar to those of king cobra venom, and its 3FTxs belongs to either Type-I alpha-neurotoxins or weak toxins of orphan-II subtype. It is remarkable that both king cobra and *W. aegyptia* cause rapid deaths of the victims, and a close evolutionary relationship between them is speculated [11].

Nucleases; enzymatic components which hydrolyze nucleic acids and derivatives are found in many different venoms from front- fanged snakes. More recently, Sales and Santoro, (2008) studied such enzymes in 28 crude venoms of animals found

*To whom all correspondence should be addressed.

in Brazil. Higher levels of ATPase, 5'-nucleotidase, ADPase, phosphodiesterase and DNase activities were observed in snake venoms belonging to Bothrops, Crotalus and Lachesis genera than to Micrurus genus [12].

Extracellular PDE's known as exonuclease, exist in venoms and their route in envenomation is mostly by attacking nucleic acids through removal of mononucleotide units from polynucleotide chain in stepwise fashion [13]. Therefore the enzyme may be useful for determining the sequences of both ribo and deoxyribooligo-nucleotides and identifying α (alpha) and (gamma) terminal nucleotides.

The biochemical, diagnostic and therapeutic uses of snake venom enzymes have led to intensive attempts to study the occurrence, purification and characterization of these enzymes [1].

The majority of methods required several ion exchange steps with different functionalities in order to remove contaminating nucleases from phosphodiesterases [14-17]. So there is a potential to develop a methodology for the isolation and purification of phosphodiesterase enzyme from snake venoms which should involve single step and / or minimum steps with reasonable good results, time saving and economical. Since there are significant gaps *e.g.* very little is known about the contribution of these enzymes to sequelae of envenomation, *in vivo* activity, amino acid or cDNA sequence, degree of homology of enzymes from various species of snakes and the absolute relation of exonuclease and endnuclease activities. The gaps in the understanding of basic biochemical, biological properties of venom phosphodiesterases which should be filled by doing such studies extensively on purified enzyme preparations.

Results and Discussion

The crude venom of *O. hannah* was fractionated by native preparative PAGE into many fractions. Only the fraction no.1 showed the PDE-1 activity. Fraction no.1 appeared as single band on native analytical PAGE (Fig. 1) and on SDS-PAGE (Fig. 2) which means the enzyme is homogenous. The enzyme was purified 2 fold over crude venom with a specific activity of 4.1 U/mg. Recovery of PDE-1 activity was 33 % whereas the protein yield of enzyme was 5.0 %. The purified PDE-1 was free

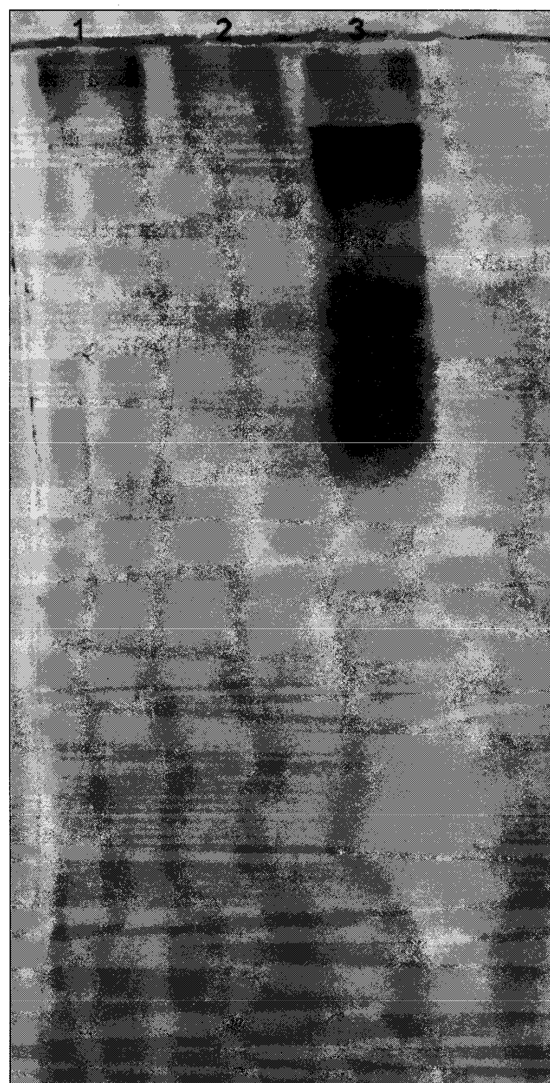

Fig. 1: Analytical native PAGE of purified PDE-1 from *O. hannah* venom along with crude venom.
(1) Purified PDE (30 μg); (2) Purified PDE (30 μg); (3) Crude venom (200 μg).

from 5'-nucleotidase activity. The non-specific alkaline phosphatase activity was also negligible.

The snake venom PDE-1 has been isolated from a number of species, including *C. adamentus* [18-22]; *A. acutus* [23]; *A. halys blomhoffii* [24]; *A. pisivorous* [25]; *B. atrox* [17, 26]; *C. durissus terrificus* [21]; *C. ruber ruber* [27]; *T. mucrosqua-*

matus [28]; *V. palestinae* [29]; *V. aspis* [30]; *C. cerastes* [31] and *B. alternatus* [32]. The purity of PDE-1 obtained by the methods described in the above mentioned reports varied considerably particularly in the early attempts at purification, the main reason is the difficulty in removing the contaminating 5′-nucleotidase and alkaline phosphatase. In contrast to other current methods for obtaining PDE-1 from snake venoms, our procedure is single step. Our procedure apparently provided PDE-1 free of these contaminants as shown by the single protein on native analytical PAGE and SDS-PAGE and by the absence of 5′-nucleotidase and alkaline phosphatase in the enzyme preparation. More over our procedure requires slab gel to be run for 8-10 hours only whereas the preparative gel procedure of Ballario *et al.* required 22 hours to get rid off 5′-nucleotidase and two other unknown proteins. The yield of protein (5.0 %) was also fair enough as reported for many others venoms PDE-1 purified by a variety of protocols [17, 21, 22, 26-28, 30, 32, 33].

The purified enzyme yielded a single band on SDS-PAGE with an estimated molecular mass of

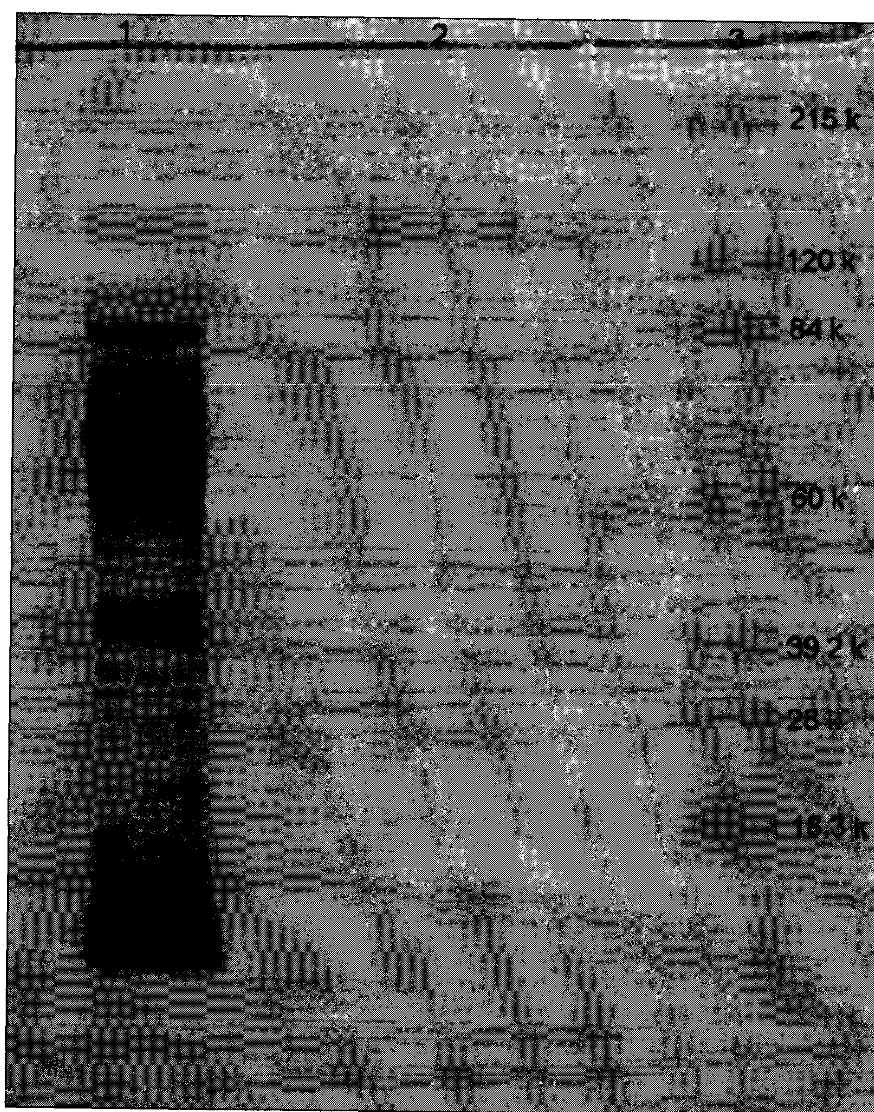

Fig. 2: SDS-PAGE of purified PDE-1 from *O. hannah* venom along with crude venom.
(1) Crude venom (300 μg); (2) Purified PDE-1(30 μg); (3) Pre-stained proteins molecular weight marker mix.

148 KDa following Coomassie brilliant blue staining (Fig. 2).The purified enzyme when treated with *β*-mercaptoethanol did not alter the position and still showed single band on SDS-PAGE (result not shown), indicating that the enzyme was a single chain polypeptide. The isoelectric point for *O. hannah* PDE-1 was calculated to be 7.5.

The snake venom PDE-1 are generally high molecular weight (90-150 KDa) glycoprotein comprising of a single chain polypeptide with iso-electric points ranging from 7.4-10.5 [21, 22, 27, 28, 30, 32, 34]. The molecular mass of *O. hannah* PDE-1 (148 KDa) is close to the other snake venom PDE's mass. The molecular mass being not affected by *β*-mercaptoethanol indicates that the protein is a single chain polypeptide, as is generally the case for venom PDE-1. Although there are reports that PDE-1 from *C. rubber, ruber* venom exist as a homo-dimer with subunits of 49 KDa [27], PDE-1 from *C. viridis oreganus* venom exists as a homo-dimer with subunits of 57 KDa [15] and PDE-1 from *C. mitchelli* pyrrhus venom exists as a homodimer with subunits of 55 KDa [14]. Throughout the whole purification procedure and in SDS-PAGE, only one fraction of activity and one protein band were obtained respectively for *O. hannah* PDE-1 which indicates that this enzyme probably does not occur in isoforms as reported about PDE-1 of *V. palestinae* [29] and PDE-1 of *T. flavoviridis* [35].

The optimum temperature for *O. hannah* PDE-1 activity was found to be 60 °C, with activity decreasing at > 65 °C (Fig. 3). Energy of activation (Ea) was calculated from an Arrhenius plot to be 164 0. The maximum *O. hannah* PDE-1 activity in Tris buffer was obtained over the pH 10.0 (Fig 4). The *O. hannah* PDE-1 is a glycoprotein having 24 % of carbohydrate contents.

Halim *et al.* [31] reported an energy of activation (Ea) 0.913 for *C. cerastes* PDE-1 which is lower as compared to *O. hannah* PDE-1. Many of these enzymes rapidly loose activity (within 1-4 minutes) at temperatures > 65-70 °C as reported by many workers [17, 21, 27, 28, 30, 31]. This property is also found to be the same for *O. hannah* PDE-1. The pH optima of *O. hannah* PDE-1 is in agreement as reported by others [17, 29, 32]. The *O. hannah* PDE-1 was found to be a glycoprotein. The PDE-1 from *T. flavoviridis* contained about 24 % carbohydrate [35]; *C. adamentus* PDE-1 contains 9.2

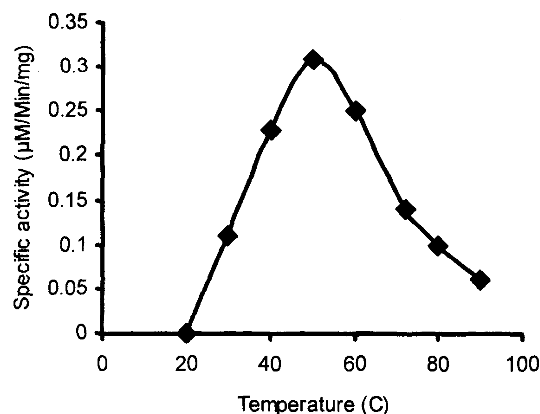

Fig. 3: Effect of temperature on *O. hannah* PDE-1 at 2 mM BpNPP and pH 9.0. Enzyme activity was tested at different temperatures in the range between 20^0 and 90^0C. A substrate control was included with each test to correct for auto substrate destruction at different temperatures. Each point represents the mean of five values.

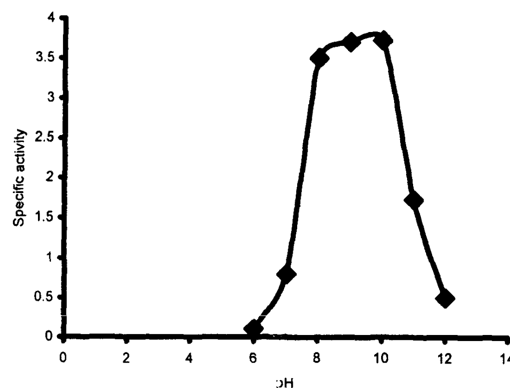

Fig. 4: Effect of pH on *O. hannah* PDE-1 activity at 37^0C. The effect of pH on enzyme activity was tested in the range between 6 and 12 using 0.11M Tris-HCl buffer. A substrate control was included at every pH tested. Each point represents the mean of three independent experiments.

% neutral and 1.9 % amino sugars [36]. A PDE-1 from *B. atrox* [26] and *C. ruber ruber* [27] reported to contain carbohydrate.

The V_{max} and K_m of *O. hannah* PDE-1 calculated from Lineweaver-Burk plot were 1.53 μM/min/mg and 2.6×10^{-3} M, respectively (Fig. 5).

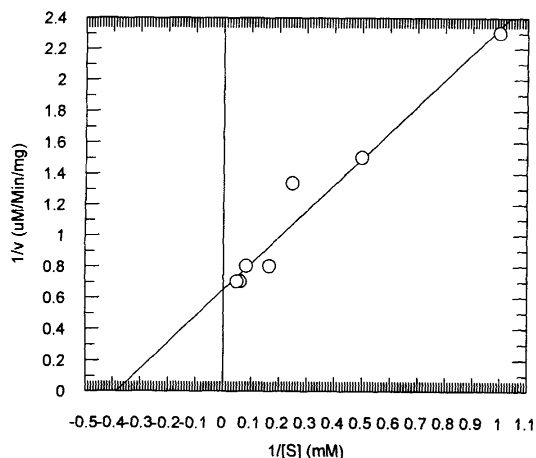

Fig. 5: Lineweaver-Burk plot for *O. hannah* PDE-1 activity. The effect of varying substrate concentration on enzyme activity was tested and K_m for the enzyme was determined from Lineweaver-Burk plot. The K_m was calculated using the GraFit software package, version 3.0. The points represents the mean ± SEM of 4-5 determination, each in duplicate.

The K_{cat} and K_{sp} values are 9.2^{s-1} and 58.8 M^{-1} Min^{-1} respectively.

Cysteine caused a non-competitive inhibition; K_i was calculated to be 8.2×10^{-3} M (Fig. 6). The IC_{50} was calculated to be 3.9 mM (Fig. 7). ADP caused a competitive inhibition; the K_i was calculated to be 1.0×10^{-3} M (Fig. 8). The IC_{50} determined was 12.0 mM (Fig. 9).

Glutathione, *o*-phenanthroline, cysteine and ADP at a concentration of 2.5-10 mM inhibited the PDE-1 activity (18-35 %), (40-62 %), (2-50 %) and (92-100 %), respectively. The EDTA at a concentration of 0.5-10mM inhibited the PDE-1 activity (50-88 %) Table1. Addition of 10 mM of Ca^{+2} reactivated PDE-1 by only 8 %.

Table-1: Effect of different concentrations of inhibitors on *O. hannah* PDE-1. (Percent activity)*

Inhibitor	0.5mM	2.5mM	5.0mM	10.0mM
Cysteine	------	60 ± 2	57 ± 2	42 ± 1
ADP	------	82 ± 3	71 ± 2.5	65 ± 2
Glutathione	------	98 ± 3	79 ± 2.5	50 ± 1.5
o-phenanthroline	------	8 ± 0.25	2.0 ± 0.1	0.0
EDTA	50 ± 1.5	23 ± 0.75	15 ± 0.5	12 ± 0.5

* Enzyme activity in the presence of Ca^{+2} or Mg^{+2} in the assay mixture is taken as 100 %. All assays were done in triplicate.

The K_m value is in the same order as reported by others *e. g.* 5.6×10^{-3} M and 8.3×10^{-3} M by using same substrate [27, 28] L.B. Dolapchiev *et al.* [36] reported K_{cat} values 1.9-40 $^{s-1}$ for PDE-1 from *C. adamentus* using ATP as substrate while S.E. Pollack and D.S. Auld [37] reported the values in the range 200-600 $^{s-1}$ for snake venom PDE-1 using nucleotide analog as substrate. The only report about K_{sp} is by S.E. Pollack and D.S. Auld [37] which mentioned a value in the range 18-66 $\mu M^{-1} S^{-1}$ for nucleotide analog. The values for K_{cat} and K_{sp} mentioned above are quite different from *O. hannah* PDE-1, this is may be due to using different substrates.

Halim *et al.* [31] reported a non-competitive inhibition by cysteine, K_i calculated was 3.346×10^{-3} M whereas they reported a competitive inhibition for ADP and the K_i was calculated to be 0.47×10^{-3} M. An IC_{50} of 7.5 mM has been reported for cysteine by Razzell and a competitive type of inhibition was also observed with ADP and AMP [38].

Recently Choudhary et al have reported a K_i up to 1.15×10^{-3} M and IC_{50} up to 1.0 mM for biscoumarin derivatives [39]. The *O. hannah* PDE-I exhibited similar type of behavior towards the inhibitors as reported above by other workers.

The *O. hannah* PDE-I was inhibited by EDTA etc. These findings are in agreement with the observations that metal chelators such as EDTA, EGTA and o-phenanthroline generally inhibit PDE-I activity and indicate that these proteins are metalloenzymes. Moreover, this activity is also inhibited by cysteine suggesting that the s—s bond is essential for activity [27, 28, 31, 32, 35].

The study with chelating agents indicates the requirement of divalent metal ions for activity. The Ca^{+2} did not affect the activity of *O. hannah* PDE-I at all. Mg^{+2} (15 mM) caused a 70 % increase in activity of PDE-I. Zn^{+2} (10 mM) showed an inhibitory effect, as it caused a 23 % decrease in activity Table-2.

The enzyme was active in the absence of any addition of divalent ions and in fact is only slightly stimulated by high concentration of Mg^{+2}. It is suggested that the necessary cation is already present on enzyme [40].

Snake venom PDE-1 show negligible activity towards p-nitro-phenyl phosphate or mono-phosphate nucleotides. *Di-* and *tri*-phosphate

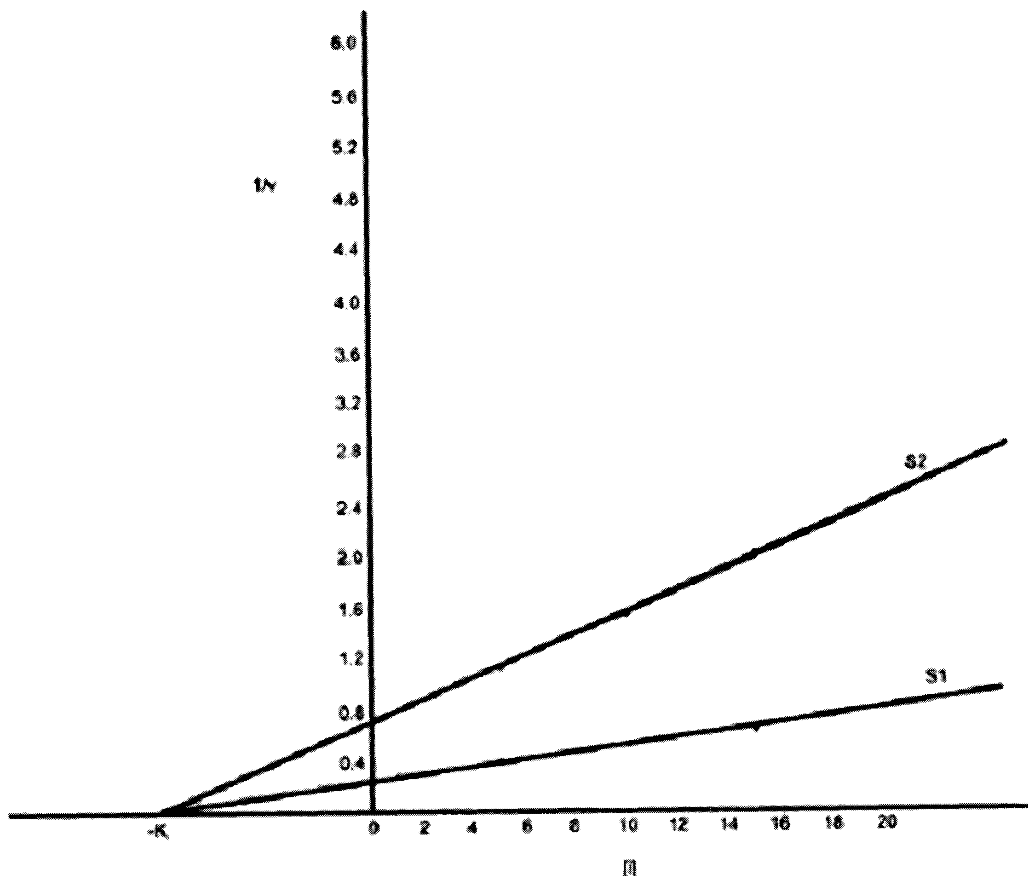

Fig. 6: Determination of K_i for cysteine according to Dixon (non-competitive inhibition). Dixon plot for *O. hannah* PDE-1 at two different concentrations of substrate.

Table-2: The effect of divalent metal ions on *O. hannah* PDE-1 activity.*

Addition	Concentration (mM)	Specific activity (μM/min/mg)
	None	5.28 ± 0.25
	3	5.29 ± 0.25
Ca^{+2}	5	5.30 ± 0.25
7	10	5.30 ± 0.25
		5.33 ± 0.25
	None	0.88 ± 0.1
Zn^{+2}	5	088 ± 0.1
	7	0.7 ± 0.1
	10	0.68 ± 0.1
	None	0.88 ± 0.1
	5	0.94 ± 0.1
Mg^{+2}	10	1.1 ± 0.1
	15	1.4 ± 0.1
	20	0.94 ± 0.1

*Note: All assays were done in triplicate.

nucleotides, DNA, RNA and many derivatives of these native molecules can serve as substrate for phosphodiesterase. *O. hannah* PDE-1 hydrolyzed bis-p-nitro-phenyl phosphate, ATP, cyclic 3′-5′-AMP and thymidine 5′-monophosphate *p*-nitro-phenyl ester. *O. hannah* PDE-1 hydrolyzed thymidine 5′ mono-phosphate *p*-nitro-phenyl ester most readily, the specific activity was 11.0 Units/mg (10 fold) while cyclic 3′-5′-AMP was least readily hydrolyzed substrate with a specific activity 0.13 Units/mg Table-3.

Table-3: Hydrolysis of different substrates by *O. hannah* PDE-1.

Name of substrate	Activity (U/L)	Sp. Activity (Units/mg)
ATP	103 ± 4.0	5.15 ± 0.2
C 3'-5'-AMP	6.5 ± 0.25	0.13 ± 0.01
BpNPP	34 ± 1	4.1 ± 0.15
T5'PpNP ester	113 ± 5	11 ± 0.3

Activity levels of various nucleases in crude venom are usually distinguished by the ability to

catalyze the hydrolysis of specific synthetic substrate. *O. hannah* PDE-1 hydrolyzed thymidine 5′-monophosphate *p*-nitro-phenyl ester most readily (10 fold) while cyclic 3′-5′-AMP was least readily hydrolyzed substrate. Many workers reported that the *p*-nitrophenyl esters are most readily hydrolyzed chromogenic substrates [16]. According to Suzuki *et al.* [41] the cyclic 3′-5′-AMP is also readily hydrolyzed by PDE-1 isolated from *N.n. atra*, *T. flavoviridis* and *A. halys blomhoffii* venom and was better substrate based on reaction rate. Whereas cyclic 3′-5′-AMP was the least hydrolyzed substrate by *O. hannah* PDE-1.

Snake venom PDE-1 has been studied extensively but few have investigated the biological activity of this unique venom component. The *O. hannah* PDE-1 up-to 4.0 mg/Kg i.p was not lethal in mice. The animals showed some symptoms of intoxication like hypo-activity and extension of hind limbs during first hour of a 24 hours experiment.

The *O. hannah* PDE-1 did not show lethality while Russell *et al.* [42] demonstrated an LD_{50} of 3.08-4.65 mg/Kg (i.v), in mice which is fairly toxic.

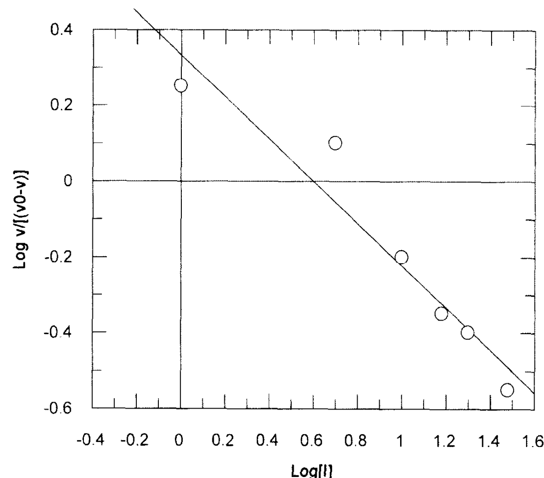

Fig. 7: *O. hannah* PDE-1 inhibition by cysteine. The transformed data is represented in the form of a hill plot, where V and V0 are the reaction rates for experimental and control system respectively. The Hill coefficient is 0.33 and slope was -0.56 respectively. Each point is the mean of three independent determinations.

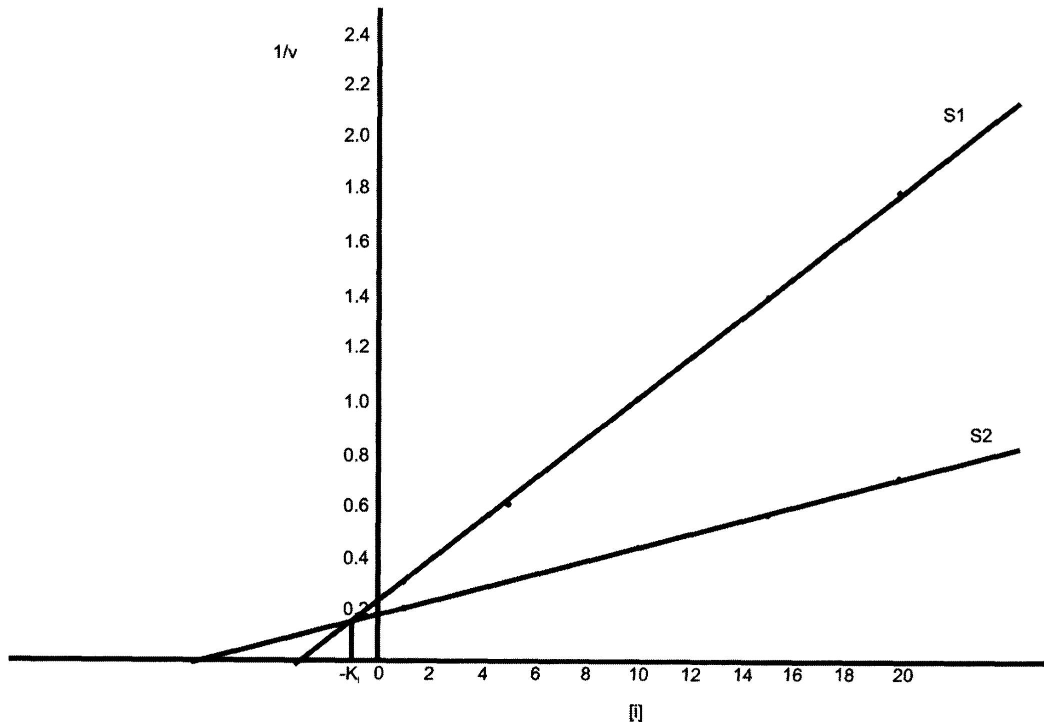

Fig. 8: Determination of K_i for ADP according to Dixon (competitive inhibition). Dixon plot for *O. hannah* PDE-1 at two different concentrations of substrate.

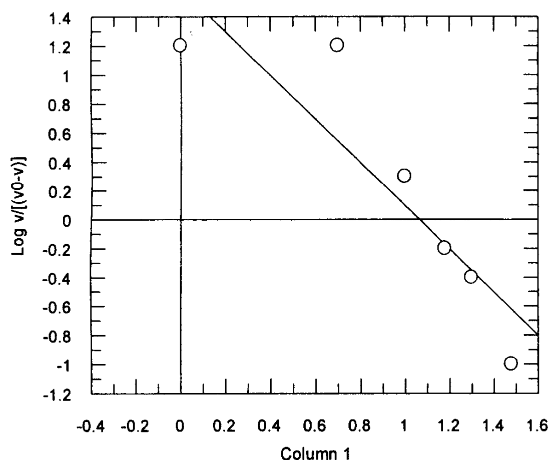

Fig. 9: *O. hannah* PDE-1 inhibition by ADP. The transformed data is represented in the form of a hill plot, where V and V0 are the reaction rates for experimental and control system respectively. The Hill coefficient is 1.53 and slope was -1.46 respectively. Each point is the mean of three independent determinations.

These actions may be due to the enzyme or they may be due to the presence of some other proteins in the preparation as several bands were observed on disc gel electrophoresis.

The *O. hannah* PDE-1 exhibited an anticoagulant effect as it significantly increased the normal clotting time of normal citrated human plasma, whereas the crude venom showed coagulant effect Table-4.

Table-4: Effect of the *O. hannah* crude venom and purified PDE-1 on coagulation of normal human plasma (means ± SD of three measurements).

Coagulation parameters	Clotting time (s)
Plasma + $CaCl_2$	175 ± 10
Crude venom	85 ± 3
Purified PDE-I	360 ± 5

The *O. hannah* PDE-1 showed strong anticoagulant effect. Ouyang and Huang [43] reported platelet aggregation inhibiting activity of venom PDE-1 isolated from *A. acutus* but a specific biological role was not assigned. It is possible that a synergistic interaction with hemorrhagic proteases and fibrinogenases found in the same venom occurs during envenomation, interfering with normal haemostatic mechanisms and promoting blood loss leading to circulatory collapse. The role of venom PDE-1 to the sequelae following envenomation is unclear, reduction of cAMP levels and production of toxic secondary metabolites may be the factors contributing to prey death [16].

In conclusion it is suggested that the properties of *O. hannah* PDE-1 isolated in this study were similar to those of other snake venom's PDE. The purification procedure described here is simple, rapid and reproducible should prove beneficial in providing pure protein for investigation into the contribution of this enzyme to the biological activities of *O. hannah* venom and of venom PDE-1 in general.

Experimental

O. hannah crude venom was purchased from Venom Supplies Pty Ltd,Tanunda, South Australia. Bis-*p*-nitrophenyl phosphate, p-nitro-phenyl thymidine-5'-phosphate, cyclic 3′, 5′-AMP, ATP, 1,10-phenanthroline and L- glutathione were from Sigma-Aldrich Co. Acrylamide was obtained from Merck. N,N-methylene bisacrylamide and coomassie brilliant blue R250 were purchased from Fluka Chemie. Ammonium persulphate, sodium dodecyl sulphate (SDS) and Tris (hydroxymethyl) methylamine were from BDH Chemicals. Standard proteins (BlueRanger Pre-stained Protein Molecular Weight Marker Mix) were from Pierce Biotechnology, Inc. Ampholine (pH 3.5-10.0) was from Pharmacia. Glycerol from Winlab Ltd. and glycin and *N,N,N,N*-Tetramethyl ethylenediamine were from Riedel-dekhen AG. All other chemicals used were of analytical grade.

Purification of PDE-I Enzyme by Native Preparative PAGE

The crude venom (freeze dried powder) of *O. hannah* (30 mg/ml) was dissolved in distilled water and centrifuged at 5,000 g for 15 minutes to remove any turbidity and then it was diluted with sampling buffer before use. Crude venom (10mg) was subjected to preparative 10 % native polyacrylamide gel electrophoresis [44]. The slab gel was prepared as described by Laemmli *et al.* with minor modifications [45]. Stock solutions for small pore gels contained 3 M Tris-HCl buffer, pH 9.5. Stock solutions for large pore gels (concentrating gel) contained 0.5 M Tris-H_3PO_4 buffer, pH 9.0. The

reservoir buffer was 0.05 M Tris-glycine, pH 9.5. The protein sample was dissolved in sampling buffer (containing 0.05 M Tris-glycine, pH 9.5, 20 % sucrose and 0.5 % bromophenol blue). Slab was run at 8 °C with a constant voltage of 100 V. Two guide strips were cut from both sides of the slab gel and stained with Coomassie Brilliant Blue R-250 for 30 minutes and then de-stained with the de-staining solutions consisting of water: glacial acetic acid: methanol in the ratio of (68: 7: 25), respectively. The locations of the protein bands on the unstained gel were then carefully marked and removed.

Recovery of Proteins by Electro-elution

The separated venom fractions were eluted from gel using a procedure described by Walker *et al.* [46]. The eluted protein fractions were dialyzed against distilled water, lyophilized and stored at 4 °C. The fraction obtained was analyzed for purity on analytical native (non-SDS) PAGE as described under the fractionation procedure.

Determination of Protein Concentration

The concentration of protein in the fraction thus obtained was determined by using the method of Lowry *et al.* [47].

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purified venom fraction (20 μg) along with crude venom (200 μg) and standard proteins were electrophoresed on SDS-PAGE at 10 % acrylamide concentration according to method of Laemmli *et al.* [45]. The samples were boiled with sample buffer (containing 0.05 M Tris-glycine, pH 9.5, 20 % sucrose and 0.5 % bromophenol blue) in the presence of 0.1 % SDS. The standard molecular weight proteins used were Myosin (215 kDa), phosphorylase b (120 kDa), bovine serum albumin (84 kDa), ovalbumin (60 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28 kDa), and lsozyme (18.3 kDa).

Note: The covalently bound dye alters the apparent molecular weight (M.W.) of the proteins relative to unstained proteins and tends to produce broader bands (Pierce Biotechnology, Inc.).

Isoelectric Focusing (IEF)

For isoelectric focusing of purified *O. hannah* PDE-1 (25 μg) we used an LKB2117 Multiphor system according to the instructions of the manufacturer. An ampholine gel with a pH range of 3.5-10.0 was used. NaOH (1.0 M) was employed as cathode solution and 1M phosphoric acid as anode solution. The gel was focused at 20 W for 90 minutes. The gel was fixed in 12 % TCA solution and washed repeatedly in de-staining solution (25 % ethanol, 8 % acetic acid) and stained in a solution of 0.4 % Coomassie Brilliant Blue R-250. Immediately after the process, the lower part of the gel was cut into pieces at 0.5cm intervals, suspended in 10mL distilled water and the pH gradient determined from cathode to anode.

Assays of Phosphodiesterase Activity

Assay- I

The phosphodieseterase activity was determined according to spectrophotometeric method of Sulkowski and Laskowski [48]. The assay mixture contained in 1 ml: 5 μ moles bis-p-nitrophenyl phosphate, 10 μ moles $MgCl_2$, 100 μ moles Tris-HCl, pH 9.0. Pre-incubation at 37 °C lasted for 5 minutes; enzyme (10 μg) was added and incubated for the next 5 minutes. The reaction was stopped by 2 ml 0.1 N NaOH; the absorbency was read at 400 nm, using 17,600 as molar extinction coefficient; activity was expressed in μ moles$\times$min$^{-1}\times$ml^{-1}.

Assay II

The assay is essentially that of Razell and Khorana [40] where the reaction velocity is determined by an increase in absorbance at 400 nm resulting from the hydrolysis of *p*-nitro-phenyl thymidine-5′-phosphate. One unit hydrolyzes one micromole of *p*-nitro-phenyl thymidine-5′-phosphate per minute at pH 8.9, and 37 °C under specified conditions. The reaction mixture contained in 1 ml: 0.9 ml of 0.11 M Tris-HCl buffer, pH 8.9 containing 0.11 M NaCl and 15 mM $MgCl_2$ and 0.1 ml of 5 mM *p*-nitro-phenyl thymidine-5′-phosphate. Pre-incubation at 37 °C lasted for 5 minutes, 10 μl enzymes (10 μg) was added and incubated for the next 5 minutes.

Assay III

The standard assay of Butcher and Sutherland [49] which consisted of measuring the release of inorganic phosphate with use of an excess of 5′-nucleotidase. This reaction mixture contained 0.01 mM of cyclic 3′, 5′-AMP or 0.002 M ATP (as

the case may be) 2 μ moles of $MgSO_4$, and 36 μ moles of Tris-HCl buffer, pH 7.5 with a suitable dilution of the phosphodiesterase (50 μg) sample being tested in a total volume of 0.9 ml. This mixture was incubated at 37 °C for 3 hours in case of cyclic 3′, 5′-AMP and 30 minutes in case of ATP. After the first 170 minutes of incubation in case of cyclic 3′, 5′-AMP and 20 minutes in case of ATP, 0.1 ml of a *C. atrox* venom solution was added containing 0.1mg Of venom in 0.001 M Tris-HCl, pH 7.5. The entire reaction was terminated by the addition of 0.1 ml of cold 55 % trichloroacetic acid. After addition of trichloroacetic acid, the precipitate was removed by centrifugation, and aliquots of the supernatant fluids were analyzed for inorganic phosphate by the method of Fiske and SubbaRow [50]. The *C. atrox* venom contained a potent 5'-nucleotidase that hydrolyzed the 5′-AMP formed in the phosphodiesterase reaction to adenosine and phosphate. The venom, when used in this concentration, was without effect on cyclic 3′, 5′-AMP. One unit of enzyme was defined as that amount that caused the release of inorganic phosphate per μmole of cyclic 3′, 5′-AMP or ATP as the case may be.

Assay for 5'-Nucleotidase Activity

The 5′-Nucleotidase was determined by the modified method of Sinsheimer and koerner [18]. The reaction mixture contained: 0.1 ml of 1 M glycine buffer, pH 9.0, 0.1 ml of 0.1 M $MgCl_2$, 0.3 ml of 0.01 M AMP, 0.1 ml of enzyme (10 μg) solution and water to a total volume of 1 ml. The mixture was incubated for 15 minutes at 37 °C. The liberated phosphate was determined according to Fiske and SubbaRow [50]. One unit was defined as the amount of enzyme liberating 1 μ mole of inorganic phosphate per minute at 37 °C.

Assay for Alkaline Phosphatase Activity

The ALP activity was determined according to the spectrophotometric method of Hausamen *et al.* [51] at 25 °C using *p*-nitrophenyl phosphate as substrate. The assay mixture (1.2 ml) contained 0.9 M diethanolamine (DEA) at pH 9.8, (0.5 mM magnesium sulfate and 10 μg purified PDE-I as the source of enzyme). The blank was also run under the same conditions and with the same components except for that the enzyme was omitted. One unit of enzyme activity is defined as the amount of ALP that catalyzes the hydrolysis of 1 μmole of pNPP per min per mg of venom protein under standard condition.

Carbohydrate Contents

Carbohydrate content of the purified sample was measured according to the method of DUBOIS *et al.* [52]. The galactose was used as standard.

Effect of pH

The effect of pH on enzyme activity was tested in the range between 6.0 and 12.0 using 0.11 M Tris-HCl buffer. A substrate control was included at every pH tested.

Effect of Temperature

Enzyme activity was tested at different temperatures in the range between 20^0 and 90^0C. A substrate control was included with each test to correct for auto substrate destruction at different temperatures. The activation energy (Ea) for the hydrolysis of bis-*p*-nitrophenyl phosphate by *O. hannah* venom PDE-I was determined by plotting the log of PDE-I activity Vs 1/T (Arrhenius plot). The value of the negative slop of this plot was put in to the following equation to give the activation energy, Ea

$$\text{Slop} = \frac{Ea}{2.3R} \quad \text{-----} [53]$$

whereas R is the Boltzman constant and its value is equal to 1.987calories/deg/mole.

Effect of Metal Ions

Enzyme assay was carried out in the absence and presence of either Mg^{++}, Ca^{++} or Zn^{++}. The concentrations of 5.0, 10.0, 15.0 and 20.0 mM of Mg^{++} were used whereas concentration of 5.0, 7.0 and 10.0 mM Zn^{++} or Ca^{++} were used.

Measurement of Kinetic Constants

The effect of varying substrate concentration on enzyme activity was tested and K_m for the enzyme was determined from Lineweaver-Burk plot [53]. The K_m was calculated using the Grafit software package, version 3.0 [54].This was repeated in the presence of different inhibitors and the type of inhibition and K_i were determined. For competitive and non-competitive inhibitors the K_i were determined by plotting 1/v against i (inhibitor's concentration) according to the graphical method of Dixon [55].

Estimation of IC_{50}

The transformed data log $V/(V_o-V)$ (where V_o = Velocity in the absence of inhibitor and V in the presence of inhibitor) versus log inhibitor concentration were plotted for the determination of IC_{50} for each type of inhibitor.

Lethality Test

Swiss albino male mice (15-20 gm) obtained from the College animal house. The animals were fed on a commercial pellet diet and water *ad libitum*. The purified venom enzymes (0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg body weight) were injected interperitoneally to mice to determine their lethal effect and the LD_{50} will be established. There were eight animals in each group. The control group animals were injected with normal saline.

Determination of Re-calcified Plasma Coagulation Times

Re-calcified plasma clotting time was determined by a Biomeriux apparatus (Option 2) following the manufacturer's instructions. Normal citrated fresh human plasma (100 μl) obtained from volunteers was incubated with (100 μl) crude venom (400 μg) or (50 μg) of purified enzyme at 37 °C for 4 minutes. Then clotting time was determined after the addition of 0.025 M calcium chloride (100 μl).

References

1. A. T. Tu, *Chemistry and molecular biology. in: Venoms*, Wiley & Sons, New York, p. 2 (1977).
2. W. B. Elliott, *Chemistry and immunology of reptilian venoms*, C. Gans, (Eds.), In: Biology of the Reptilia. Vol. **8.** Academic Press, London, p. 163 (1978).
3. S. Iwanaga and T. Suzuki, *Enzymes in snake venoms*, Lee C.Y. Lee (Eds.), In Snake venoms, Handbook *Exp. Pharmacology.*, Vol. **52.** Springer-Verlag, Berlin, p. 61 (1979).
4. K. F. Stocker, *Composition of snake venoms*, K. F. Stocker (Eds.), In: Medical use of snake venom proteins. CRC Press, Boca Raton. Ann Arbor, Boston, p. 33 (1990b).
5. D. Mebs, *Use of toxins in neurobiology and muscle research*, K. F. Stocker (Eds.), In: Medical use of snake venom proteins. CRC Press, Boca Raton. Ann. Arbor, Boston, p. 57 (1990).
6. W. Vogt, *Snake venom constituents affecting the compliment system*, K.F. Stocker (Eds.), In: Medical use of snake venom proteins, CRC Press, Boca Raton. Ann. Arbor, Boston, p. 79 (1990b).
7. R. B. Zingali, *Toxin Rev.*, **26**, 25 (2007).
8. M. A. McLane, X. Zhang, J. Tian and C. P. Straub, *Toxin Rev.*, **26**, 47 (2007).
9. H. H. Chang and S. J. Lo, *Toxin Rev.*, **26**, 189 (2007).
10. K. S. Mohan and C. Yu, *Toxin Rev.*, **26**, 99 (2007).
11. H. Y. Tsai, Y. M. Yang and I. H. Tsai, *Toxicon.*, **51**, 1245 (2008).
12. B. P. Sales and M. L. Santoro, *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.*, **147**, 85 (2008).
13. R. L. P. Adams, J. T. Knowper and D. P. Leader. *The Biochemistry of Nucleic Acids*, R. L. P. Adams (Eds.), Chapman and Hall, New York. p. 87 (1986).
14. S. Perron, S. P. Mackessay and R. M. Hyslop, *J. Colo. Wyo. Acad. Sci.*, **25**, 21 (1993).
15. S. P. Mackessay., Ph.D. Dissertation. *Venoms as trophic adaptation: an ultra-structural investigation of the venom apparatus and biochemical characterization of the proteolytic enzymes of northern Pacific rattlesnake Crotalus viridis oreganos,* Washington State University, (1989).
16. S. P. Mackessy, *Phosphodiesterases, Ribo-nucleases and Deoxyribo-nucleases*, G. S. Bailey (Eds.), in: Enzymes from Snake Venoms, Alaken Inc. Fort Collins, Colorado, p. 361 (1998).
17. W. Bjork, *J. Biol. Chem.*, **238**, 2487 (1963).
18. R. L. Sinsheimer and J. F. Koerner, *J. Boil. Chem.*, **198**, 293 (1952).
19. M. Privat de Garilhe and M. Laskowski, *Biochim. Biophys. Acta.*, **18**, 370 (1955).
20. G. M. Richards, D. J. Tutas, W. J. Wechter and M. Sr. Laskowski, *Biochemistry*, **6**, 2908 (1967).
21. G. R. Phillips, *Hoppe Seylers Z. Physiol. Chem.*, **356**, 1085 (1975).
22. M. Sr. Laskowski, *Meths. Enzymol.*, **65**, 276 (1980).
23. H. Sugihara, T. Nikai, Y. Komori, H. Katada and N. Mori, *Jap. J. Trop. Med. Hyg.*, **12**, 247 (1984).
24. T. Tatsuki, S. Iwanaga and T. Suzuki, *J. Biochem.*, **77**, 831(1975).
25. G. C. Butler, *Phosphodiesterase from snake venoms*, S. P. Colowick and N.O. Kaplan, (Eds.), in: Methods in Enzymology, Vol. **2**, Academic press, New York- London, p. 561 (1955).

26. A. M. Frischauf and F. Eckstein, *Eur. J. Biochem.*, **32**, 479 (1973).
27. N. Mori, T. Nikai and H. Sugihara, *Int. J. Biochem.*, **19**, 115 (1987).
28. H. Sugihara, T. Nikai, M. Naruse, M. Kishida and N. Mori, *Int. J. Biochem.*, **18**, 203 (1986).
29. Z. Levy and A. Bdolah, *Toxicon.*, **14**, 389 (1976).
30. P. Ballario, M. Bergami and F. Pedone, *Anal. Biochem.*, **80**, 646 (1977).
31. H. Y. Halim, E. A. Shaban, M. M. Hagag, E. W. Daoud and M. F. Al-Asmar, *Toxicon.*, **25**, 1199 (1987).
32. A. A. Valerio, A. C. Corradini, P. C. Panunto, S. M. Mello and S. Hyslop, *J. Protein. Chem.*, **21**, 495 (2002).
33. F. Felix, J. Potter and M. Laskowski, *J. Biol. Chem.*, **235**, 1150 (1960).
34. S. S. Stoynov, A. T. Bakalova, S. I. Dimvo, A. V. Mitkova and L. B. Dolapchiev, *FEBS Lett.*, **409**, 151 (1997).
35. R. M. Kini and T. V. Gowda, *J. Chromatogr.*, **291**, 299 (1984).
36. L. B. Dolapchiev, R. A. Vessileva and K. S. Koumanov, *Biochim. Biophys. Acta*, **622**, 331 (1980).
37. S. E. Pollack and D. S. Auld, *Analyt Biochem.*, **127**, 81 (1982).
38. W. E. Razzell, *Phosphodiesterases*. S. P. Colowick and N. O. Kaplan, (Eds.), in: Methods in Enzymology, Vol. **6**, Academic press, New York- London, p. 236 (1963).
39. M. I. Choudhary, N. Fatima, K. M. Khan, S. Jalil, S. Iqbal and Atta-Ur-Rahman, *Bioorg. Med. Chem.*, **14**, 8066 (2006).
40. W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).
41. T. Suzuki, S. Iwanaga and K. Nitta, *J. Pharm. Soc. Japan*, **80**, 1040 (1960).
42. F. E. Russell, F. W. Buess and M. Y. Woo, *Toxicon.*, **1**, 99 (1963).
43. C. Ouyang and T. F. Haung, *Toxicon.*, **24**, 1099 (1986).
44. S. S. Al-Saleh, H. K. Ghneim, H. Y. Haddad and S. U. Khan, *Cell Biochem. Funct.,* **20**, 153 (2002).
45. U. K. Laemmli, *Nature*, **227**, 680 (1970).
46. J. E. Walker, A. C. Auffert and A. Carne, *Eur J. Biochem.*, **123**, 253 (1982).
47. O. H. Lowry, N. J. Rosebrough, A. L. Faar and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
48. E. Sulkowski and M. Sr. Laskowski, *Biochimica et Biophysica Acta,* **240**, 443 (1971).
49. R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.*, **237**, 1244 (1961).
50. C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).
51. T. U. Hausamen, R. Hlger, W. Rick and W. Gross, *Clin. Chim. Acta*, **15**, 241 (1967).
52. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Roberts and F. Smith, *Analyt. Biochem.*, **28**, 350 (1956).
53. I. H. Segel, *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme System*, John Wiley and Sons, New York, p. 933, (1975).
54. R. J. Leatherbarrow, GraFit version 3.0. Erithacus Software Ltd. Staines. U. K. (1992).
55. M. Dixon, *Biochem. J.*, **55**, 170 (1953).