CHAPTER V

PURIFICATION AND CHARACTERIZATION OF A NOVEL APYRASE (RUVIAPYRASE) ENZYME FROM THE VENOM OF RUSSELL'S VIPER (*DABOIA RUSSELII*) OF WESTERN INDIA (WI) ORIGIN

5.1 Results

5.1.1 Purification of an apyrase by multi-dimensional chromatography

5.1.1.1 Fractionation of WI RVV through GF followed by anion-exchange chromatography

Fractionation of WI RVV on a HiLoad 16/600 Superdex 75 pg gel filtration column resolved it into 10 peaks as shown in Fig. 4.2. The peak GF1 was characterized with highest ATPase as well as ADPase activities, and a second separation of WI RVV GF1 on a HiTrap Q FF anion-exchange column resolved it into 17 peaks (Fig. 5.1). Among them, the peak 8 (WI RVV GF1 HiQ-8) exhibited highest ATPase as well as ADPase activities.

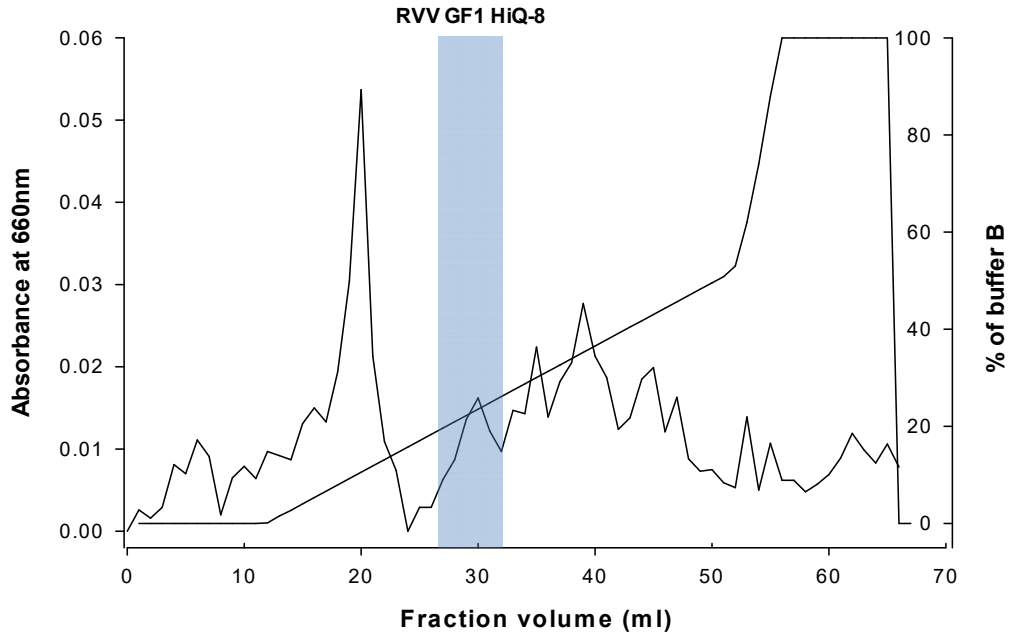


Fig. 5.1. Fractionation of WI RVV GF1 on an FPLC HiTrap Q FF anion exchange column. The column was washed with two CV of buffer A (20 mM Tris-HCl, pH 7.4) to elute the unbound proteins and the bound proteins were eluted by a 0 to 50% linear gradient of buffer B (20 mM Tris-HCl, pH 7.4 containing 1.0 M NaCl) for 40 min at a flow rate of 1.0 ml/min. The peak showing highest ATPase as well as ADPase activity is highlighted.

5.1.2 Assessment of purity and molecular mass of RVV apyrase

5.1.2.1 RP-HPLC analysis of purified RVV apyrase

The RP-HPLC analysis of peak WI RVV GF1 HiQ-8 yielded a single major peak with a retention time of 7.0 min (Fig. 5.2).

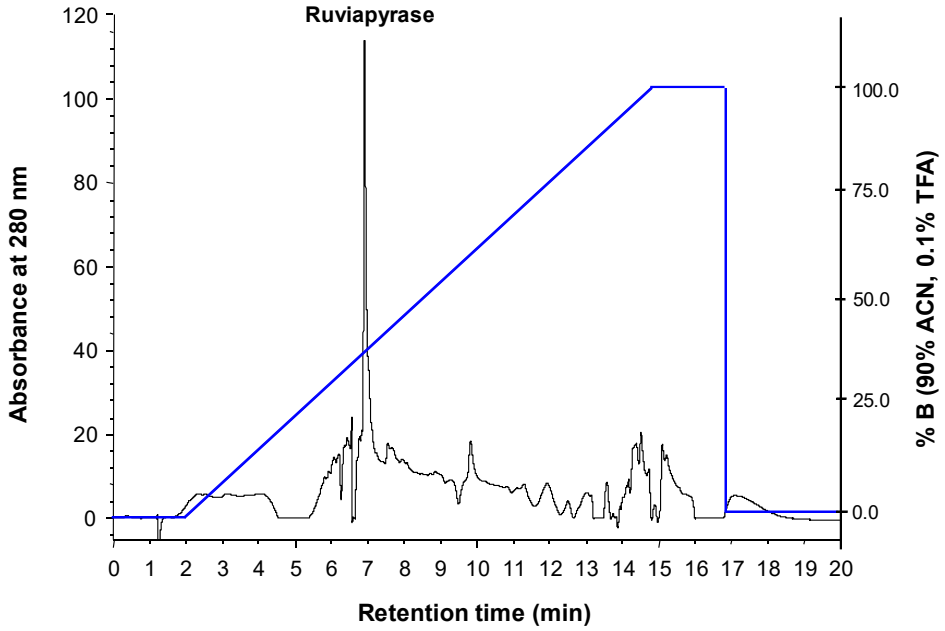


Fig. 5.2. RP-HPLC analysis of WI RVV GF1 HiQ-8 on a C_{18}-RP-HPLC column (2.1 × 150 mm, 3 µm). The column was washed with solvent A (0.1% TFA) for 2 min, followed by elution of bound proteins with a linear gradient over 12 min from 0 to 100% of solvent B (90% ACN in H_2O containing 0.1% TFA).

5.1.2.2 SDS-PAGE analysis of purified RVV apyrase

The homogeneity of the enzyme preparation was assessed by 12.5% SDS-PAGE analysis where it displayed a single protein band with the apparent molecular weight of 79.4 kDa under both reduced and non-reduced conditions (Fig. 5.3). This purified enzyme from WI RVV was named Ruviapyrase (Russell's viper apyrase). A summary of the purification of Ruviapyrase is shown in Table 5.1.

Table 5.1. A summary of purification of Ruviapyrase from WI RVV. Values are mean ± SD of triplicate determinations.

Purification steps	Total protein (mg)	Yield of protein (%)	ATPase activity		Purification (fold)	ADPase activity		Purification (fold)
			Total activity (Units*) × 10^4	Specific activity (Units/mg) × 10^4		Total activity (Units*) × 10^4	Specific activity (Units/mg) × 10^4	
Crude RVV	161.0 ± 8.05	100	201.7 ± 3.3	1.2 ± 0.05	1.0	87.2 ± 2.8	0.5 ± 0.02	1.0
GFC (GF-1)	10.0 ± 0.50	6.2 ± 0.31	14.7 ± 0.6	1.5 ± 0.05	1.2 ± 0.04	7.0 ± 0.2	0.7 ± 0.03	1.3 ± 0.04
AEX (HiQ-8)	0.7 ± 0.03	0.5 ± 0.02	1.5 ± 0.06	2.1 ± 0.07	1.6 ± 0.06	0.9 ± 0.01	1.3 ± 0.02	2.3 ± 0.07

***** Unit of ATPase and ADPase activity is defined as micromoles of Pi released per min at 37 °C.

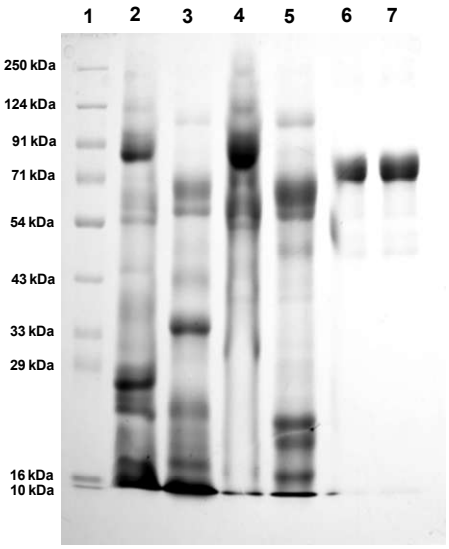


Fig. 5.3. Determination of purity and molecular mass of Ruviapyrase by 12.5% SDS-PAGE analysis under reduced and non-reduced conditions. Lane 1, protein molecular markers; lanes 2, 4 and 6 represent crude RVV (80 µg), RVV GF1 (60 µg), and Ruviapyrase (40 µg) under non-reduced condition; lanes 3, 5 and 7 represent crude RVV (80 µg), RVV GF1 (60 µg), and Ruviapyrase (40 µg) under reduced condition.

5.1.2.3 MALDI-TOF-MS analysis of Ruviapyrase

The purity of the enzyme was further evaluated by MALDI-TOF-MS analysis and the molecular weight of the purified enzyme was found to be 83041.02 Da (Fig. 5.4).

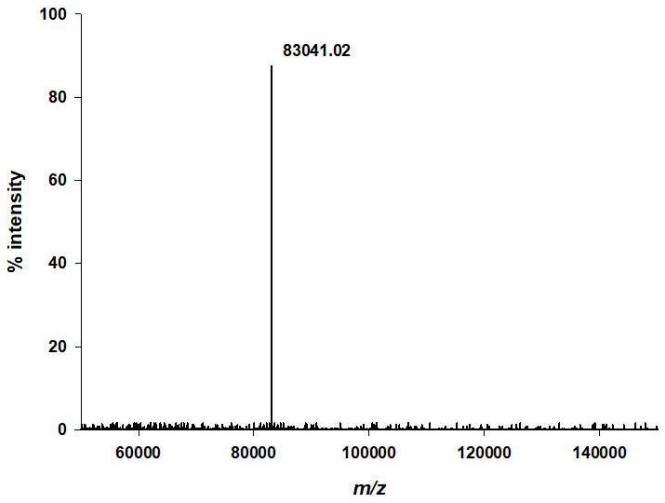


Fig. 5.4. MALDI-TOF-MS spectrum of Ruviapyrase showing a signal at m/z 83041.02 corresponding to a single species with a molecular mass of 83.0 kDa. Two microgram

of the purified enzyme was mixed with 0.5 µl of α-cyano-4-hydroxycinnamic acid matrix, dried and analyzed on a Bruker Daltonics UltrafleXtreme mass spectrometer at 40000 resolutions full width at half maximum.

5.1.3 Mass spectrometry identification of Ruviapyrase

The tryptic and semi-tryptic fragments of Ruviapyrase could not be matched to any Viperidae entries of the NCBI database by MS/MS search. However, a few *de novo* sequences of the enzyme demonstrated sequence similarity with apyrase entries from different organisms submitted to the NCBI database (Table 5.2). Notably, a *de novo* peptide sequence `ATGEVLNRHCEWVKVID` (m/z 985.9514), that shared 81% identity and 94% sequence coverage with a soluble calcium-activated nucleotidase 1 (accession no. XP_015272480.1) from *Gekko japonicus*, indicated the presence of a putative conserved domain of the Apyrase superfamily (Table 5.2).

5.1.4 Biochemical characterization

5.1.4.1 Catalytic activity and stability of Ruviapyrase

Ruviapyrase exhibited dose-dependent ATPase and ADPase activities when assayed by biochemical method (Fig. 5.5a).

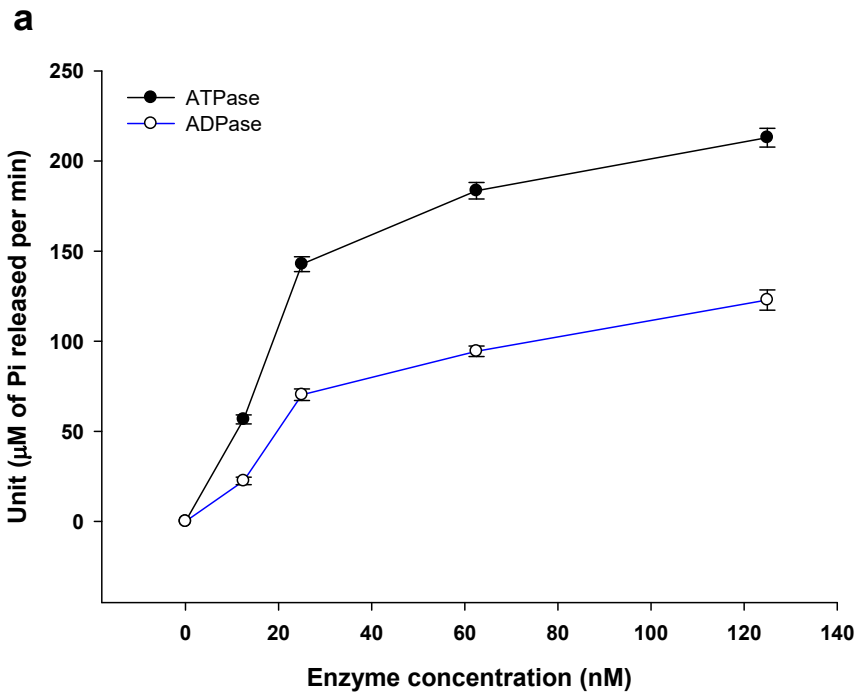


Fig. 5.5a. Dose-dependent ATPase and ADPase activities of Ruviapyrase determined by biochemical analysis. Values are mean ± SD of triplicate determinations.

Table 5.2. Results of *de novo* sequencing and subsequent BLASTp search of the *de novo* peptides generated from Ruviapyrase by PEAKS 7.0 software. The table includes relevant *de novo* peptide with respective protein hits along with their ALC score, mass, charge (z) and ppm. In addition, total score, query coverage, E-value and percent identity of the protein hits obtained from BLASTp search are also shown.

***De novo* peptide**	**ALC (%)**	**z**	**Mass (Da)**	**ppm**	**BLAST similarity**	**Accession no**	**Source organism**	**Total score**	**Query coverage**	**E-value**	**% Identity**
MNRGNGGGLVRK	57	2	1274.7	0.5	Soluble calcium-activated nucleotidase 1-like	XP_014238353.1	*Trichogramma pretiosum*	53.2	50%	1.1000	100%
DWLLTGKVFYSGLK	73	2	1667.9	-8.7	Soluble calcium-activated nucleotidase 1-like	XP_015752245.1	*Acropora digitifera*	41.8	57%	1.1000	83%
ATGEVLNRHCEWVKVID	57	2	1969.9	4.5	Soluble calcium-activated nucleotidase 1	XP_015272480.1	*Gekko japonicus*	40.9	94%	7.00E-08	81%
TVSNGTLYLLQQLPK	83	2	1717.9	0.1	Soluble calcium-activated nucleotidase 1-like	XP_008472404.1	*Diaphorina citri*	37.3	53%	0.6100	100%
ECESWLLFALQFWR	69	2	1865.9	5.4	Salivary apyrase	AAO06829.1	*Anopheles stephensi*	32.2	64%	0.3100	86%
SEQQFVVKVSLK	50	2	1433.8	0.0	Apyrase	KPI98864.1	*Papilio xuthus*	30.5	50%	6.5000	100%
KVFQKAGCPQK	56	2	1290.7	7.7	Apyrase	KZC05761.1	*Dufourea novaeangliae*	26.7	54%	16.0000	100%
DAGSSHTSH	82	2	897.6	1.2	Ectonucleoside triphosphate diphosphohydrolase 1 isoform 3	NP_001157650.1	*Homo sapiens*	26.1	88%	0.0030	100%
LMTHYLGLGLR	55	2	1288.7	9.1	ATP-diphosphohydrolase 2	ABI79456.1	*Schistosoma mansoni*	21.4	54%	0.2200	100%
DFYTFDSEGLVR	72	2	1447.7	0.7	Apyrase-3	AEP27181.1	*Heligmosomoides polygyrus bakeri*	20.2	50%	0.7700	83%
SHTMGFR	50	2	850.4	4.1	Apyrase	ETN57989.1	*Anopheles darlingi*	17.2	57%	3.1000	100%
LRSLESFEEAECRK	63	2	1753.8	8.7	Soluble calcium-activated nucleotidase 1 isoform X1	XP_012611646.1	*Microcebus murinus*	17.2	50%	13.0000	86%
KHGRKKK	53	2	880.6	4.3	Apyrase	EPB72521.1	*Ancylostoma ceylanicum*	15.5	57%	13.0000	100%

The RP-HPLC analysis of nucleotide hydrolytic activity of Ruviapyrase suggested the conversion of both ATP and ADP to AMP in a time-dependent manner; however, the latter was not degraded further (Fig. 5.5b).

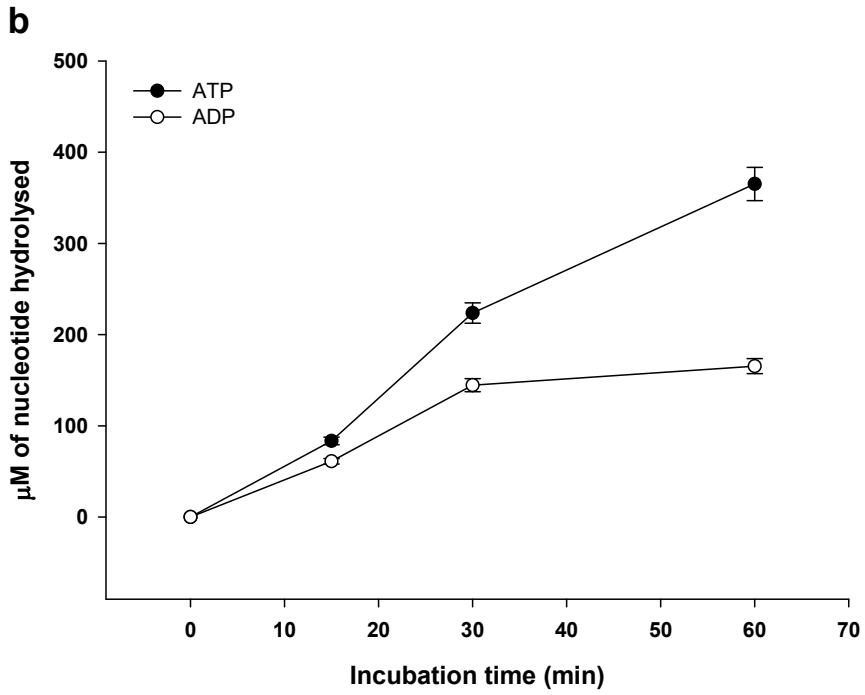


Fig. 5.5b. RP-UHPLC analysis of kinetics of ATP and ADP hydrolysis by Ruviapyrase on an Acclaim 300 C_{18} column (4.6 × 150 mm, 3 µm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.0 (solvent A). The bound nucleotides were eluted with the following multi-step gradient of 100% methanol (solvent B): 0% B for 1.4 min, 0-12.5% B in 3.6 min, 12.5% B for 1.5 min, and 12.5-0% B in 2.5 min. The elution of nucleotides at a flow rate of 1 ml/min was monitored at 254 nm. The micromoles of ATP/ADP hydrolyzed were calculated from the area under standard curve of the respective nucleotides. Values are mean ± SD of triplicate determinations.

Both ATPase as well as ADPase activities of Ruviapyrase diminished rapidly at physiological conditions (37 °C, pH 7.4) and it lost >80% of its enzyme activity within 2 h of incubation at 37 °C. On the contrary, the same activities of crude RVV were relatively more stable ($p<0.05$) compared to the purified enzyme (Fig. 5.6).

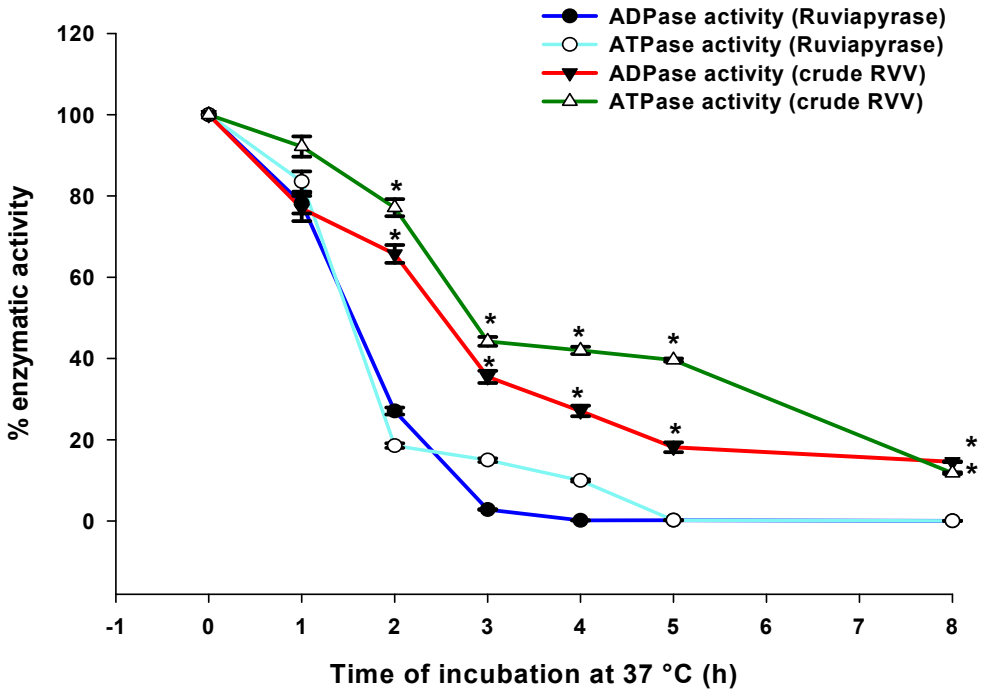


Fig. 5.6. A comparison of ATPase and ADPase activities of Ruviapyrase and WI RVV determined by biochemical analysis. The experiment was performed at mammalian physiological conditions (37 °C, pH 7.4). Values are mean ± SD of triplicate determinations. Significance of difference with respect to ATPase and ADPase activities of Ruviapyrase, *$p < 0.05$.

5.1.4.2 Substrate specificity of Ruviapyrase

Ruviapyrase demonstrated preferential hydrolysis of ATP over ADP with a 1.6 fold higher specific activity towards the former substrate. Nevertheless, Ruviapyrase could not cleave AMP and BNPP thereby suggesting it is devoid of 5′- NT and PDE activities (Table 5.3). Further, the ATPase/ADPase activity ratio of Ruviapyrase at 30 min was found to be 1.55 by RP-HPLC analysis, thereby suggesting a good correlation of apyrase activity measured by biochemical and RP-HPLC methods.

Table 5.3. Substrate specificity of Ruviapyrase against ATP, ADP, AMP, and BNPP substrates. Values are mean ± SD of triplicate determinations. Significance of difference with respect to ADP hydrolysis by Ruviapyrase, *p < 0.05.

Substrate (1.5 mM)	Specific activity (Unit per mg protein)
BNPP	0.0
AMP	0.0
ADP	12578.1 ± 371.2
ATP	20677.1 ± 545.3*

5.1.4.3 Effect of metal ions and chemical inhibitors on the enzymatic activity of Ruviapyrase

The effect of metal ions on the catalytic activities of Ruviapyrase is summarized in Table 5.4. Zn^{2+} significantly inhibited the ATPase and ADPase activities of Ruviapyrase, while ATPase and ADPase activities were enhanced by 1.14 and 1.43 folds, and 1.16 and 1.20 folds, in presence of Mg^{2+} and Ca^{2+} ions, respectively.

Table 5.4. Effect of Mg^{2+}, Ca^{2+}, and Zn^{2+} ions on ATPase and ADPase activities of Ruviapyrase (25 nM). Values are mean ± SD of triplicate determinations.

Metal (final concentration 2 mM)	ATPase activity	ADPase activity
None	100%	100%
Mg^{2+}	114.2 ± 0.06%	116.8 ± 0.02%
Zn^{2+}	28.9 ± 0.01%	37.7 ± 0.02%
Ca^{2+}	143.7 ± 0.07%	120.8 ± 0.06%

ATPase and ADPase activities of Ruviapyrase were inhibited by 5 mM EDTA, 2 mM DTT and 5 mM IAA to comparable extents (Fig. 5.7); however PMSF at a concentration of 2 mM failed to inhibit the catalytic activity of Ruviapyrase (Fig. 5.7).

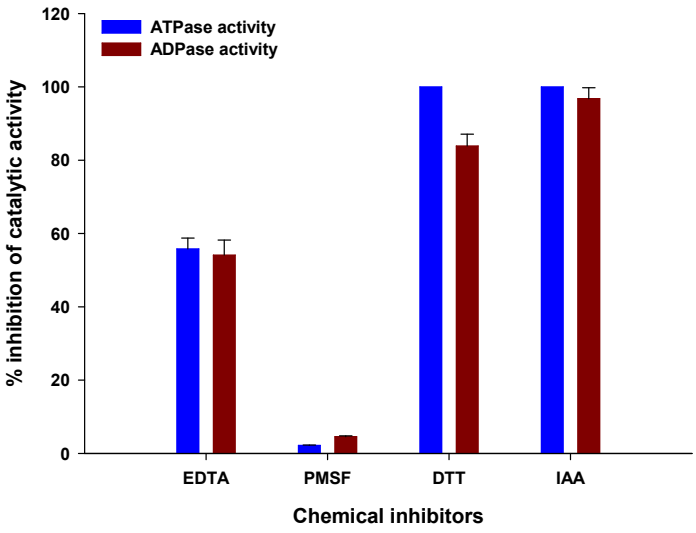
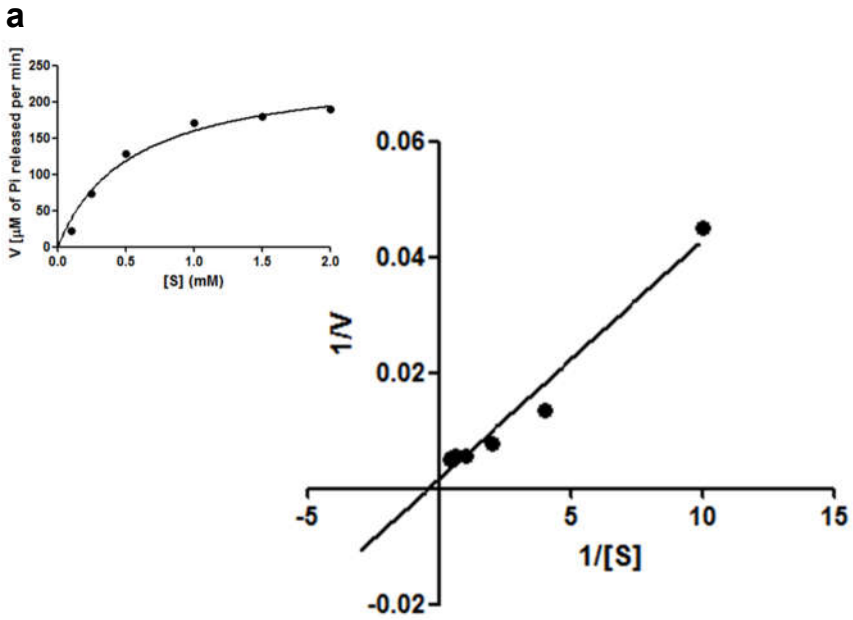


Fig. 5.7. Effect of chemical inhibitors on catalytic activities of Ruviapyrase. The chemical inhibitors were EDTA (5 mM), PMSF (2 mM), DTT (2 mM), and IAA (5 mM). Values are mean ± SD of triplicate determinations.

5.1.4.4 Kinetic parameters of Ruviapyrase

The Michaelis-Menten constant (*Km*) of Ruviapyrase against ATP and ADP were calculated at 2.5 and 5.7 µM, respectively and the *Vmax* value of ATP and ADP hydrolysis by Ruviapyrase were determined at 615.0 and 372.8 micromoles of Pi released per min, respectively (Figs. 5.8a,b; Table 5.5).



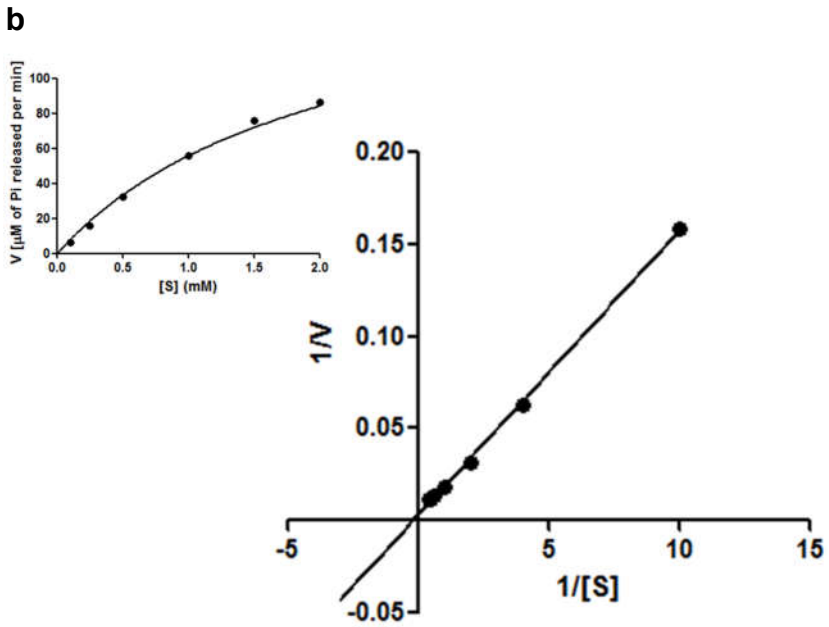


Fig. 5.8. Determination of *Km* and *Vmax* of Ruviapyrase (25 nM) against **a.** ATP and **b.** ADP (0.1-2.5mM). Lineweaver-Burk plots and Michaelis-Menten plots (insets) of Ruviapyrase were generated using GraphPad Prism 5.0 software.

In addition, the turnover numbers (*Kcat*) of Ruviapyrase against ATP and ADP were found to be 24,600 and 14,914 per min, respectively, whereas the specificity constant or kinetic efficiency of the enzyme against ATP (9685.0) was 3.7 folds higher compared to ADP (2584.8) under identical experimental conditions (Table 5.5).

Table 5.5. Kinetic parameters of Ruviapyrase (25 nM) against ATP or ADP substrate. Values are mean ± SD of triplicate determinations. *Km* and *Vmax* values were calculated using GraphPad Prism 5.0 software, while *Kcat* and Specificity constant were calculated using equations 6 and 7, respectively as described in section 3.2.6.3.4.

Kinetic parameter	ATP	ADP
Km	2.5 ± 0.1µM	5.7 ± 0.2 µM
Vmax	615.0 ± 19.1 µM min^{-1}	372.8 ± 15.6 µM min^{-1}
Kcat	24600.0 ± 321.8 min^{-1}	14914.0 ± 192.8 min^{-1}
Specificity constant	9685.0 ± 321.4	2584.8 ± 102.1

5.1.4.5 Carbohydrate content and glycosylation of Ruviapyrase

From a standard curve of D-glucose, the neutral carbohydrate content of Ruviapyrase was estimated to be 24.4 µg per mg of protein (equivalent to 2.4% of the total protein). The SDS-PAGE analysis of PNGase F-treated Ruviapyrase demonstrated a well-resolved protein band at ~33 kDa, while the native enzyme migrated as a single broad band of ~79.4 kDa (Fig. 5.9). The percentage of N-linked oligosaccharides in Ruviapyrase calculated from the molecular masses of the native and PNGase F treated enzyme was found to 58.4%. Further, Concanavalin A bound Ruviapyrase was eluted with 1 M glucose, thereby suggesting it is a highly glycosylated enzyme.

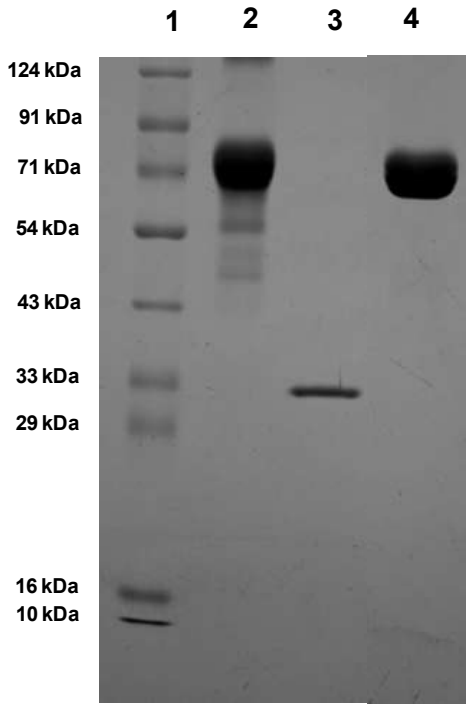


Fig. 5.9. Determination of N-linked glycosylation in Ruviapyrase. Lane 1, protein molecular markers; lane 2, Ruviapyrase (40 µg, denatured) incubated at 37 °C for 4 h; lane 3, Ruviapyrase (40 µg, denatured) treated with PNGase F (500 units) for 4 h at 37 °C and lane 4, native Ruviapyrase (40 µg).

5.1.4.6 Spectrofluorometric interaction between ATP/ADP and Ruviapyrase

With increasing concentration of Ruviapyrase (0.25-2.5 nM), a decrease in λ_{max} of ATP and ADP at 380 nm was observed in the spectrofluorometric analysis (Figs. 5.10a,b). The change in the maximum fluorescence intensities (ΔF) of ATP and ADP in

presence of the enzyme was fitted to a one site-specific binding curve, and the *Kd* values for the interaction of Ruviapyrase with ATP and ADP were calculated at 0.92 ± 0.03 nM and 1.25 ± 0.05 nM, respectively (Figs. 5.11a,b).

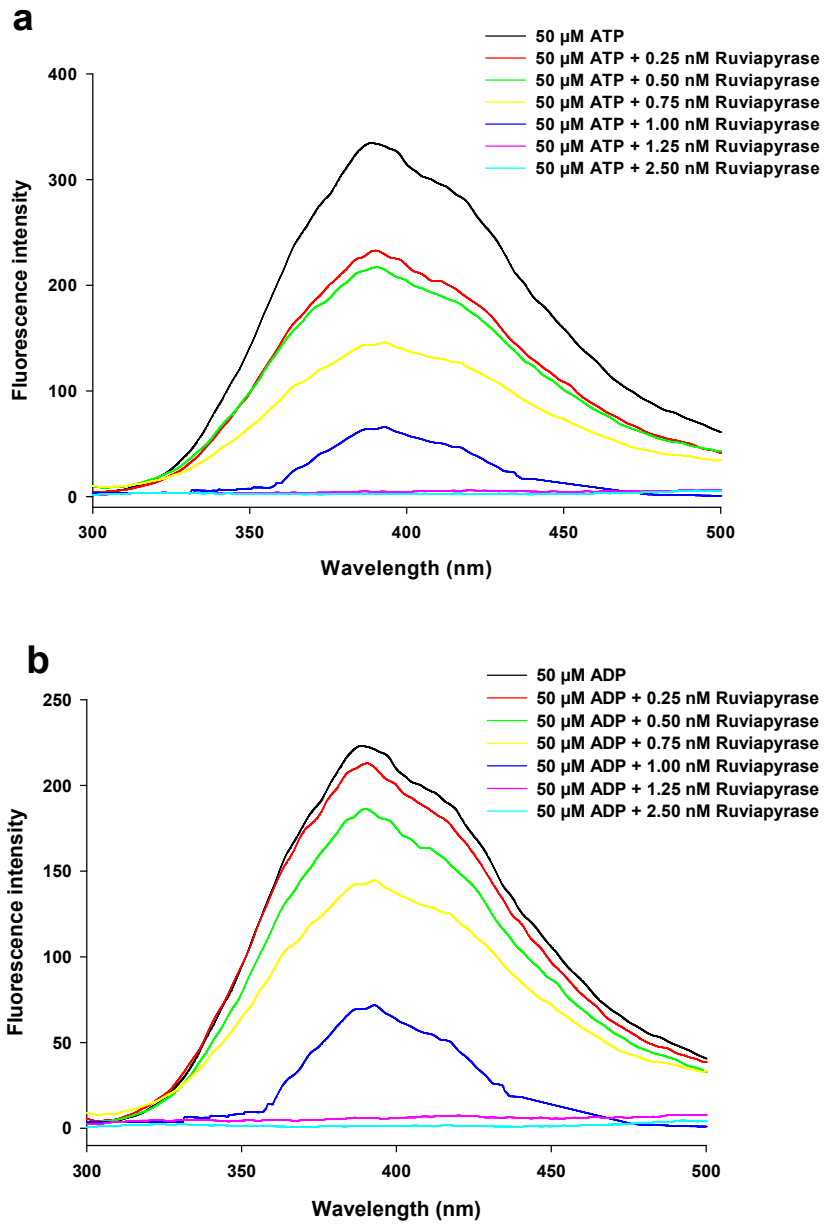


Fig. 5.10. Spectrofluorometric interaction of ATP or ADP with Ruviapyrase. The decrease in the maximum fluorescence intensity (λ_{max}) of **a.** ATP (50 µM) and **b.** ADP (50 µM) in presence of different concentrations (0.25-2.5 nM) of Ruviapyrase.

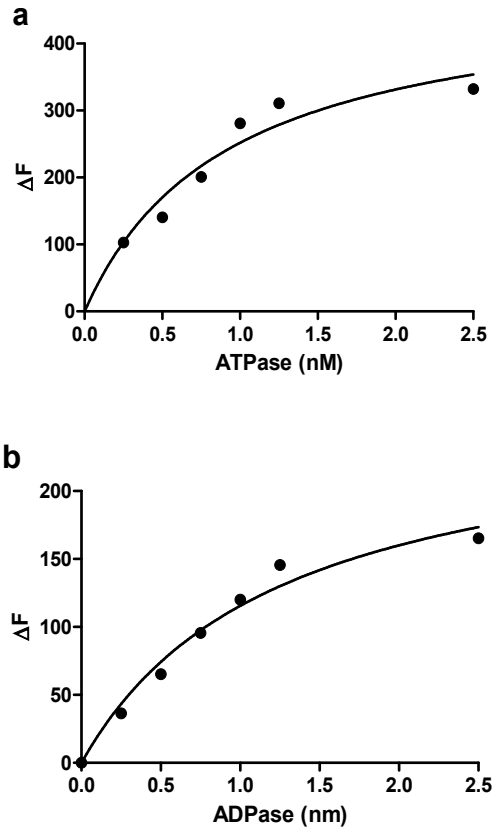


Fig. 5.11. One site-specific binding curve showing the change in the maximum fluorescence intensity (λ_{max}) of **a.** ATP (50 μM) and **b.** ADP (50 μM) by different concentrations (0.25-2.5 nM) of Ruviapyrase.

5.1.5 Pharmacological characterization

5.1.5.1 Assessment of cytotoxicity of Ruviapyrase on MCF-7 cells and erythrocytes

Ruviapyrase at a concentration of 125 nM was non-toxic to MCF-7 cell and was devoid of haemolytic activity (Table 5.6).

Table 5.6. Cytotoxicity and haemolytic activities of Ruviapyrase. Haemolytic activity was assessed against 5% washed erythrocytes (goat) where absorbance of erythrocytes treated with 0.1% Triton X-100 was considered as 100% activity. Values are mean ± SD of triplicate determinations.

Pharmacological property	Value
Cytotoxicity against MCF-7 cells (125 nM) (% cell death)	2.6 ± 0.1
Haemolytic activity (25 nM) (% lysis)	0.1 ± 0.005

5.1.5.2 Plasma clotting activity, platelet modulation and effect on ADP-induced platelet aggregation by Ruviapyrase

Ruviapyrase (25 nM) was found to be marginal anticoagulant (10 ± 0.3 units). Further, at a concentration of 120 nM, Ruviapyrase demonstrated deaggregation of PRP (antiplatelet activity) and it dose-dependently inhibited the ADP-induced platelet aggregation (Fig. 5.12).

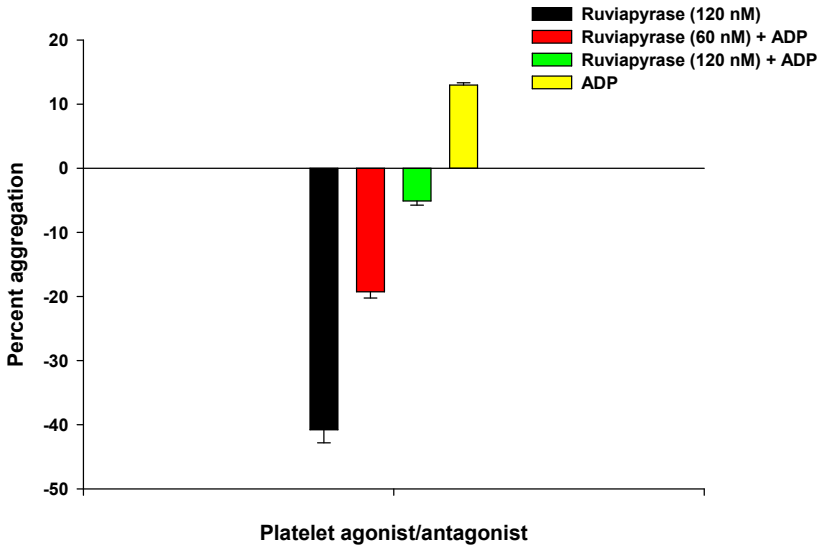


Fig. 5.12. Effect of Ruviapyrase on platelet-rich plasma (PRP) and ADP-induced platelet aggregation. PRP (100 µl) was pre-incubated with 60 and 120 nM of the Ruviapyrase for 5 min prior to the addition of ADP (30 µM) or 1X PBS, pH 7.4 (control). The aggregation induced by ADP was considered as 100% activity and other values were compared with that. Values are mean ± SD of triplicate determinations.

5.1.6 Immunological characterization

5.1.6.1 Neutralization of enzyme activity of Ruviapyrase by commercial Indian PAV and MAV

At a Ruviapyrase: antivenom ratio of 1:5, PAV (Bharat Serums and Vaccines Ltd.) and MAV (Vins BioProducts Ltd.) exhibited moderate neutralization (~40 to 70%) of ATPase and ADPase activities of Ruviapyrase (Fig. 5.13); however, at 1:10 ratio the catalytic activities of the enzyme were completely neutralized. In addition, the platelet deaggregation property exhibited by Ruviapyrase was also well neutralized by both the antivenoms (Fig. 5.13).

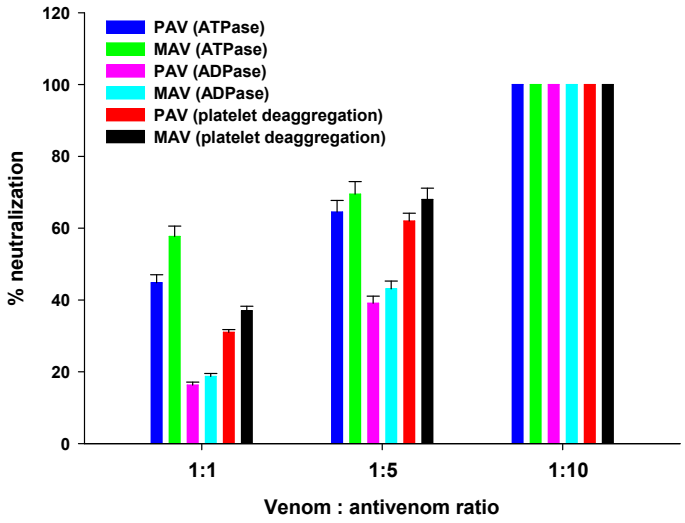


Fig. 5.13. Neutralization of ATPase, ADPase, and platelet deaggregation activities of Ruviapyrase by commercial PAV and MAV. Ruviapyrase (1 µg) was pre-incubated with PAV or MAV at 1:10 (protein: protein) ratio for 30 min at 37 ˚C followed by assay of enzymatic activities and platelet deaggregation activities. Values are mean ± SD of triplicate determinations.

5.1.6.2 Immuno cross-reactivity of Ruviapyrase towards commercial PAV and MAV

ELISA depicted that Ruviapyrase was recognized by the commercial PAV as well as MAV, which corroborates the neutralization results (Fig. 5.14). Nevertheless, the immuno-recognition of Ruviapyrase by MAV was significantly higher ($p<0.05$) compared to PAV (Fig. 5.14).

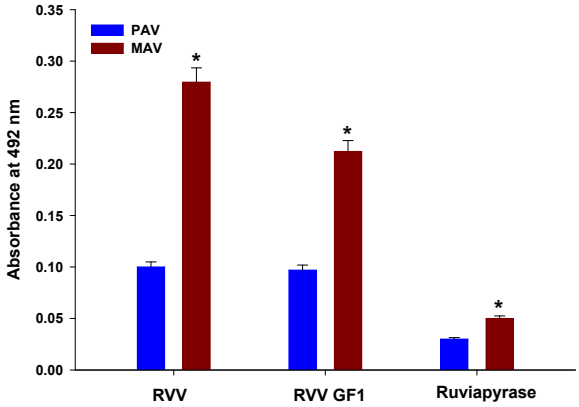


Fig. 5.14: Immuno cross-reactivity of venom components with commercial MAV and PAV by ELISA. The immuno cross-reactivity of RVV or RVV GF1 or Ruviapyrase

(100 ng) with commercial MAV and PAV (200 ng) was studied by ELISA as described in section 3.2.4.1. Values are mean ± SD of triplicate determinations. Significance of difference with respect to cross-reactivity of PAV, *$p < 0.05$.

5.2 Discussion

Nucleotide hydrolyzing enzymes such as phosphodiesterase, 5′-nucleotidase, ADPase, ATPase, and alkaline phosphatase, are ubiquitously present in snake venoms including RVV [1-4]; however, they are poorly characterized due to their low abundance in venom, poor stability, and sensitivity to denaturing agents [5]. These enzymes can cleave a variety of nucleotide molecules *viz*. ATP, ADP and AMP, and release purines from their respective substrates. Purines are a class of multi-toxin that play a pivotal role in prey immobilization via activation of A1 (neuronal), A2 (vascular smooth muscle) and A3 (mast cell) receptors and inducing vascular permeability and hypotension [6,7].

Ruviapyrase is a high molecular weight anionic (at physiological pH) protein that exhibits characteristic features of apyrase enzymes. Mass spectrometry analysis can be a useful tool for identification of snake venom proteins; however, it is limited by the paucity of existing database [3,8]. Therefore, although ATPase and ADPase activities of RVV was confirmed by biochemical assays, albeit due to the absence of protein sequences of these snake venom enzymes they could not be identified by LC-MS/MS analysis [3]. On the contrary *de novo* sequencing of proteins is database independent and can be a convenient way of determining the identity of novel proteins [9]. The presence of a putative conserved domain of apyrase superfamily in Ruviapyrase suggests that the purified enzyme belongs to apyrase family (EC 3.6.1.5).

It was hypothesized that ATPase is a toxic component of venom, as it was thought to produce shock symptoms by the depletion of ATP [10]. It is logical to understand that immobilization of the prey or victim could be achieved by depletion of their cellular harmony (ATP) so rapidly that the agile prey loses its ability to evade. Therefore, the transient stability of Ruviapyrase under physiological conditions indicates that this class of enzymes may have evolved in snake venoms to rapidly accomplish its task of depletion of their cellular harmony (ATP). However, further *in vivo* studies on Ruviapyrase are warranted to establish the exact role of this enzyme in

prey immobilization. Interestingly, the catalytic activity of Ruviapyrase declines more rapidly compared to the same activity in crude venom which suggests possible interactions of this enzyme with other RVV components for better stability.

It has been reported that Ca^{2+} and Mg^{2+} ions act as cofactors in mammalian ATPases or other nucleotidases [11]. The ATPase and ADPase activities of Ruviapyrase were found to be marginally enhanced in presence of divalent cations which corroborates the earlier finding [11]. In addition, the catalytic activity of Ruviapyrase was significantly inhibited by EDTA and DTT that suggests the important role of metal ions in the catalytic activity and presence of disulfide linkages in the enzyme. Further, IAA also inhibited the catalytic activity of Ruviapyrase by modification of cysteine residues involved in intramolecular disulfide bond formation [12].

Preferential hydrolysis of ATP and ADP over AMP suggests that Ruviapyrase can cleave phospho-anhydride bonds present in ATP and ADP, while it failed to catalyze the hydrolysis of phosphoester bond present in AMP. Apyrase enzymes are classified based on the ATPase/ADPase ratio which is defined as a ratio between the rates of hydrolysis of ATP and ADP [13,14]. Enzymes with ATPase/ADPase ratio of ~10 or more (high ratio) are classified under apyrase A, while those with a ratio of ~1 (low ratio) are grouped as apyrase B [13,14]. The ATPase/ADPase ratio of Ruviapyrase was found to be 1.6 suggesting it belongs to class apyrase B.

The concentration of ATP (2-8 μM/l) in mammalian blood is significantly higher than that of ADP (0.13-0.26 μM/l) [15,16]. In addition, ATP released from the erythrocytes due to manifestation of intravascular haemolysis in RV bite patients [1] may also be a target for Ruviapyrase. Therefore, although Ruviapyrase can hydrolyse both ATP and ADP, its ATPase activity may have a major contribution to the pathophysiology of RV envenomation.

Strong affinity of Ruviapyrase towards Concanavalin A matrix suggests the presence of α-D-mannosyl and α-D-glucosyl residues in this enzyme [17]. Further, Ruviapyrase contained neutral carbohydrates and significant amounts of N-linked oligosaccharide. This is in good agreement with previous studies demonstrating glycosylation as a common feature of apyrase enzymes purified from other biological sources [18,19]. In addition, several snake venom enzymes are associated with glycosylation and the importance of this post-translational modification in maintaining

the catalytic activity and stability of human brain ecto-apyrase [19] and snake venom thrombin-like enzyme [20] has already been reported. Unfortunately, due to transient stability of Ruviapyrase at 37 °C, the exact role of glycosylation on catalytic activity and/or stability of Ruviapyrase could not be ascertained.

Usually high molecular weight venom proteins (>50 kDa) serve as better immunogen than their low molecular weight counterparts and during the process of raising antivenoms in horses, sufficient antibodies are produced against these components. Subsequently, commercial antivenoms can effectively neutralize the enzymatic properties and pharmacological effects of these high molecular weight venom proteins [3]. Therefore, the catalytic activity and platelet deaggregation property of Ruviapyrase were well neutralized by commercial PAV and MAV. In addition, immuno-recognition of Ruviapyrase by commercial antivenom suggests that the enzyme may not pose a serious threat in RV bite victims if commercial antivenom is immediately administered. Further, the enzyme is also devoid of toxicity against MCF7 cells and erythrocytes; however, it inhibited ADP-induced platelet aggregation. ADP, a key platelet agonist can induce shape change and platelet aggregation for maintaining physiological haemostasis [22]. Therefore, inhibition of ADP-induced platelet aggregation by Ruviapyrase may lead to platelet dysfunction and increased risk of bleeding in RV envenomed patients [1].

Bibliography

[1] Mukherjee, A. K., Ghosal, S., and Maity, C. R. Some biochemical properties of Russell's viper (*Daboia russelli*) venom from Eastern India: correlation with clinico-pathological manifestation in Russell's viper bite. *Toxicon*, 38(2): 163-175, 2000.

[2] Mukherjee, A. K. and Maity, C. R. Biochemical composition, lethality and pathophysiology of venom from two cobras-- *Naja naja* and *N. kaouthia*. *Comp Biochem Physiol B Biochem Mol Biol*, 131(2): 125-132, 2002.

[3] Mukherjee, A. K., Kalita, B., and Mackessy, S. P. A proteomic analysis of Pakistan *Daboia russelii russelii* venom and assessment of potency of Indian polyvalent and monovalent antivenom. *J Proteomics*, 144: 73-86, 2016.

[4] Sales, P. B. and Santoro, M. L. Nucleotidase and DNase activities in Brazilian snake venoms. *Comp Biochem Physiol C Toxicol Pharmacol*, 147(1): 85-95, 2008.

[5] Marcus, A. J., Broekman, M. J., Drosopoulos, J. H., Islam, N., Alyonycheva, T. N., Safier, L. B., Hajjar, K. A., Posnett, D. N., Schoenborn, M. A., Schooley, K. A., Gayle, R. B., and Maliszewski, C. R. The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *J Clin Invest*, 99(6): 1351-1360, 1997.

[6] Aird, S. D. Ophidian envenomation strategies and the role of purines. *Toxicon*, 40(4): 335-393, 2002.

[7] Dhananjaya, B. L. and D'Souza, C. J. The pharmacological role of nucleotidases in snake venoms. *Cell Biochem Funct*, 28(3): 171-177, 2010.

[8] Patra, A., Kalita, B., Chanda, A., and Mukherjee, A. K. Proteomics and antivenomics of *Echis carinatus carinatus* venom: Correlation with pharmacological properties and pathophysiology of envenomation. *Sci Rep*, 7(1): 17119, 2017.

[9] Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty-Kirby, A., and Lajoie, G. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun Mass Spectrom*, 17(20): 2337-2342, 2003.

[10] Zeller, E. A. The formation of pyrophosphate from adenosine triphosphate in the presence of a snake venom. *Arch Biochem*, 28(1): 138-139, 1950.

[11] Zimmermann, H. Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol*, 362(4-5): 299-309, 2000.

[12] Saikia, D., Bordoloi, N. K., Chattopadhyay, P., Choklingam, S., Ghosh, S. S., and Mukherjee, A. K. Differential mode of attack on membrane phospholipids by an acidic phospholipase A_2 (RVVA-PLA_2-I) from *Daboia russelli* venom. *Biochim Biophys Acta*, 1818(12): 3149-3157, 2012.

[13] Molnar, J. and Lorand, L. Studies on apyrases. *Arch Biochem Biophys*, 93: 353-363, 1961.

[14] Traverso-Cori, A., Traverso, S., and Reyes, H. Different molecular forms of potato apyrase. *Arch Biochem Biophys*, 137(1): 133-142, 1970.

[15] Jabs, C. M., Ferrell, W. J., and Robb, H. J. Plasma ADP levels: direct determination with luciferase luminescence using a biometer. *Clin Biochem*, 11(5): 190-193, 1978.

[16] Gorman, M. W., Feigl, E. O., and Buffington, C. W. Human plasma ATP concentration. *Clin Chem*, 53(2): 318-325, 2007.

[17] Kobata, A. and Endo, T. Immobilized lectin columns: useful tools for the fractionation and structural analysis of oligosaccharides. *J Chromatogr*, 597(1-2): 111-122, 1992.

[18] Plesner, L. Ecto-ATPases: identities and functions. *Int Rev Cytol*, 158: 141-214, 1995.

[19] Smith, T. M. and Kirley, T. L. Glycosylation is essential for functional expression of a human brain ecto-apyrase. *Biochemistry*, 38(5): 1509-1516, 1999.

[20] Mukherjee, A. K. and Mackessy, S. P. Biochemical and pharmacological properties of a new thrombin-like serine protease (Russelobin) from the venom of Russell's Viper (*Daboia russelii russelii*) and assessment of its therapeutic potential. *Biochim Biophys Acta*, 1830(6): 3476-3488, 2013.

[21] Mukherjee, A. K. The pro-coagulant fibrinogenolytic serine protease isoenzymes purified from *Daboia russelii russelii* venom coagulate the blood through factor V activation: role of glycosylation on enzymatic activity. *PLoS One*, 9(2): e86823, 2014.

[22] Jin, J. and Kunapuli, S. P. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci U S A*, 95(14): 8070-8074, 1998.