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A proteomic analysis of Pakistan *Daboia russelii russelii* venom and assessment of potency of Indian polyvalent and monovalent antivenom

Ashis K. Mukherjee [a,b,*], Bhargab Kalita [a], Stephen P. Mackessy [b]

[a] Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur, 784028, Assam, India
[b] School of Biological Sciences, University of Northern Colorado, Greeley, CO 80639-0017, USA

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ABSTRACT

To address the dearth of knowledge on the biochemical composition of Pakistan Russell's Viper (*Daboia russelii russelii*) venom (RVV), the venom proteome has been analyzed and several biochemical and pharmacological properties of the venom were investigated. SDS-PAGE (reduced) analysis indicated that proteins/peptides in the molecular mass range of ~56.0–105.0 kDa, 31.6–51.0 kDa, 15.6–30.0 kDa, 9.0–14.2 kDa and 5.6–7.2 kDa contribute approximately 9.8%, 12.1%, 13.4%, 34.1% and 30.5%, respectively of Pakistan RVV. Proteomics analysis of gel-filtration peaks of RVV resulted in identification of 75 proteins/peptides which belong to 14 distinct snake venom protein families. Phospholipases A_2 (32.8%), Kunitz type serine protease inhibitors (28.4%), and snake venom metalloproteases (21.8%) comprised the majority of Pakistan RVV proteins, while 11 additional families accounted for 6.5–0.2%. Occurrence of aminotransferase, *endo*-β-glycosidase, and disintegrins is reported for the first time in RVV. Several of RVV proteins/peptides share significant sequence homology across Viperidae subfamilies. Pakistan RVV was well recognized by both the polyvalent (PAV) and monovalent (MAV) antivenom manufactured in India; nonetheless, immunological cross-reactivity determined by ELISA and neutralization of pro-coagulant/anticoagulant activity of RVV and its fractions by MAV surpassed that of PAV.

Biological significance: The study establishes the proteome profile of the Pakistan RVV, thereby indicating the presence of diverse proteins and peptides that play a significant role in the pathophysiology of RVV bite. Further, the proteomic findings will contribute to understand the variation in venom composition owing to different geographical location and identification of pharmacologically important proteins in Pakistan RVV.

1. Introduction

Russell's Viper (*Daboia russelii*) is one of the most medically important snakes in Southeast Asian countries and is responsible for a tremendous amount of snakebite-induced morbidity and mortality in these regions [1,2]. *Daboia russelii* is distributed throughout the Indian subcontinent and much of Southeast Asia, including Sri Lanka, Pakistan, Myanmar, southern China and Taiwan [3]. Variation in venom composition is a well-established phenomenon that can be observed at the individual, population and geographic levels, and so clinical manifestations of snakebite depend upon the qualitative composition as well as the quantitative distribution of different components of venom proteins [2,4,5].

Although venom of Russell's Vipers from different geographic origins of Indian subcontinent are well characterized [2,4,6–8], with the exception of recent analyses of individual components [9–13], there is little information on composition, biochemical properties and pharmacological effects of RVV from Pakistan. Furthermore, snake venom is a largely untapped natural resource of novel pharmacologically active molecules [14], and only limited attempts have been made to explore the biomedically important proteins/peptides from Pakistan RVV. During the last ten years, the field of proteomics has evolved rapidly and is now being utilized to analyze venom proteomes, giving rise to a new field termed venomics [15]. Using venomic methods, researchers around the globe have demonstrated the efficiency of current techniques to identify pharmaceutically important venom proteins and establish a better correlation of geographic variation in venom composition with clinical manifestations in snakebite patients. The present study is the first report describing proteome composition of Pakistan RVV using a proteomic approach. In addition, some biochemical and pharmacological properties of this venom and its fractions have also been characterized.

Successful treatment of envenoming by snakes is critically dependent on the ability of antivenoms to reverse venom-induced lethality, necrotic and other adverse pharmacological effects in victims. Variation in venom composition may result in significant differences in the neutralizing potency of the same antivenom against different populations

* Corresponding author at: Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India.
E-mail addresses: akm@tezu.ernet.in (A.K. Mukherjee), stephen.mackessy@unco.edu (S.P. Mackessy).

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of the same snake species [16,17]. Notably, several low molecular mass, non-enzymatic components of venom were shown to be poorly neutralized by commercial antivenom, and this lack of efficacy greatly complicates hospital management of snakebite patients [12,18]. Therefore, in this study an attempt has been made to assess the potency of commercial polyvalent (PAV) and monovalent antivenom (MAV) against Pakistan RVV and its fractions. The results of the present study set the basis for the development of effective antivenoms on an immunologically sound basis, which in turn will lead to better hospital management of Russell's Viper bite patients.

2. Materials and methods

Russell's Viper (*D. russelii russelii*) venom (snakes of Pakistan origin), pooled from 3 snakes, was a gift from Kentucky Reptile Zoo. Pre-cast NuPAGE Novex® Bis-Tris mini gels, buffers and Mark 12 unstained molecular mass standards were obtained from Life Technologies (Invitrogen Inc.), USA. Protein concentration standard reagents were purchased from BioRad Inc., USA. Kits for thromboplastin time (APTT) and prothrombin time (PT) was purchased from Tulip Diagnostics Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade or better and procured from Sigma-Aldrich, USA. Lyophilized monovalent antivenom produced against crude Russell's Viper venom, and polyvalent antivenom (against *Naja naja*, *D. russelii russelii*, *Bungarus caeruleus*, *Echis carinatus*), were obtained from Vins Bioproducts Limited, India, (Batch no. 30AS11001; expiry April, 2015; protein concentration 0.5 mg/ml) and Bharat Serum and Vaccines Limited, India, (Batch no. A05315029; expiry January 2019; protein concentration 0.5 mg/ml), respectively.

2.1. Fractionation of crude RVV through gel filtration chromatography

Lyophilized *D. russelii russelii* venom (200 mg) dissolved in 1.5 ml of 25 mM HEPES buffer containing 100 mM NaCl and 5 mM $CaCl_2$ (pH 6.8) was fractionated on a Bio Gel P-100 gel filtration column (2.8 × 80 cm) previously equilibrated with the same buffer [9]. The flow rate was adjusted to 6 ml/h and fractions of 3.0 ml were collected at 4 °C. Protein was monitored at 280 nm, and peaks were characterized for enzyme activity, pharmacological properties, and immunological cross-reactivity with commercial PAV and MAV (see below).

2.2. Determination of molecular masses of venom proteins through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular mass of the proteins present in crude RVV and gel filtration fractions was determined by SDS-PAGE analysis (12.5% NuPAGE Novex® Bis-Tris mini gels) under both reduced and non-reduced conditions. Proteins were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 and destaining with methanol/acetic acid/water (40:10:50). Approximate molecular masses of proteins were determined from a plot of log MW of standards vs. migration distance. The RVV protein bands of reduced and non-reduced SDS-PAGE were scanned and band intensities were analyzed using ImageJ software. The cumulative intensity of RVV protein bands in SDS-PAGE was considered as 100% and the relative intensity of individual protein band was calculated by comparing with that.

2.3. Identification of Pakistan RVV proteome by LC-MS/MS analysis of gel filtration fractions and determination of relative abundance of proteins/peptides

For LC-MS/MS analysis of trypsin digested peptide fragments of the gel filtration fractions of RVV, our previously described procedures were followed [12,19]. Briefly, 40 μg of lyophilized sample [gel filtration (GF) fractions], reduced with 10 mM dithiothreitol followed by alkylation with 50 mM iodoacetamide (in the dark), was subjected to in-solution digestion with ProMega sequencing-grade trypsin (50 ng/μl in 25 mM ammonium bicarbonate containing 10% acetonitrile) for 18 h at 37 °C. The digested peptides were Zip-Tip concentrated and reconstituted in 20 μl of 0.1% (v/v) formic acid in 2% acetonitrile and 1 μl of the same was subjected to nano-UHPLC-MS/MS analysis. The ion source was ESI (nano-spray), fragmentation mode was collision-induced dissociation (y and b ions), MS scan mode was FT-ICR/Orbitrap, and MS/MS scan in the range from 500 to 2000 m/z was linear ion trap. For collision-induced dissociation (CID) MS/MS analysis, the doubly or triply-charged ions were selected.

For fixed and variable modifications, carbamidomethylation of cysteine residues and oxidation of methionine residues, respectively, were selected. The percent mass error tolerance and fragment mass error tolerance were set to 12 ppm and 0.8 Da, respectively. The data were searched against the UniProt Swiss-Prot database (non-redundant database with reviewed proteins) and against Viperidae venom from NCBI using PEAKS 7.0 software. In addition, non-tryptic peptide sequences with an average local confidence (ALC) of ≥55% were also derived directly from the MS/MS spectrum (*de novo* sequencing) using PEAKS software. Only matching proteins and peptides showing a −10lgP value ≥30 and ≥20 were considered for identification purposes. The false discovery rate was kept very stringent (0.8%) which was verified manually. These filtration parameters does not allow false identification of proteins and discarded low score venom proteins.

The abundance of venom proteins and peptides identified in Pakistan RVV by LC-MS/MS analysis was determined by spectral count method that has been used for measuring protein abundance in label-free shotgun proteomics [20,21]. The relative abundance of a protein in a particular chromatographic fraction was determined by the method described by Tan et al. [8] however, with the following modifications (Eq. (1)).

$$\begin{aligned} &Relative abundance of\ a\ protein/peptide\text{“}X\text{”} in\ a\ particular chromatographic fraction\text{“}Y\text{”} \\ &= \frac{Mean spectral count of\ X\ in fraction\ Y}{Total mean spectral counts of\ all\ proteins/peptides in\ Y} \times Percent protein content of\ Y\ (\%) \end{aligned} \quad (1)$$

Further, the MALDI-ToF-MS analysis demonstrated presence of degraded RVV proteins of mass less than 1 kDa in GF-11 and GF-12 chromatographic fractions. Therefore, these fractions were not subjected to LC-MS/MS analysis and the protein content of these two fractions was not considered for percent protein calculation for the remaining chromatographic fractions (GF1 to GF-10).

2.4. Amidolytic activity assay

The amidolytic activity was determined by mixing 50 μl of 1.0 mM various chromogenic substrates (see below) with 10 μg of crude RVV or GF fraction; total volume was adjusted to 375 μl with 100 mM HEPES (pH 8.0) containing 100 mM NaCl [9]. The following chromogenic substrates were utilized in amidolytic activity assays (all from Sigma-Aldrich): *N*-Benzoyl-L-Phe-L-Val-L-Arg-p-nitroanilide hydrochloride (substrate for thrombin), *N*-Benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride (substrate for plasma kallikrein) and D-Val-Leu-Lys-p-nitroanilide dihydrochloride (substrate for plasmin). The unit of amidolytic activity was defined as μmol of 4-nitroaniline released per minute by crude RVV or GF fractions under the assay conditions. For every experiment, a control was run in parallel where instead of enzyme, an equivalent volume of buffer was used.

2.5. Assay of esterase activity

Esterolytic activity was assayed by a spectrophotometric method as described previously using Nα-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as a substrate at pH 8.0 [9]. One unit of BAEE esterase activity was defined as an increase in absorbance of 0.01 at 244 nm

during the first 5 min of the reaction at 37 °C [9]. For every experiment, a control was run in parallel where instead of enzyme, an equivalent volume of buffer was added to the reaction mixture. Specific activity was expressed as units of BAEE activity/mg protein. Phosphodiesterase (PDE) activity was determined by a modification of the method of Björk [22]. The reaction was initiated by adding 5 μg crude RVV or GF peaks in a reaction mixture containing bis-p-nitrophenyl phosphate (final concentration 0.4 mM) in a total volume of 375 μl of 0.1 M Tris-HCl, 10 mM $MgCl_2$, pH 9.0. After 30 min incubation at 37 °C, the reaction was terminated by adding 0.1 N NaOH-10 mM disodium EDTA solution. After 10 min, absorbance was measured at 400 nm against the reagent blank. Specific activity was expressed as $\Delta A_{400\ nm}$/min/mg protein.

2.6. Assay of enzyme activity and protein content

The proteolytic activity of crude RVV or GF peaks (5 μg/ml) was assessed against human plasma fibrinogen (fraction I). The reaction mixture containing specified amount of venom protein and substrate were incubated for 3 h at 37 °C [23]. One unit (U) of protease activity was defined as 1.0 μg of tyrosine equivalent liberated per min per ml of enzyme [9]. Venom metalloprotease activity was determined spectrophotometrically at 450 nm using azocasein as a substrate, and specific activity was expressed as $\Delta A_{450\ nm}$/min/mg protein [24].

Crude RVV as well as chromatographic fractions were screened for phospholipase A_2 (PLA_2) activity by incubating a specified amount of protein (5 μg/ml) with 50 μl of 3.0 mM 4-nitro-3-(octanoyloxy) benzoic acid (Sigma) in acetonitrile as a substrate [25]. Specific activity of PLA_2 was defined as nmol product formed/min/mg protein. L-amino acid oxidase (LAAO) activity was assayed using L-kynurenine as a substrate [26]. The unit of LAAO activity was defined as nmol kynurenic acid produced/min under the assay conditions and specific LAAO activity was expressed as unit/mg protein.

ATPase and AMPase activities were assayed by adding crude RVV (10 μg) or venom fractions (5 μg) to a reaction mixture (500 μl) containing 0.5 mM ATP or AMP in 20 mM Tris-HCl, pH 7.4 [2]. After incubation for 30 min at 37 °C, the reaction was terminated by addition of 10 μl of ice cold 10% TCA and liberated P_i was determined spectrophotometrically at 695 nm using a UV–Vis MultiSkan 165 GO multi plate reader (Thermo Scientific, Waltham, MA, USA) [27]. One unit of ATPase/AMPase activity was defined as μM of P_i released per min.

Protein content of crude RVV and fractions was determined by using commercial reagents (Bio-Rad protein assay kit, Bio-Rad, California, USA). From a standard curve of BSA, the concentration of unknown protein was determined at 540 nm.

2.7. Assessment of pharmacological properties and in vitro cytotoxicity

The anticoagulant activity (Ca^{2+}-clotting time) of crude RVV or venom fractions was determined against 300 μl of goat platelet-poor plasma [11,28]. One unit of coagulant or anticoagulant activity was defined as a one second decrease or increase in clotting time of PPP in the presence of crude venom/venom fractions (compared with the clotting time of control plasma) [11]. Effect of crude RVV or venom fractions on partial thromboplastin time (APTT) and prothrombin time (PT) of PPP was determined by using commercial diagnostic kits and following the instructions of the manufacturer [28]. The fibrinogen clotting activity of crude RVV or venom fractions was determined by using a BBL-Fibrinosystem, as described earlier [9].

The hemolytic activity of crude RVV or venom fractions was determined against 5% (v/v) washed erythrocytes [29]. Three ml of erythrocytes suspension was treated with 10 μg/ml of crude RVV or fractions, or 0.1% triton X-100 (100% activity), for 90 min at 37 °C. Hemolytic activity, if any, of crude RVV or fractions was expressed as shown in Eq. (2)

$$\text{Percent hemolytic activity} = \frac{A540\text{expt}}{A540\text{triton}} \times 100(\%) \quad (2)$$

In the above equation, $A540_{expt}$ is supernatant absorbance at 450 nm of erythrocytes treated with venom or fractions, and $A540_{triton}$ is the supernatant absorbance of erythrocytes treated with 0.1% triton X-100 (100% activity).

Dose-dependent in vitro cytotoxicity against Colo-205 (human colorectal adenocarcinoma) and MCF-7 (human breast adenocarcinoma) was assayed by adding crude RVV or fraction (10 μg/ml) to the culture medium containing 1×10^5 cells/ml. Venom-induced cytotoxicity was assayed by an MTT-based method and was expressed as percent cell death as determined from a standard curve of control cells [9].

2.8. Determination of immunological cross-reactivity by enzyme-linked immunosorbent assay (ELISA)

One hundred ng of crude RVV or venom fractions (protein content) were added (in triplicate) to wells of a 96-well ELISA plate and the plate was incubated overnight at 4 °C. After washing the wells for three times with wash buffer (1× PBS containing 0.05% Tween-20), the wells were blocked with 5% fat-free milk powder in PBS. The washing step was repeated three times, and then 200 ng (protein content) of MAV or PAV in 100 μl PBS was added to each well; only PBS was added to control wells. After incubation for 2 h at room temperature (~23 °C), excess (unbound) antibodies were removed by 3× washing with wash buffer. This was followed by addition of rabbit anti-horse IgG conjugated with horseradish peroxidase (HRP) (1:2000) as the secondary antibody to detect the bound primary antibodies. The reaction was allowed to proceed for 2 h at room temperature and then 100 μl substrate (1× TMB/H_2O_2) was added and the plate was incubated at room temperature in the dark for 30 min. The reaction was stopped by adding 50 μl 2 M H_2SO_4 to each well and the color developed was measured at 492 nm against blanks in Multiskan GO (Thermoscientific, USA) microplate reader.

2.9. Neutralization of pro-coagulant or anticoagulant effects of crude RVV and fractions by commercial antivenom

Crude RVV or gel filtration fractions (1 μg) showing pro- or anticoagulant activity were incubated with commercial PAV or MAV at 1:10 (protein: protein) ratio for 30 min at room temperature (~23 °C) prior to the assay of Ca^{2+}-clotting time of PPP [11]. The Ca^{2+}-clotting time of CRVV/fractions in absence of antivenom was considered as 100% activity and other values were compared with that. The results were expressed as percent neutralization of pro-coagulant/anticoagulant activity of RVV or fractions by antivenom (PAV or MAV).

2.10. Statistical analysis

Student's *t*-test using Sigma Plot 11.0 for Windows (version 7.0) was used to determine the significance of difference of enzymatic and anticoagulant/coagulant activities between crude RVV and GF fractions. A value of $p \leq 0.05$ was considered significant.

3. Results and discussion

3.1. SDS-PAGE analysis and biochemical characterization of crude RVV and GF fractions

Fractionation of crude Pakistan RVV via gel filtration chromatography resulted in the separation of 12 protein peaks (GF1 to GF 12) (Fig. 1A). The relative abundances of crude RVV proteins (reduced)

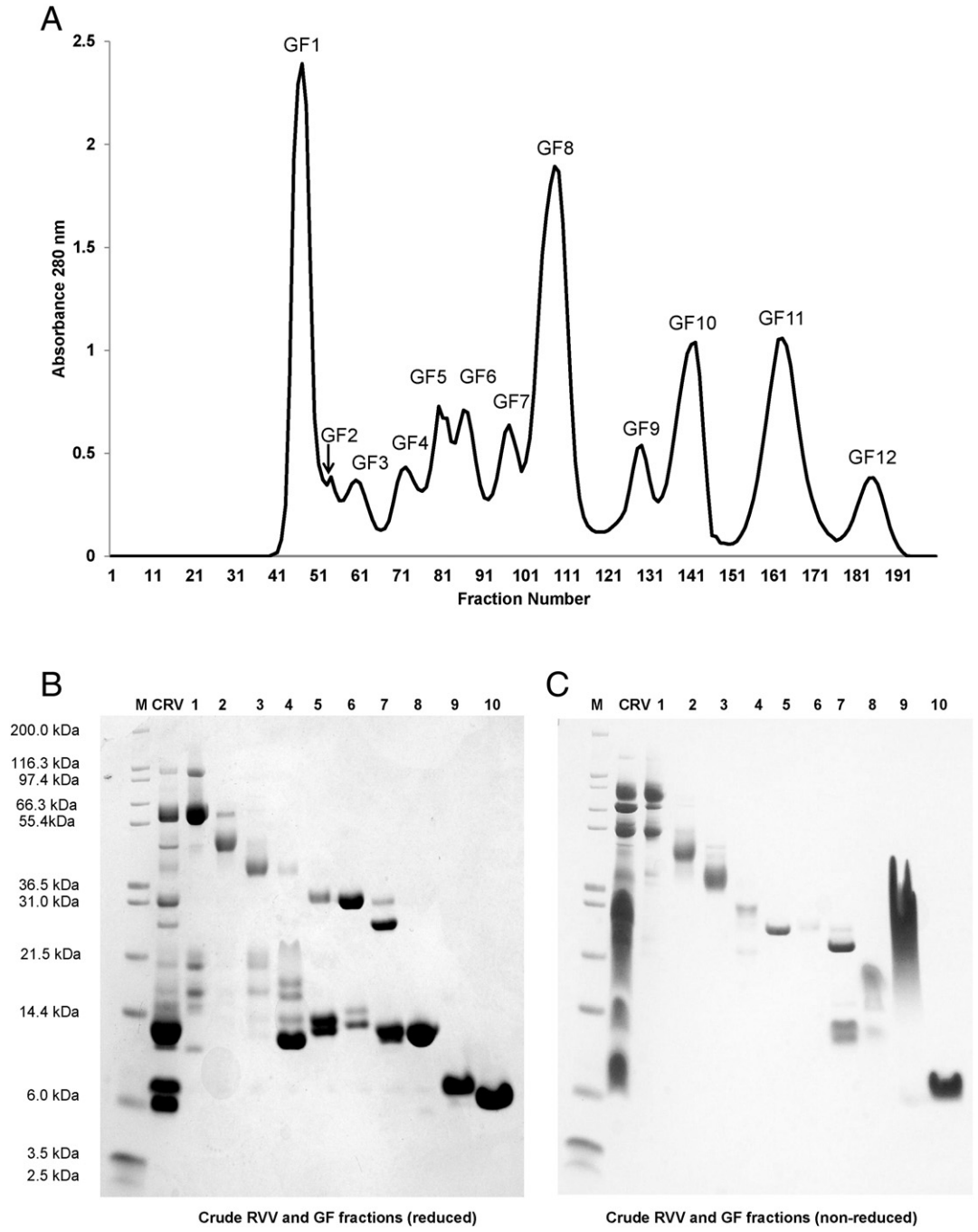

Fig. 1. A. Fractionation of Pakistan Russell's Viper (*D. russelii russelii*) venom (200 mg dry weight) on size-exclusion BioGel P-100 column (2.8 × 80 cm). The flow rate was 6 ml/h at 4 °C. (This figure was originally published as Fig. 1A by Mukherjee, A.K., Mackessy, S.P., 2013, Biochim. Biophys. Acta-Gen. Subj. 1830, 3476–3488. Reproduced with permission from the journal). B. 12.5% SDS-PAGE analysis of crude RVV and its gel-filtration fractions under reduced conditions. Lane M contains protein molecular markers. C. 12.5% SDS-PAGE analysis of crude RVV and its gel-filtration fractions non-under reduced conditions. Lane M shows protein molecular markers.

were determined by densitometry scanning of SDS-PAGE bands (Supplementary Fig. S1). The published reports describing mass of RVV proteins were used as references for the identification of reduced SDS-PAGE protein bands as shown in Supplementary Fig. S1 [8–13,30–32]. The relative abundance of RVV proteins/peptides determined by SDS-PAGE and LC-MS/MS analysis was found to be nearly identical. For example, SDS-PAGE analysis demonstrates relative abundance of PLA_2 enzymes was ~34.0% of RVV protein (Supplementary Fig. S1) whereas LC-MS/MS analysis shows that PLA_2 enzymes constitute ~33.0% of the RVV proteome (see below). Similarly, by SDS-PAGE analysis the relative abundances of KSPIs and disintegrins were determined to be 30.5% (Supplementary Fig. S1) and this value correlates well with the relative abundances (~29.0%) of the above proteins determined by LC-MS/MS analysis (see below). The relative abundance of a high molecular weight protein (104.7 kDa) representing PDE in SDS-PAGE was determined at 0.8% (Supplementary Fig. S1) whereas by LC-MS/MS analysis its relative abundance was found at 0.6% of RVV protein (see below). The above data advocate a good correlation between the two different analysis methods to determine the relative abundance of RVV proteins.

SDS-PAGE analysis of gel filtration fractions of Pakistan RVV under reduced and non-reduced conditions revealed a diversity of venom proteins (Fig. 1B, C). Determination of abundance of RVV proteins by densitometry scanning of SDS-PAGs under reduced and non-reduced conditions suggested that proteins in the molecular mass range of

Table 1
Percent yield and some enzyme activity of crude RVV and gel filtration (GF) fractions. Values are mean ± S.D. of three replicates.

Crude RVV or GF peak	% yield (based on protein content)	Fibrinogenolytic activity* (U/mg)	Metalloprotease activity (U/mg)	PLA_2 activity (U/mg)	LAAO activity (U/mg)	ATPase activity (U/mg)	AMPase activity (U/mg)	BAEE-esterase (U/mg)	PDE activity (U/mg)
Crude RVV	100	1.5 ± 0.2	0.2 ± 0.03	7.8 ± 1.0	3.2 ± 0.5	4.1 ± 0.8 × 10^3	12.3 ± 1.4 × 10^3	1.9 ± 0.8 × 10^3	1.2 ± 0.1
GF-1	17.9 ± 1.3	8.1 ± 1.1	3.4 ± 0.2	0.4 ± 0.3	9.8 ± 1.1	10 ± 1.3 × 10^3	19.5 ± 1.7 × 10^3	2.1 ± 0.9 × 10^3	3.4 ± 0.4
GF-2	4.6 ± 0.8	4.2 ± 0.8	1.5 ± 0.1	0	0	3.1 ± 0.7 × 10^3	2.5 ± 0.2 × 10^3	1.0 ± 0.1 × 10^3	0
GF-3	6.4 ± 0.9	2.0 ± 0.1	1.1 ± 0.1	0.3 ± 0.04	0	1.2 ± 0.1 × 10^2	1.1 ± 0.1 × 10^3	4.9 ± 0.4 × 10^3	0
GF-4	7.8 ± 1.0	1.2 ± 0.1	0.5 ± 0.01	0.2 ± 0.03	0	0	0	5.0 ± 0.8 × 10^2	0
GF-5	6.1 ± 0.5	0	0	1.3 ± 0.1	0	0	0	0	0
GF-6	6.9 ± 0.7	0	0	2.8 ± 0.1	0	0	0	0	0
GF-7	7.7 ± 1.0	0	0	5.2 ± 0.3	0	0	0	0	0
GF-8	13.3 ± 1.1	0	0	22.0 ± 1.4	0	0	0	0	0
GF-9	3.2 ± 0.3	0.0	0	0.3 ± 0.04	0	0	0	0	0
GF-10	5.2 ± 0.8	0.8 ± 0.1	0	1.2 ± 0.1	0	0	0	0	0
GF-11	2.3 ± 0.1	0	0	0	0	0	0	0	0
GF-12	1.2 ± 0.1	0	0	0	0	0	0	0	0

* Incubated for 3 h at 37 °C.

>40 kDa, 14–40 kDa, and <14 kDa contribute approximately 15.5%, 19.8%, and 64.6% (reduced) (Fig. 1B) and 28.6%, 48.6%, and 24.9% (non-reduced), respectively of RVV proteins (Fig. 1C). By SDS-PAGE, no protein bands could be detected in peaks GF-11 and GF-12 and mass spectroscopic analysis of these peaks showed peptides of less than 1 kDa mass (data not shown). SDS-PAGE analysis of reduced proteins (Fig. 1B) indicated the following approximate mass ranges of Pakistan RVV proteins: GF-1 (10–116 kDa), GF-2 (48–116 kDa), GF-3 (14.4–45 kDa), GF-4 (10–40 kDa), GF-5 (13–36 kDa), GF-6 (14–36.5 kDa), GF-7 (14–36 kDa), GF-8 (13–14.4 kDa), GF-9 (6.1–7.9 kDa), and GF-10 (5.6–6.1 kDa). However, SDS-PAGE analysis under non-reducing conditions showed that many of the RVV proteins in the native state were either self-aggregated [10], consisted of multiple subunits or interacted with other venom protein(s) to form higher molecular mass complexes (Fig. 1C).

The SDS-PAGE (reduced) analysis indicated that proteins/peptides in the molecular mass range of ~56.0–105.0 kDa, 31.6–51.0 kDa, 15.6–30.0 kDa, 9.0–14.2 kDa and 5.6–7.2 kDa contribute approximately 9.8%, 12.1%, 13.4%, 34.1% and 30.5%, respectively of Pakistan RVV (Fig. 1B). The elution of relatively low molecular mass proteins (10–21 kDa range) along with high molecular mass proteins (> 50 kDa) in the GF-1 fraction provided strong evidence of protein-protein interactions among venom proteins (Figs. 1B, C). These interactions are unlikely to be non-specific (due to ionic interactions or non-ionic weak interactions among proteins) because the elution buffer contained relatively high salt concentrations. Instead, interactions are likely occurring in defined stoichiometric ratios, forming stable protein complexes which enhance toxicity or pathophysiological effects relative to individual components of these complex, as observed previously [33,10] (Mukherjee A.K., unpub. obs.).

The total yield (based on protein content) of venom fractions was 82.6% of the crude RVV loaded onto the GF column (Table 1). The percent protein content of GF-1 was highest and the fractions GF-11 and GF-12 were lowest. The enzymatic activity levels displayed by GF chromatographic peaks were dependent on qualitative and quantitative distribution of different enzymes in these peaks. Therefore, low abundance of a particular enzyme in a peak, for example PLA_2 enzymes in GF-3 to GF-6 and GF-9 to GF-10, may not allow its catalytic activity to be well detected. Based on biochemical analyses, peak GF-1 demonstrated the highest fibrinogenolytic, metalloprotease, LAAO, ATPase, AMPase, and PDE activities (Table 1). The occurrence of these enzymes in GF-1 was also evident from the presence of protein bands in their corresponding molecular weight ranges in SDS-PAGE (Fig. 1B). These are primarily higher molecular weight snake venom enzymes (>50 kDa) and therefore they eluted near the void volume of the GFC [9,11,13]. The PLA_2 and BAEE-esterase enzymes eluted in peak GF-8, and peaks GF-3 and GF-4, respectively (Table 1), in accordance with their molecular masses (Fig. 1B) [30,31]. However, low to very low levels of PLA_2 activity were observed in almost all of the GF fractions of RVV (Table 1), although by CBB staining such low quantity of PLA_2 was either not detected or faintly detected in all the GF fractions by SDS-PAGE analysis (Fig. 1B). Our result suggests interaction of PLA_2 enzymes with other components of the venom as has been noted previously [10,30].

The amidolytic activity toward various chromogenic substrates showed that the proteases present in peak GF-2 were very specific for hydrolyzing substrates for thrombin and plasma kallikrein mainly due to presence of Russelobin [9] and other proteases (Fig. 1B). The peak GF-3 followed by GF-4 contained the highest amount of plasmin-like enzyme(s) (Table 2) and enzymes in these peaks may also be responsible for showing BAEE-esterase activity (Table 1). These enzyme activities in snake venom are frequently seen in higher molecular mass serine proteases, and lower levels of these activities were also noted in the first three fractions (GF-1 to GF-3). Crude Pakistan RVV showed higher activity towards the substrates for thrombin, a fibrinogenolytic

Table 2
Amidolytic activity of crude RVV and gel filtration fractions. Values are mean ± S.D. of three replicates.

Crude RVV/GF peak	Thrombin-like (U/mg)	Kallikrein-like (U/mg)	Plasmin-like (U/mg)
Crude RVV	0.4 ± 0.3	4.2 ± 0.5	0.1 ± 0.01
GF-1	0.5 ± 0.3	2.7 ± 0.6	0
GF-2	3.2 ± 0.4	20.8 ± 1.4	0.2 ± 0.01
GF-3	1.2 ± 0.2	1.9 ± 0.2	0.5 ± 0.1
GF-4	0.7 ± 0.1	0.4 ± 0.1	0.4 ± 0.01
GF-5	0.1 ± 0.01	0	0.1 ± 0.01
GF-6	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
GF-7	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
GF-8	0	0	0
GF-9	0	0.6 ± 0.1	0
GF-10	0	0	0
GF-11	0	0	0
GF-12	0	0	0

Table 3
Summary of different venom proteins identified in Pakistan RVV by ESI-LC-MS/MS analysis of the gel filtration peaks. The distribution of the proteins in different GF peaks are shown in the last column of the table.

Sl. no.	Protein	Accession no.	−10logP	% COVERAGE	Homology with protein from	GF peak(s)
		Enzymatic proteins				
Phospholipase A_2 (PLA_2)						
1	Chain A, Crystal Structure Of The Complex Of A Group Iia Phospholipase A2	gi|71042571	333.56	61	*Daboia russellii pulchella*	GF-1,4,5,6,8
2	Basic phospholipase A2 Drk-b1	gi|408407668	159.95	18	*Daboia russelii russelii*	GF-5
3	Basic phospholipase A2 VRV-PL-V	gi|81174981	147.00	60	*Daboia russellii russellii*	GF-1
4	Acidic phospholipase A2 Drk-a2	gi|408407660	143.78	57	*Daboia russellii russellii*	GF-1,3,4,5,6,7,8,10
5	Acidic phospholipase A2 RV-7	gi|400714	141.57	72	*Daboia siamensis*	GF-1
6	phospholipase A2-IV	gi|87130860	129.65	37	*Daboia russelii russelii*	GF-1,4,5,9
7	Chain A, anticoagulant class II phospholipase A2	gi|157834128	125.63	61	*Daboia russelii russelii*	GF-1,4,5,6,7,8,9,10
8	Chain H, structure of daboiatoxin	gi|149241831	119.64	47	*Daboia siamensis*	GF-1
9	Ammodytin I1(C) isoform	gi|50874274	111.95	38	*Vipera aspis zinnikeri*	GF-1
10	Basic phospholipase A2	gi|71912227	108.07	40	*Daboia russelii limitis*	GF-1,4,5,8
11	Ammodytin I1(B) isoform	gi|50874252	103.38	40	*Vipera ammodytes ruffoi*	GF-1
12	Basic phospholipase A2 Drk-b2	gi|408407671	97.27	26	*Daboia russelii russelii*	GF-1,4,5,6,8,10
13	Chain A, first crystal structure of A C49 monomer Pla2	gi|48,425,253	94.72	10	*Daboia russelii pulchella*	GF-1,4
14	Ammodytin I2(A) variant	gi|50874438	91.11	29	*Vipera aspis aspis*	GF-1
15	Phospholipase A2/PLA2	gi|1839638	87.56	39	*Daboia russelii*	GF-4,6,8,9,10
16	phospholipase A2-II	gi|87130856	73.80	12	*Daboia russelii russelii*	GF-4
17	phospholipase A2	gi|478779	42.84	9	*Daboia russelii russelii*	GF-8
Snake venom metalloprotease (SVMP)						
1	Factor X activator heavy chain	gi|300079900	230.89	38	*Daboia russelii russelii*	GF-1,2,3,4,5,6,7,8,10
2	Chain A, crystal structure of Russell's viper venom metalloproteinase heavy chain	gi|162329887	216.39	49	*Daboia russelii russelii*	GF-1
3	coagulation factor X activating enzyme light chain	gi|251205	203.13	54	*Daboia russelii russelii*	GF-1
4	Factor X activator light chain 2	gi|300079896	177.15	68	*Daboia russelii russelii*	GF-1
5	Factor X activator light chain 2	gi|380765752	136.84	49	*Daboia russelii russelii*	GF-1
6	Chain B, crystal structure of Russell's viper venom metalloproteinase light chain	gi|162329888	136.84	57	*Daboia siamensis*	GF-1
7	Factor X activator light chain 2	gi|50980285	134.38	24	*Macrovipera lebetina*	GF-1
8	Coagulation factor X activating enzyme heavy chain	gi|251204	124.28	14	*Daboia russelii*	GF-3,4
9	Zinc metalloproteinase-disintegrin-like VLAIP-A	gi|82228619	115.74	11	*Macrovipera lebetina*	GF-1
10	Metalloproteinase, partial	gi|297593790	111.64	7	*Echis carinatus sochureki*	GF-1
11	Metalloproteinase, partial	gi|297593990	109.05	11	*Echis pyramidum leakeyi*	GF-1
12	Metalloproteinase MP-2, partial	gi|62547937	80.48	5	*Bitis arietans*	GF-1
13	Metalloproteinase, partial	gi|297593820	53.15	11	*Echis carinatus sochureki*	GF-1
Snake venom serine protease (SVSP)						
1	Serine beta-fibrinogenase precursor	gi|306756036	134.93	21	*Macrovipera lebetina*	GF-1
2	Factor V activator RVV-V gamma	gi|134130	129.7	30	*Macrovipera lebetina*	GF-1
3	Serine beta-fibrinogenase-like protein precursor	gi|311223824	120.14	30	*Daboia siamensis*	GF-1,2
4	Serine protease VLSP-3	gi|380875417	111.86	12	*Macrovipera lebetina*	GF-1
5	Venom serine proteinase-like protein 2	gi|13959655	102.88	14	*Macrovipera lebetina*	GF-1
6	Serine protease VLSP-1	gi|381141431	95.67	19	*Macrovipera lebetina*	GF-1
7	Thrombin-like enzyme gyroxin B1.7	gi|380875412	75.22	10	*Crotalus durissus terrificus*	GF-1,2
8	Serine protease KN14 precursor	gi|33307092	66.31	18	*Trimeresurus stejnegeri*	GF-1
9	Venom serine proteinase-like protein	gi|13959640	51.87	6	*Protobothrops jerdonii*	GF-1
Phosphodiesterase (PDE)						
1	Phosphodiesterase	gi|586829527	224.81	41	*Macrovipera lebetina*	GF-1
2	Phosphodiesterase	gi|547223111	170.48	19	*Ovophis okinavensis*	GF-1
Nucleotidase (NUC)						
1	5′-nucleotidase, partial	gi|586829529	166.41	47	*Macrovipera lebetina*	GF-1
2	Snake venom 5′-nucleotidase	gi|395455152	154.53	18	*Gloydius brevicaudus*	GF-1
L-amino acid oxidase (LAAO)						
1	L-amino-acid oxidase	gi|395406796	210.38	47	*Daboia russellii russellii*	GF-1
2	L-amino acid oxidase	gi|538260091	116.79	11	*Ovophis okinavensis*	GF-1
Aminotransferase (AMT)						
1	Glutaminyl cyclase	gi|547222991	67.52	9	*Protobothrops flavoviridis*	GF-1
Hyaluronidase						
1	Truncated hyaluronidase	gi|113203693	43.07	5	*Echis carinatus sochureki*	GF-1
		Non-enzymatic proteins				
Kunitz-type serine protease inhibitor (KSPI)						
1	Protease inhibitor	gi|377657516	330.51	52	*Daboia russelii russelii*	GF-4,6,7,8,9,10
2	Kunitz-type serine protease inhibitor 2	gi|123907425	156.30	64	*Daboia russelii russelii*	GF-1,4,5,6,7,8,10
3	Trypsin inhibitor-5 precursor	gi|159883526	123.32	46	*Daboia siamensis*	GF-1
4	Protease inhibitor	gi|377657518	111.07	55	*Daboia russelii russelii*	GF-1,7,8,10
5	Kunitz-type serine protease inhibitor 2	gi|125041	88.96	17	*Daboia siamensis*	GF-9
6	Kunitz protease inhibitor-IV	gi|87130868	88.87	48	*Daboia russelii russelii*	GF-1,8
7	Kunitz-type serine protease inhibitor B1	gi|239977245	85.44	46	*Daboia siamensis*	GF-1
8	*Vipera russelli* proteinase RVV-V homolog 2	gi|298351879	53.06	87	*Daboia russelii russelii*	GF-4

Table 3 (*continued*)

Sl. no.	Protein	Accession no.	−10logP	% COVERAGE	Homology with protein from	GF peak(s)
Snaclec						
1	P68 beta subunit	gi|300490476	209.51	74	*Daboia russelli limitis*	GF-1
2	Snaclec 4	gi|73620112	207.05	55	*Daboia siamensis*	GF-1
3	C-type lectin-like protein subunit 2	gi|67043475	203.13	46	*Daboia siamensis*	GF-1
4	C-type lectin-like protein subunit 5	gi|67043481	189.79	64	*Daboia siamensis*	GF-1
5	P68 alpha subunit	gi|300490474	187.43	55	*Daboia russelli limitis*	GF-1
6	Snaclec A14	gi|218526485	163.77	47	*Macrovipera lebetina*	GF-1
7	Snaclec coagulation factor X-activating enzyme light chain 2	gi|73621140	136.84	49	*Daboia siamensis*	GF-1
8	Dabocetin alpha subunit	gi|300490466	135.69	40	*Daboia siamensis*	GF-1
9	Dabocetin beta subunit	gi|300490464	119.15	41	*Daboia russelli russelli*	GF-1,4,5,7,8
10	P31 alpha subunit	gi|300490486	111.23	35	*Daboia russelii russelii*	GF-1
11	P31 beta subunit	gi|300490484	92.38	21	*Daboia siamensis*	GF-1
Cysteine-rich secretory protein (CRISP)						
1	Cysteine-rich secretory protein Dr.-CRPK	gi|190195321	125.77	41	*Daboia russellii russellii*	GF-1,4,5
2	Cysteine-rich secretory protein Da-CRPa, partial	gi|190195337	86.14	12	*Deinagkistrodon acutus*	GF-1
3	Cysteine-rich secretory protein Dr.-CRPB, partial	gi|190195323	63.97	10	*Daboia russellii russellii*	GF-1,4,6
Vascular endothelial growth factor (VEGF)						
1	VR-1 precursor	gi|223711929	88.87	33	*Daboia russellii russellii*	GF-1,4,9
2	Snake venom vascular endothelial growth factor toxin ICPP	gi|48429241	69.53	23	*Macrovipera lebetina*	GF-1
3	Snake venom vascular endothelial growth factor	gi|521752303	31.22	8	*Crotalus horridus*	GF-1
Nerve growth factor (NGF)						
1	Venom nerve growth factor	gi|400499	106.57	32	*Daboia russellii russellii*	GF-1
Disintegrin						
1	Catroriarin, partial	gi|110610039	74.31	7	*Crotalus atrox*	GF-1
Uncharacterized protein						
1	Unnamed protein product	gi|19574645	31.22	8	*Agkistrodon piscivorus piscivorus*	GF-1

enzyme, and for plasma kallikrein; RVV displayed comparatively lower activity towards the plasmin substrate, indicating that fibrinolytic serine proteases with plasmin-like activity are less abundant in RVV (Table 2). Alternatively, this may also be due to enzyme promiscuity toward various pNA substrates. Collectively, these data (Tables 1 and 2) indicate that multiple higher molecular mass serine proteases and metalloproteases account for the observed high proteolytic activity of RVV from Pakistan [2,9,31]. These results are also consistent with the observed coagulopathic toxicity of RVV following human envenomations [2].

3.2. LC-MS/MS analysis of RVV GF peaks

Snake venoms contain many enzyme and non-enzymatic proteins and peptides which exert toxic or lethal effects in prey or snakebite victims [34]. The shotgun proteomic approach, coupled with protein database searches and protein assembly algorithms, has been shown to surpass other MS-based proteomic systems in terms of number and diversity of proteins identified and in dynamic range for detection [35]. The ESI-LC-MS/MS analysis has provided an overview of presence of different proteins and peptides in GF fractions of Pakistan RVV (Table 3 and Supplementary Table S1). The relative abundance of proteins and peptides of Pakistan RVV identified by LC-MS/MS analysis is shown in Fig. 2. The alignment of MS/MS-derived peptide sequences with the homologous protein/peptide from the Viperidae snake venom database is shown in Supplementary Fig. S2. By LC-MS/MS analysis of tryptic digested proteins (as well as non-tryptic peptides identified by *de novo* sequencing) of gel-filtration fractions, we have identified 75 proteins/peptides from NCBI databases in the Pakistan RVV proteome which belong to 14 distinct snake venom protein families. In comparison, using a proteomics approach (2D electrophoresis followed by ESI-MS/MS analysis of gel spots), Risch et al. (2009) identified 89 proteins distributed in 6 protein families in the venom of *D. russelii siamensis* from Myanmar [6]. MS/MS analyses of trypsin-digested gel filtration peaks resulted in the identification of 63 different proteins and peptides belonging to 12 protein families in RVV from southern India [7]. Recently, Tan et al. (2015) had identified 41 proteins belonging to 11 snake venom protein families in the venom of *D. russelii russelii* of Sri Lankan origin by 1D SDS-PAGE of crude venom followed by in-gel trypsin digestion of protein bands and their mass spectroscopic analyses [8].

For a better understanding of the biochemical and pharmacological properties of RVV proteome and to correlate the above properties with the clinical manifestations of Russell's Viper bite, we have categorized the RVV proteome into two major groups: enzymatic and non-enzymatic proteins. Fig. 2 shows that PLA_2 (32.8%), followed by snake venom metalloprotease (SVMP) (21.8%) are found as the most abundant enzymatic proteins whereas Kunitz-type serine protease inhibitors (KSPI) (28.4%) represent the most abundant non-enzymatic proteins of Pakistan RVV. The relative abundance of Pakistan RVV proteins and peptides determined by LC-MS/MS analysis (Fig. 2) demonstrated a good correlation with the relative intensities of the protein/peptides determined by reduced SDS-PAGE analysis of RVV (Supplementary Fig. S1). A brief description of the different proteins and peptides identified in Pakistan RVV follows below.

3.3. Enzymatic proteins in Pakistan RVV

RVV proteins identified in this group are LAAO, snake venom serine protease (SVSP), SVMP, PDE, 5′-nucleotidase (AMPase), aminotransferase, hyaluronidase and PLA_2. Enzymatic assays and/or LC-MS/MS analyses demonstrated that most of these proteins are higher molecular mass enzymatic proteins and are eluted in GF-1 and GF-2 peaks; however, highest PLA_2 activity was detected in peak GF-8 (Table 1), which is according to molecular mass of majority of PLA_2 isoenzymes (Fig. 1B) [30].

Viperidae snake venoms are the richest known source of LAAO [36]. Snake venom L-amino acid oxidases (SV-LAAO) are thermolabile, homodimeric enzymes with a molecular weight in the range of 60–150 kDa [13,36,37]. The reported pharmacological properties of SV-LAAO include edema-induction, platelet modulation, apoptosis induction, anticoagulant effects, hemolytic activity and hemorrhagic effects

[38,39]. By peptide mass fingerprinting analysis we have identified 2 LAAO isoenzymes in GF-1 fraction of Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). According to LC-MS/MS analysis, these LAAOs corresponding to molecular mass of ~66 kD (Fig. 1B, Supplementary Fig. S1) [11] comprise about 0.6% of the Pakistan RVV proteome (Fig. 2). One of the LAAOs (Accession no. gi|395406796) found in Pakistan RVV was also reported in RVV from Southern India (7.9%) and Sri Lanka (5.2%) [7,8]. However, the relative abundance of LAAOs in RVV from Southern India RVV (7.9%) and Sri Lanka (5.2%) [7,8] is significantly higher as compared to Pakistan RVV (Fig. 2).

RVV is a rich source of proteolytic enzymes [40]. Proteolytic enzyme profiling, in addition to comparison of levels of proteolytic activity in venom from different subspecies of RV, has shed light on the toxicological contribution of proteases to RV envenomations [2,40]. Snake venom proteases have been broadly classified into two catalytic types – SVSPs and SVMPs [40,41]. RVV is rich in both SVSPs and SVMPs, and they affect the hemostatic system in victims by proteolytic cleavage of several different blood coagulation factors [31,42,43]; thrombin-like activity is prominent [9,44,45]. In in vitro conditions, crude RVV displays procoagulant activity owing to the predominance of pro-coagulant proteases [2]. In addition, snake venom proteases account for hemorrhage, necrosis and muscular degeneration in victims [40].

SVMPs are highly toxic components of snake venom that interfere with the blood coagulation cascade and also degrade the basement membrane and extracellular matrix of victims, leading to excessive bleeding [42,43,46]. LC-MS/MS analysis showed that SVMP is the second most abundant enzymatic protein family of Pakistan RVV, comprising 21.8% of the venom proteome (Fig. 2) and most of the SVMPs were detected in GF-1 fraction. RVV-X, an SVMP reported from RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2) can potentially activate blood coagulation factor X [47]. It is a heterodimer of a heavy chain of molecular weight 59 kDa and heterogeneous light chains of molecular weights 18 and 21 kDa [47], and corresponding protein bands have also been detected by SDS-PAGE analysis of GF-1 (Fig. 1C). The enzymatic assay also shows predominance of SVMPs in GF-1 (Table 1). By proteomic analysis, 3 heavy chain and 5 light chain isoforms of RVV-X were identified in the proteome of RVV from Pakistan (Table 3, Supplementary Table S1, Supplementary Fig. S2). In addition, a single homolog of VLAIP, a heterodimeric αβ-fibrinogenase isolated from *Macrovipera* (formerly *Vipera*) *lebetina* [48] was also identified in Pakistan RVV (Table 3, Supplementary Table S1, and Supplementary Fig. S2). Interestingly, 5 out of 13 SVMPs identified in Pakistan RVV (Table 3) share sequence homology with SVMPs in RVV from Southern India [7]. Nevertheless, only one SVMP (Accession no. gi|82228619) was found to be common between RVV from Sri Lanka [8] and Pakistan which is a good example of geographical variation in venom composition.

SVSPs exhibit fibrinogenolytic or fibrinolytic activity, and in some instances they may show fibrin(ogen)olytic activity [40]. The molecular mass of these proteins is typically in the range of 23–33 kDa [15,31]; however, Russelobin is a high molecular mass (51.3 kDa) serine protease (Fig. 1B, Supplementary Fig. S1) reported from Pakistan RVV [9]. Some of the SVSPs demonstrate fibrinogen clotting activity and they are termed "snake venom thrombin-like enzymes" (SVTLEs) [9,31,45]. Another class of SVSPs is similar to mammalian kallikrein and cleaves kininogen, thereby releasing bradykinin; these SVSPs are known as "kallikrein-like proteases" [49,50]. In addition to these biological activities, some SVSPs have been reported to activate Factor V, Protein C and/or plasminogen [31,51,52]. By LC-MS/MS analysis we have identified 9 SVSPs mostly in GF-1 fraction (Table 3, Supplementary Table S1, Supplementary Fig. S2) and they comprise about 3.2% of Pakistan RVV (Fig. 2). SVSPs such as serine β-fibrinogenase-like protein precursor and thrombin-like enzyme gyroxin B1.7 detected in GF-2 fraction (Table 3) indicated the elution of thrombin-like serine protease(s) in this peak (Table 2) which is according to our previous report [9].

Three isoforms of RVV-V, a serine protease that activates blood coagulation factor V, viz. RVV-Vα, RVV-Vβ and RVV-Vγ have been reported previously from RVV [52]. A single homologue of RVV-Vγ was identified in RVV from Pakistan (Table 3, Supplementary Table S1, Supplementary Fig. S2). The serine proteases from Pakistan RVV showed sequence similarity with fibrinogenases from venom of *Macrovipera lebetina* and *Daboia siamensis*, VLSP-1 and 3 from *Macrovipera lebetina* venom, and gyroxin-like *B*1 serine protease from *Crotalus durissus terrificus*, indicating that SVSPs share significant sequence homology across subfamilies (Supplementary Fig. S2); however, the primary sequence similarity of SVSPs may not always be correlated with their biological activity or pharmacological potency [9,31,44,53]. A comparative analysis shows that percent contribution of SVSPs in Pakistan RVV (3.2%) is significantly lower compared to RVVs from Southern India (17.5%) and Sri Lanka (16.0%) [7,8]. Noteworthy, two of the SVSPs identified in Pakistan RVV (accession no. gi|380875417 and gi|13959655) were also found in RVVs from southern India [7] and Sri Lanka [8].

Nucleotidases (AMPase, ADPase, and ATPase) present in snake venom can cleave a wide range of nucleotide molecules in presence or

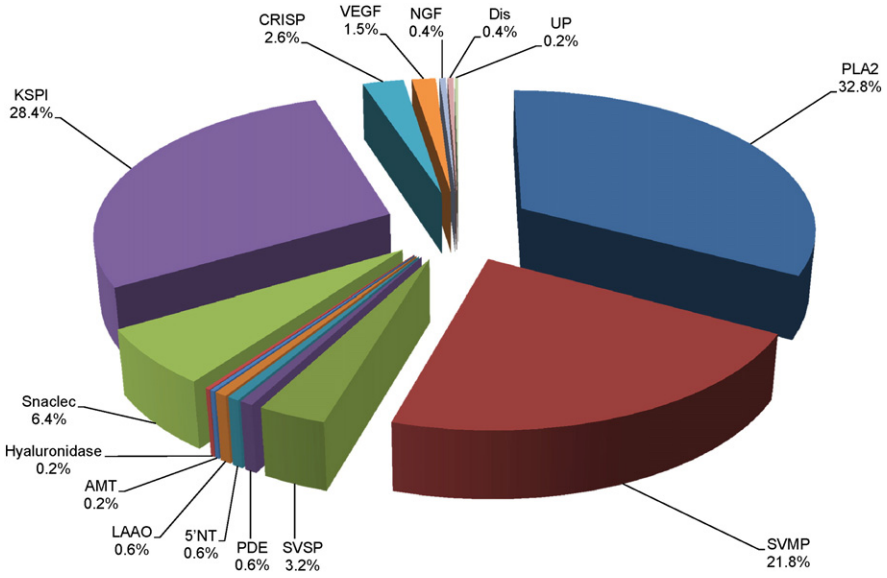

Fig. 2. A comparison of relative composition (expressed as percent of total identified proteins by LC-MS/MS analysis) of different enzymatic and non-enzymatic protein/peptide families of Pakistan RVV. The details of each protein/peptide are listed in Table 3 and in supplementary Fig. S2.

absence of divalent metal ions [54,55]. They generally exist as multimeric forms of around 260 kDa, and their monomeric molecular weight ranges from 60–70 kDa [56,57]. High molecular weight NT enzymes were eluted in GF-1 followed by GF-2 fractions (Table 1, Fig. 1B) and they represent a minor component (0.6%) of Pakistan RVV (Fig. 2). By LC-MS/MS analysis, peptides showing sequence homology with 2 NT enzymes reported from venoms of Viperidae snakes have been identified in GF-1 fraction of Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). Recently, presence of 2 and 3 NT enzymes in RVV from Southern India [7] and Sri Lanka [8], respectively have been reported. However, only one NT enzyme (accession no. gi|586829529) identified in Pakistan RVV share sequence homology with NT enzyme present in Southern India RVV [7]. Little information on the pharmacological properties of NT enzymes is available, but this group of enzymes present in Pakistan RVV may affect hemostasis by modulating platelet function via the action of adenosine released upon enzymatic cleavage of nucleotides [58,59]. Failure to identification of ATPase enzyme(s) eluted in GF-1 to GF-3 fractions (Table 1) by LC-MS/MS analysis may presumably be explained by the fact that proteomic identification of proteins/peptides from a particular sample is database dependent. Because ATPase has not been purified and sequenced from snake venom, the presence of this enzyme could not be ascertained by LC-MS/MS analysis; however, biochemical analysis of crude RVV and GF-1 to GF-3 fractions provided evidence of this enzyme activity in Pakistan RVV (Table 1).

Snake venom PDEs are single-chain high molecular weight enzymes (100–140 kDa) with basic pI and are readily inhibited by EDTA [60,61]. PDE sequentially hydrolyses phosphodiester bond from the 3′ terminus of polynucleotides and affects blood coagulation and modulation of platelet function in victims [62,63]. In Pakistan RVV we have identified 2 PDE enzymes (Table 3, Supplementary Table S1, Supplementary Fig. S2) which comprise 0.6% of the RVV proteome (Fig. 2). By SDS-PAGE analysis of crude RVV (reduced) the relative intensity of a 104.7 kDa protein band probably representing PDE was determined at 0.8% (Supplementary Fig. S1) which corroborates well with LC-MS/MS data. Furthermore, by enzymatic assay the PDE activity was detected only in GF-1 chromatographic fraction which is substantiated by SDS-PAGE (Fig. 1B) and LC-MS/MS analyses (Table 3). Latter analysis suggested that one of the PDE isoforms (accession no. gi|586829527) occurring in Pakistan RVV was also reported from Southern India RVV [7]; however the single PDE identified in RVV from Sri Lanka was different from PDEs existing in Pakistan and Southern India RVV samples [8].

Snake venom hyaluronidase, a neglected class of *endo*-β-glycosidases, has long been labeled as 'spreading factor', and their molecular mass ranges from 33 to 110 kDa [64]. Hyaluronidases degrade hyaluronic acid (*endo*-β-*N*-acetyl-D-hexosamine) present in extracellular matrix of local tissue and promote local hemorrhagic effects as well as enzyme-mediated distribution of the venom following injection, and therefore these enzymes likely have a significant contribution to Pakistan RVV-induced toxicity [64,65]. There is no report on the purification and characterization of hyaluronidase from RVV, and proteomic analysis did not reveal the presence of this enzyme in venom of *D. russelii russelii* from southern India [7], Sri Lanka [8] or in venom of *D. russelii siamensis* from Myanmar [8]. However, by LC-MS/MS analysis we have identified the occurence of one hyaluronidase isoform showing sequence similarity with hyaluronidase enzyme from *Echis carinatus sochureki* venom in GF-1 fraction of Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). The hyaluronidase activity detected by zymogram of crude *D. russelii russelii* venom under non-reduced conditions indicated the molecular mass of this protein was approximately 60 kDa [64] which substantiates the elution of this protein in GF-1 fraction of Pakistan RVV (Fig. 1B, C).

Aminotransferase (AMT) or glutaminyl-peptide cyclotransferase (ϑ-glutaminyl cyclase) enzymes with molecular weights ranging from 33–40 kDa are highly conserved from yeast to humans [66]. In snake venoms, their role has been assigned to protect the N-terminus of biologically active peptides such as the bradykinin potentiating peptides (BPPs), some metalloproteases, three-finger toxins of rear-fanged snakes [55], and the B and C chains of the acidic subunit of crotoxin homologs [67]. The presence of AMTs in Viperidae snake venom has been well documented [68], but no evidence has been presented demonstrating an active role of this enzyme in envenomations. AMT has not been reported previously from RVV from Southern India, Sri Lanka or Myanmar [6–8], but proteomic analysis of Pakistan RVV has documented the presence of this enzyme which comprised 0.2% of venom (Fig. 2) and eluted in GF-1 peak (Table 3). Only a single AMT was identified in Pakistan RVV and interestingly, it demonstrated sequence similarity with AMT from venom of *Protobothrops flavoviridis* a Viperidae family of snake endemic to Japan suggesting protein sequence similarity across Viperidae subfamilies (Table 3, Supplementary Table S1, Supplementary Fig. S2).

PLA_2, one of the most abundant and best characterized superfamilies of snake venom enzymes, catalyzes the hydrolysis of phospholipids at their sn-2 position to liberate lysophospholipids and free fatty acids [69]. The PLA_2 superfamily has broadly been classified into four major groups; the snake venom PLA_2s, with molecular weight ranging from 10 to 15 kDa, are classified under Groups I and II of the secretory PLA_2 ($sPLA_2$) [69]. PLA_2 enzymes display diverse pharmacological effects like neurotoxicity, cardiotoxicity, myotoxicity, necrosis, anticoagulant, hypotensive, hemolysis, hemorrhage, edema and membrane damage by several different mechanisms and PLA_2s are therefore considered important toxic enzymes of snake venoms [28–30,70,71].

Like other RVV proteomes [7,8] PLA_2s also represent the most abundant group of protein present in the venom of Pakistan *D. russelii russelii* and constitute 32.8% of the RVV proteome (Fig. 2) which was also according to SDS-PAGE analysis of crude RVV (Fig. 1B). By LC-MS/MS analysis 17 PLA_2 isoforms have been identified from Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). Although PLA_2 activity has been detected across all the GF peaks; however, its maximum activity was observed in GF-8 (Table 1). LC-MS/MS analysis also identified maximum number of PLA_2 enzymes in GF-8 fraction (Table 3). SDS-PAGE analysis of GF 8 proteins showed a broad but single protein band of 13.1–14.4 kDa range (Fig. 1C) representing PLA_2 isoenzymes [30,69]. Recently, Sharma et al. [7] and Tan et al. [8] have identified 15 and 4 PLA_2 isoenzymes in venom of *D. russelii russelii* from Southern India [7] and Sri Lanka [8], respectively. However, Risch et al. [6] have reported 22 PLA_2 isoenzymes in venom of *D. russelii siamensis*. These results indicate geographic as well as subspecies-specific differences in venom composition among Russell's Vipers. Daboiatoxin, a major PLA_2 toxin in venom of *D. russelii siamensis* from Myanmar [6], was reported to be absent in *D. russelii russelii* venom from southern India [7] and Sri Lanka [8]; however, a single isoform of this PLA_2 was identified in Pakistan RVV proteome (Table 3, Supplementary Table S1, Supplementary Fig. S2) reinforcing geographical variation in venom composition. Remarkably, PLB enzyme reported in RVV from Sri Lanka [8] could not be identified in Pakistan RVV.

3.4. Non-enzymatic proteins of RVV

RVV is rich in several proteins and peptides which do not show enzymatic activity but have a profound role in venom-induced toxicity and lethality. Some non-enzymatic proteins/peptides, such as snaclec, KSPIs, a low molecular mass pro-angiogenic peptide, a dual inhibitor of thrombin and FXa, and a cytotoxin-like peptide have been purified and characterized from RVV [10–12,18,19,72].

KSPIs are 60 amino acid peptides (molecular weight 6–10 kDa) that bind to the active site of serine proteases via 6 conserved cysteine residues, termed the Kunitz motif [15,73]. KSPIs have been reported to inhibit serine proteases, block ion-channels, and act on coagulation, fibrinolysis and inflammation [10,12,74–76]. LC-MS/MS analysis shows that KSPIs constitute 28.4% of the Pakistan RVV proteome (Fig. 2) which is corroborated by SDS-PAGE analysis of RVV (Supplementary

Fig. S1) and therefore, they represent the most abundant non-enzymatic proteins of this venom. Interestingly, KSPIs are detected in almost all GF peaks of Pakistan RVV (Table 3) suggesting their interaction with other components, including venom PLA_2s and proteases [77] (Mukherjee, A. K., unpublished observation). By LC-MS/MS analysis we have identified 8 KSPI isoforms in the venom of *D. russelii russelii* from Pakistan (Table 3, Supplementary Table S1 and Supplementary Fig. S2). Recently, Sharma et al. [7] and Tan et al. [8] have reported 3 and 2 KSPIs in the venom of *D. russelii russelii* from southern India and Sri Lanka, respectively. However, no KSPI was detected in Burmese Russell's Viper (*D. russelii siamensis*) venom [6].

C-type lectin like proteins (CLPs) or snaclecs are a class of non-enzymatic, Ca^{2+} dependent proteins whose basic structure is characterized by αβ heterodimeric forms linked by disulfide bonds [78]. These subunits are further linked by covalent bonds to form multimeric $(\alpha\beta)_2$ and $(\alpha\beta)_4$ structures [11,78]. It has been found that blood coagulation factors, cell membranes and platelet receptors are primary targets for snaclecs, and therefore they are at least partly responsible for hemostatic imbalances seen in snake bite victims [11, 79]. Recently, a 66.3 kDa multimeric snaclec named RVsnaclec was purified and characterized from Pakistan RVV [11]. The peptide fragments K.GSHLLSLHNIAEADFVLK·K (*m/z* 982.5356), and M.GLNDVWNECNWGWTDGAK.L (*m/z* 1061.459) of RVsnaclec [11] were also detected in this RVV proteome (Supplementary Table S1). Proteomic analysis shows that snaclecs are the second abundant non-enzymatic protein family in Pakistan RVV (6.4%) (Fig. 2), and 11 snaclecs were identified in Pakistan RVV mostly in GF-1 peak (Table 3, Supplementary Table S1, Supplementary Fig. S2). The snaclecs represented 7.9% and 22.4% of RVV protein from Southern India [7] and Sri Lanka [8], respectively. Homologs of both the α and β subunits of Dabocetin, a heterodimeric snaclec that inhibits ristocetin-induced platelet aggregation via binding to glycoprotein Ib receptor [80], were also observed in Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). In addition, peptide sequence homologs of α and β subunits of P31 and P68 snaclecs from *D. siamensis*, as well as Snaclec A14 from *Macrovipera lebetina* venom, were also identified in Pakistan RVV proteome (Table 3, Supplementary Table S1, Supplementary Fig. S2). In the snaclec family, only Dabocetin, *P*31, and P68 were found to be common in RVVs from Pakistan, Southern India [7] and Sri Lanka [8].

Cysteine-rich secretory proteins (CRISPs) are widely distributed among venoms of colubrids, viperids and elapids [81], and we have identified 3 CRISP homologs in Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). CRISPs consist of a single polypeptide chain of 20–30 kDa with 16 conserved cysteine residues [81] and have been reported to inhibit smooth muscle contraction and cyclic nucleotide-gated ion channels [82,83]. CRISPs constituted 2.6% of the Pakistan RVV (Fig. 2) and this value corroborates well with SDS-PAGE analysis result (Supplementary Fig. S1). However, CRISPs comprised 11.0% and 0.29% of RVV from Southern India and Sri Lankan RVV, respectively [7–8] indicating a wide variation in distribution of CRISPs in venom of RV from different geographical origin. This protein was also detected in GF-1 peak (Table 3) which indicated interaction of CRISP with other components of RVV. The MS-MS derived peptide sequences homologs of Dr-CRPK and Dr-CRPB from *D. russelii russelii*, and Da-CRPa from *Deinagkistrodon acutus* were found in Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). Remarkably, Dr.-CRPK was also detected in RVV of Southern India and Sri Lanka [7–8].

Disintegrins are characterized by their unique ability to interfere with and inhibit integrin-ligand interactions; this binding is generally associated with a conserved arginine-glycine-aspartic acid (RGD) motif [84,85]. Disintegrins are typically liberated upon proteolytic cleavage of class P II metalloproteases and molecular masses of disintegrins range from 4 to 15 kDa [15]. These cysteine-rich venom components are potent inhibitors of platelet aggregation and thus can affect hemostatic balance in victims [85]. Proteomic analysis suggests that disintegrins are a very minor component (0.4%) of Pakistan RVV (Fig. 2). Interestingly, by proteomic analysis presence of this relatively low molecular mass protein was detected only in GF-1 fraction (Table 3). This observation advocates interaction of disintegrins with other proteins of venom to form a high molecular mass complex resulting its elution in GF-1 peak because the SDS-PAGE (non-reduced) analysis of GF-1 did not show presence of any low molecular mass protein in the range of disintegrins (Fig. 1C). The MS-MS derived peptide sequences from Pakistan RVV demonstrated sequence similarity to catroriarin, a disintegrin isolated from *Crotalus atrox* venoms (Table 3, Supplementary Table S1, Supplementary Fig. S2). Disintegrins were not identified in Sri Lankan or Southern India RVVs [7,8] and to the best of our knowledge this is the first report showing the presence of disintegrins in RVV.

Snake venom nerve growth factor (NGF) belongs to a family called "neurotrophic factors" and their molecular weight ranges from 25 kDa to 54 kDa [86]. Although snake venom NGFs have been reported to cause apoptosis, vascular permeability and wound healing [87], the exact mechanism of their action and the reason for their presence in snake venoms are yet to be deciphered. Presence of NGFs has already been reported in RVVs from Southern India and Sri Lanka [7,8]. NGFs constitute only 0.4% of Pakistan RVV (Fig. 2), and by proteomic analysis a single NGF showing sequence similarity with NGF from *D. russelii russelii* venom have been identified in Pakistan RVV (Table 3,

Table 4
Pharmacological properties of crude RVV and gel filtration fractions. Values are mean ± S.D. of three replicates. Significance of difference wih respect to control ($^a p < 0.05$).

Crude RVV/GF peak	Cytotoxicity*		Ca-clotting time†	APTTπ	PT¶	Fibrinogen clotting activity**	Hemolytic activity (%)¥
	MCF-7	Colo-205					
Crude RVV	14 ± 2.1 [a]	8 ± 1.1 [a]	73.0 ± 3.2[a]	25.8 ± 1.7 [a]	11.1 ± 1.0 [a]	−	1.3 ± 0.4
GF-1	26 ± 2.3 [a]	13 ± 1.9 [a]	48.5 ± 2.9 [a]	20.9 ± 2.0 [a]	8.0 ± 0.8 [a]	−	0
GF-2	0	0	60.5 ± 3.3 [a]	28.1 ± 1.3 [a]	8.6 ± 0.9 [a]	+	0
GF-3	0	0	77.8 ± 4.1 [a]	28.6 ± 0.8 [a]	8.3 ± 0.9 [a]	−	0
GF-4	0	0	131.8 ± 3.9	36.5 ± 0.8	14.0 ± 1.1	−	0
GF-5	0	0	178.3 ± 3.2 [a]	47.3 ± 1.1 [a]	18.7 ± 1.2 [a]	−	0
GF-6	0	0	155.0 ± 4.3 [a]	40.8 ± 1.6	17.5 ± 1.0 [a]	−	0
GF-7	0	0	148.0 ± 3.2 [a]	40.3 ± 1.4	17.1 ± 1.2 [a]	−	0
GF-8	0	0	152.5 ± 2.1 [a]	53.8 ± 2.1 [a]	18.5 ± 1.1 [a]	−	0
GF-9	0	0	149.3 ± 2.9 [a]	39.2 ± 0.4	17.2 ± 0.7 [a]	−	0
GF-10	0	0	147.8 ± 3.4 [a]	39.8 ± 0.4	17.3 ± 1.0 [a]	−	0

† Ca-clotting time of control PPP is 137.5 ± 5.8 s. The anticoagulant activity was assayed with 1.0 μg protein.
π Average APTT of control PPP was 38.9 ± 2.1 s. The assay was done with 1.0 μg protein.
¶ Average PT of control PPP was 14.3 ± 1.1 s. The assay was done with 1.0 μg protein.
¥ The assay was done with 10 μg/ml protein.
* Cytotoxicity (percent cell death) of 5 μg protein/ml was evaluated following incubation for 24 h at 37 °C, 5% CO_2.
** The (+) sign indicates presence whereas (−) sign indicates absence of fibrinogen clotting activity. The assay was done with 10 μg protein.

Supplementary Table S1, Supplementary Fig. S2). Interestingly, the presence of this particular isoform (bearing the same accession no) was also reported in RVV from Sri Lanka [8] but not in RVV from Southern India [7].

Snake venom vascular endothelial growth factors are one of the 7 members of the vascular endothelial growth factor (VEGF-F) family of proteins and their molecular weights range from 23–33 kDa [15]. Evidence has indicated that VEGF-F binds to receptors like KDR and Flt-1 and exhibit potent hypotensive effects and enhances vascular permeability [88]. Two VEGFs from venom of southern India *D. russelii russelii* [7] and 2 VEGF isoforms from *D. russelii siamensis* venom [6] have been identified. The VEGFs constitute 1.5% of Pakistan RVV (Fig. 2) and 3 isoforms of this protein family have been identified in peaks GF-1, 4, and 9 (Table 3, Supplementary Table S1, Supplementary Fig. S1). However, the identified VEGFs are exclusively reported in Pakistan RVV and the occurrence of identical isoforms of VEGFs was not reported in RVV from other geographical locations [6–8].

In addition to the enzymatic and non-enzymatic proteins mentioned above, an MS-MS derived peptide showing sequence similarity to a hypothetical protein (accession no. gi|19574645) from *Agkistrodon piscivorus piscivorus* venom was also identified in Pakistan RVV (Table 3, Supplementary Table S1, Supplementary Fig. S2). Due to a lack of information, the biological activity of this protein could not be assigned.

3.5. Pharmacological properties of crude RVV and chromatographic peaks

Some pharmacological properties of crude RVV and gel-filtration peaks are shown in Table 4. However, due to lack of published clinical data, the pharmacological properties of Pakistan RVV could not be correlated with the observed clinical manifestations of RV bite in Pakistan.

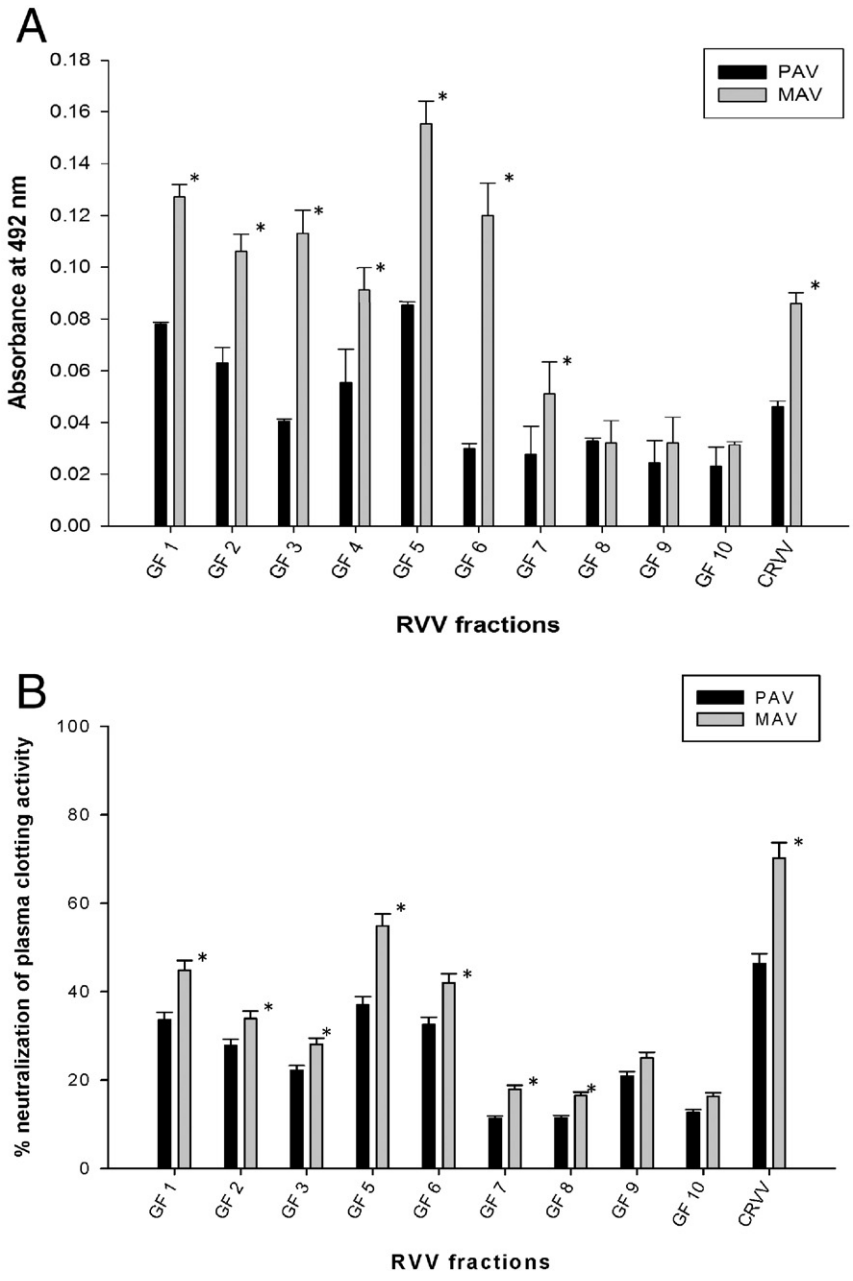

Fig. 3. A. Immunological cross-reactivity of Pakistan RVV and its GF fractions with commercial polyvalent (PAV) and monovalent (MAV) antivenom. Values are mean ± SD of triplicate determinations. B. Neutralization of pro-coagulant/anticoagulant activity of CRVV and its fractions by PAV and MAV. The experiment was conducted as described in the text. Values are mean ± SD of triplicate determinations. CRVV, crude Russell's Viper venom. Significance of difference with respect to cross-reactivity of PAV, $^{*}p < 0.05$.

The crude RVV or GF peaks did not exhibit hemolytic activity (Table 4), in accordance with our previous reports showing RVV PLA_2 enzymes do not induce direct hemolysis of washed erythrocytes [30]; however, PLA_2s may induce indirect hemolysis in the presence of phospholipids [2,29]. The cytotoxicity of peak GF-1 against Colo-205 and MCF-7 cells was found to be higher than the cytotoxicity exhibited by crude RVV under identical experimental conditions (Table 4), which may be attributed to the presence of the cytotoxic protein Rusvinoxidase (a LAAO enzyme) in this peak [13]. Only peak GF-2 showed fibrinogen clotting activity (Table 4), resulting from the elution of Russelobin (a thrombin-like serine protease) in this peak [9].

Crude RVV in in vitro demonstrated pro-coagulant activity (Table 4) due to predominance of procoagulant components in the venom; nevertheless, in vivo it displayed anticoagulant activity due to consumption of components of hemostatic system [2,42,43]. The first three GF peaks (GF-1 to GF-3) demonstrated pro-coagulant activity in decreasing potency; however, RVV proteins/peptides present in GF-5 to GF-10 peaks showed anticoagulant activity (Table 4). The proteins/peptides in GF-4 have no effect on blood coagulation cascade ($p > 0.05$ compared to control). The observed pro-coagulant activity of the fractions GF-1 to GF-3 may be due the prevalence of procoagulant SVMPs and SVSPs in these peaks (see Table 1) [9,10,40,43]; whereas predominance of PLA_2s and/or KSPI in GF-5 to GF-10 explains the anticoagulant properties of these fractions [10,12,31]. Although SDS-PAGE analysis demonstrated occurrence of proteins in the mass range of 13–14 kDa in peak GF-4 (Fig. 1B); however, enzymatic assay showed negligible PLA_2 activity in this peak (Table 1). The anticoagulant action of these PLA_2 enzymes may presumably be counter balanced by RVV pro-coagulant protease(s) showing BAEE-esterase activity in GF-4 fraction (Table 1) [31]. The anticoagulant effect of venom proteins is exerted owing to their interactions with the blood coagulation factors involved in the intrinsic, extrinsic and/or the common pathway [28,89]. A change in either PT or APTT of PPP by RVV proteins indicates their effect on extrinsic or intrinsic pathway, whereas deviations in PT and APTT indicate the impediment of the common pathway by RVV proteins [28,43]. A significant change in both PT and APTT of PPP by GF-5 and GF-8 signifies the interaction of proteins/peptides of these fractions with the coagulation factors involved in the common pathway; the remaining anticoagulant peaks of Pakistan RVV influence the extrinsic pathway at the concentration tested to exert the anticoagulant action (Table 4).

3.6. Immunoreactivity and neutralization of pro-coagulant or anticoagulant activities of crude RVV and its GF chromatographic peaks with commercial polyvalent and monovalent antivenom

Intravenous administration of antivenom is the only choice for the treatment of snakebite. However, due to the complex interplay of several crucial factors, for example, geographic and species-level variation in snake venom composition [5,34], as well as low immunogenicity of low molecular weight components of venom [10,12,18], the therapeutic efficacy of an antivenom in neutralizing all of the toxic components of a snake venom remains a challenge [90]. Therefore, in the present study antigenic cross-reactivity of commercial PAV and MAV manufactured in India with crude and gel filtration fractions of Pakistan RVV was investigated by ELISA technique.

The immunological cross-reactivity of PAV against RVV and its fraction was found to be significantly less ($p < 0.05$) as compared to cross-reactivity with MAV (Fig. 3A). This suggests that MAV is likely a better option compared to PAV for the treatment of Russell's Viper bite patients. Interestingly, the proteins/peptides present in different GF peaks were recognized by both PAV and MAV however at a different extent, indicating variation in antigenic potency among Pakistan RVV proteins/peptides. Although present study is a preliminary characterization of the antigenic property of crude RVV and its fractions; nevertheless, result of this study directs us to identify the low antigenic proteins/peptides in Pakistan RVV by second generation antivenomics approach [24].

Because of numerous pro-coagulant and anticoagulant proteins and peptides in RVV [9,43], the most common clinical symptoms of RV envenomation are hemostatic disturbances, defibrination, non-coagulable blood, and extensive hemorrhage leading to spontaneous bleeding from vital organs [2]. The neutralization potency of Indian PAV and MAV toward pro-coagulant and anticoagulant activities of Pakistan RVV and its fractions was assessed in the present study. Both PAV and MAV showed good neutralization albeit significantly lower neutralization ($p < 0.05$) towards anticoagulant activity of GF-7 to GF-10 compared to neutralization of pro-coagulant activity of proteins/peptides present in GF-1 to GF-3 peaks was observed (Fig. 3B). Furthermore, neutralization of anticoagulant or procoagulant activity of RVV fractions by PAV or MAV (Fig. 3B) corroborates well with the immunological cross-reactivity of RVV fractions with PAV/MAV determined by ELISA (Fig. 3A). Besides, the neutralization of pro- or anticoagulant activity of RVV GF fractions by MAV was found to be superior than PAV.

4. Conclusion

Present study shows that Pakistan RVV contains 75 enzymatic and non-enzymatic proteins/peptides which belong to 14 distinct snake venom protein families. Some of the proteins, for example aminotransferase, *endo*-β-glycosidase, and disintegrins are reported for the first time in RVV. The proteomic analysis has documented qualitative as well as quantitative variation in composition of Pakistan RVV as compared to the venom composition of RV from other geographical localities. The data presented in this study have also provided strong indication of protein-protein interactions among RVV proteins. Due to lack of published clinical data on Pakistan RV envenomation the in vivo pharmacological or toxicological effect of each identified protein of Pakistan RVV could not be ascertained. Nevertheless, it may be assumed that these proteins/peptides are collectively accountable for the toxic and pharmacological effects of Pakistan RV envenomation. Our study suggests that strategies should be developed for further improvement of PAV for effective snakebite treatment and MAV is the better choice for the treatment of RV envenomed patients.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.06.001.

Conflict of interest

The manuscript entitled-"A proteomic analysis of Pakistan *Daboia russelii russelii* venom and assessment of potency of Indian polyvalent and monovalent antivenom" has no conflict of interest.

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