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Purification and characterization of a low molecular weight multifunctional cytotoxic phospholipase A_2 from Russell's viper venom☆

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Abstract

A basic toxin from Russell's viper venom of 7.2 kDa (RVV-7) has been purified to homogeneity after partial unfolding by 4 M urea followed by filtration through Centricon-30 membrane. Its N-terminal sequence showed strong homology with snake venom cytotoxins. Cytotoxic activity of RVV-7 has been demonstrated with B16F10 melanoma cells. PLA_2 activity was observed in cytotoxin (CX3) from *Naja kauthia* bearing sequence homology with RVV-7. Phospholipase A_2 and trypsin inhibitory activities were also observed with RVV-7. Chemical modification and inhibition studies suggested independent functional sites for these activities. A qualitative assessment of tumor growth inhibition by RVV-7 has been made.
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Keywords: Phospholipase A_2; Low molecular weight enzyme; Russell's viper venom; Cytotoxicity; Trypsin inhibitor; Tumor growth suppression

1. Introduction

Russell's viper is an important cause of snakebite and related mortality especially among rice farmers throughout Asia. Based on the geographical origin, Russell's viper has been classified as *Daboia russelli russelli* (Indian subcontinent), *Daboia russelli pulchella* (Sri Lanka), *Daboia russelli siamensis* (Burma, Thailand, Cambodia and Southern China), *Daboia russelli formosensis* (Taiwan) and *Daboia russelli limitis* (Java, Komodo, Flores and Lomblen) [1]. To avoid a definitive alteration in the taxonomy, these five subspecies have been currently recognized as two, namely *Daboia russelli russelli* (Western form) and *Daboia russelli siamensis* (Eastern form) based on morphological analysis [2]. All population from the Indian subcontinent except those from East of the Bay of Bengal belongs to the Western form. Experience from India *versus* Thailand, Burma and Sri Lanka has revealed a fascinating geographical variation in the clinical manifestations of Russell's viper bite [3]. This variation of Russell's viper ophidism has also been observed within different parts of India [1]. While neurotoxicity and hypopituiterism are the major symptoms of such envenomation from Southern India, those from Eastern India are spontaneous systemic and dermal hemorrhages [4,5].

Russell's viper venom (RVV) in general contains a number of phospholipase A_2 (PLA_2) with isoforms [6], coagulation factor V and X activating proteases [7,8], hyaluronidase [9], nucleases, hemorrhagins and several other constituents [1]. Attempts were made to correlate regional variation of clinical manifestations with abundance of toxic components of RVV within India where it was observed that the eastern region venom is most toxic [10]. Strong hemorrhagic activity of RVV from eastern India can be explained by site-specific hemorrhagins like RVV-22 [5], RVV-73 [11] and RVV-12 [12] (the suffix indicating Mw in kDa). Comparative SDS-PAGE profile of RVV from eastern, western, southern and northern India indicates the presence of a distinct low Mw protein with high abundance in eastern and western forms [10]. Characterization of this proteins from eastern regional RVV will help understanding the mechanism of action by this venom.

Here we report one-step purification of the low Mw protein, named as RVV-7, with PLA_2 and strong cytotoxic activity. It also showed trypsin inhibitory and tumor growth retarding property.

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To our knowledge, this is the smallest PLA_2 reported so far having multiple functions.

2. Materials and methods

2.1. Materials

Russell's viper (*Daboia russelli russelli*) and *Naja kauthia* venom was collected from a licensed trophy (Mr. Dipak Mitra, Calcutta snake park) as desiccated crystals. The snake venom antiserum (AVS), manufactured by Bharat Serums and Vaccines Limited, against Cobra, Common krait, Russell's viper (*Daboia russelli russelli*) and Saw-scaled viper, was a gift from the Superintendent, M.R.S. Bangur Hospital, Department of Health, West Bengal. Each ml of the AVS neutralizes not less than 0.6 mg of Cobra, 0.45 mg of Common krait, 0.45 mg of saw-scaled viper and 0.6 mg of Russell's viper venom. Proteases (trypsin, chymotrypsin, bromelain, collagenase and papain), their substrates (azocasein, azoalbumin, tosyl-L-arginine methyl ester or TAME), 1,2-dimyristoyl-sn-glycero-3 phosphocholine or DMPC (phospholipase A_2 substrate) and Mw markers were from Sigma–Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), penicillin and streptomycin were from GIBCO Invitrogen Corporation, USA. Sephadex G-15, Sephadex G-100 and CM-Sephadex C-50 were from Amersham Biosciences, Sweden. Centrifugal filtration devices of 10 and 30 kDa cut off ranges (Centricon Ym-10 and 30) were from Amicon (Bioseparation, Millipore, USA). Other analytical grade reagents were purchased locally. Adult male inbred BALB/C or BLJ6 mice of 20 ± 2 g were from the central animal house facility of this institute (IICB). All animal experiments were performed following local legislative guidelines.

Melanoma cells (B16F10) were obtained from National Center for Cell Science, Pune, India. Serial cultures were maintained in canted neck tissue culture flasks in DMEM supplemented with 10% FCS. Cultures were grown in conventional monolayer tissue culture technique in 25 cm^2 canted neck tissue culture flasks at 37 °C in water vapor saturated with 5% CO_2/95% air mixture.

2.2. Purification of RVV-7

Dry venom crystals (43 mg, equivalent to 30 mg of protein), was suspended in 1 ml of 20 mM K-phosphate, pH 7.4 at 25 °C for 30 min and insoluble materials were removed by centrifugation ($1000 \times g$) for 10 min at 4 °C [11]. The supernatant was dialyzed against the same buffer for 6 h with three changes to remove organic molecules. This was followed by changing the dialysate to 500 ml of 4 M urea in same buffer and dialysis was continued for 3 h at 25 °C. During the second dialysis, venom proteins were partially denatured slowly at equilibrium. Thus, the toxins were not exposed to strong denaturants avoiding possibility of irreversible damages. This also prevented unwanted dilution of the sample for the following step. The dialyzed sample was applied to Centricon Ym-30 filtration unit. After centrifugation at $2000 \times g$ at 4 °C for 30 min, the filtrate was collected. The volume of retainate was made up to 1 ml by 4 M urea solution and was centrifuged. This procedure was repeated four times to improve yield. Pooled filtrate was dialyzed extensively using benzoylated dialysis tubing (cut off range 2 kDa) against 20 mM K-phosphate, pH 7.4. Protein content was estimated after Lowry et al. with BSA as reference [13]. SDS-PAGE was performed as described [14].

2.3. Gradient PAGE

Native Mw of the purified protein was estimated from electrophoretic mobility through a linear gradient of 4–40% acrylamide gel. Preliminary analysis of the protein indicated that it migrated in reversed direction under conventional electric field and thus, all kinds of PAGE were carried out with reversed electrode arrangement. In addition, better migration was observed in presence of 25 mM alanine-acetate, pH 4.2 [15] against standard protocol of 25 mM Tris-glycine, pH 8.8. Under such conditions, an empirical linear relation between log Mw *versus* migration of proteins holds good [16]. Reference markers used were BSA (66 kDa), ovulbumin (45 kDa), Soya bean trypsin inhibitor (STI) (20 kDa), lysozyme (14 kDa), cytochrome C (12.5 kDa) and aprotinin (6.5 kDa).

2.4. Denaturing PAGE

Electrophoresis in 10% PAGE in presence of 8 M urea was done either at pH 4.2 using alanine-acetate, pH 4.2 for 2 h or Tris-glycine, pH 8.8 for 3 h. Electrophoresis at low pH was done after reversing the polarity. The sample was pre-equilibrated in presence of 8 M urea for 2 h to attain equilibrium denaturation. Protein bands were visualized after treating the gels with 10% trichloroacetic acid (TCA) as fixative followed by staining with Coomassie Brilliant Blue R -250 (Sigma).

2.5. Reverse phase (RP)–HPLC

RP–HPLC of RVV-7 was performed using C_{18} μ-Bondapak column (waters, 7.8 mm × 300 mm, bead size 10 μm and porosity 125 Å) pre-equilibrated with 0.1% trifluoro acetic acid (TFA) in water. About 100 μg of protein, pre-incubated with 0.1% TFA was applied in an injector fitted with 100 μl loop. After washing with the same solvent for10 min, the protein was eluted with an initial 0–5% water-acetonitrile gradient at a flow rate of 1 ml/min for 10–30 min. This was followed by a 5–100% water-acetonitrile gradient for 30–70 min to ensure complete release of associated proteins or peptides, if any. All solvents contain 0.1% TFA. Elution was followed at 280 nm.

2.6. Size exclusion (SE)–HPLC

SE–HPLC was carried out using Waters Protein Pac-60 column (7.8 mm × 300 mm, bead size 10 μm, fractionation range 1–20 kDa). The column was equilibrated and eluted with 20 mM K-phosphate, pH 7.4 containing 0.2 M NaCl at a flow rate of 1 ml/min and was followed at 280 nm. BSA (66 kDa), lysozyme (14.5 kDa), cytochrome C (12.5 kDa) and aprotinin (6.5 kDa) were used to calibrate the column including its void volume

determination. A linear dependency of log Mw *versus* retention time (R_t) was observed.

2.7. Isoelectric focusing (IEF)

IEF was carried out in horizontal mini gel apparatus (7.5 cm × 6 cm, Genei, Bangalore, India) [17]. Gel was cast with 5.1 ml of acrylamide solution (30% acrylamide and 0.8% bis-acrylamide), 10.8 ml of 20% sucrose; 7.5 ml of 0.14% ammonium persulphate; 15 μl of TEMED; and 100 μl ampholyte, pH 3–10. Cathode and anode buffers were 1 M NaOH and 1 M phosphoric acid, respectively. A pre-electrophoresis at 500 V for 30 min developed the pH gradient. Completion of gradient formation was indicated by drop of initial current from 50 to 0 mA. Samples containing 10% glycerol were loaded in different wells and electrophoresis was carried out at 50 V for the first 30 min followed by at 100 V for 3 h. Protein bands were stained with Coomassie Brilliant Blue R-250 after treating with fixative (30% methanol, 3.5% sulfosalicylic acid and 12% TCA in water). Isoelectric point of the protein was estimated from linear extrapolation of the calibration curve relating electrophoretic mobility (R_f) from cathode to anode to p*I* of reference proteins as follows: STI (p*I* 4.5, R_f 44 mm), α-lactalbumin (p*I* 5.1, 42 mm), myoglobin (p*I* 7.1, 16 mm), trypsinogen (p*I* 9.3, 8 mm) and lysozyme (p*I* 10.5, 3.5 mm).

2.8. Mass spectrometry

RVV-7 was desalted by extensive dialysis against water at 4 °C and lyophilized. The sample was dissolved in 50% acetonitrile in water containing 0.5% formic acid and was injected in a Q-Tof Mass Spectrometer (Micro mass). The sample was analyzed under positive ionization electro spray mode at a desolvation temperature of 200 °C. Argon as a collision gas at 2 kg/cm^2 having collision energy of 10 ev was applied. Micro channel plate detectors were used. The exact molecular mass of the protein was determined after deconvolution of the raw MS spectra with MaxEnt3 algorithm.

2.9. Amino acid sequencing

N-terminal amino acid sequencing of RVV-7 was done by an Applied Biosystem, USA automated protein sequencer (model Procise–491). Following manufacturer's protocol, the band of RVV-7 containing 100 pmol of protein from 10% SDS-PAGE was electrotransferred on an Immobilon—P^{SQ} (Millipore) membrane. The transfer buffer was 10 mM 3-[cyclohexylamino-1-propanesulfonic acid] (CAPS), pH 11.0, containing 10% methanol. The transferred band was loaded on the sample cartridge. A sequence up to 20th residue was derived without any ambiguity.

2.10. Cytotoxicity assay

Cytotoxicity assay—B16F10 melanoma cells were plated at a concentration of 5×10^4 cells/well in to 96 well flat bottom tissue culture plates in DMEM supplemented with 2% FCS. Thereafter, cells were treated with increasing doses of RVV-7 for 24 h. Cell viability was assayed following MTT estimation [18]. Briefly after removing RVV-7 containing medium, MTT solution (100 μl, 1 mg/ml in DMEM) was added to the wells and incubated in CO_2 incubator at 37 °C. After 4 h of incubation, the supernatant was carefully removed and the crystals of reduced MTT were dissolved in 100 μl of 0.04 N HCl in isopropanol at 37 °C, and absorbance at 595 nm was read by an Elisa reader (Emax, molecular devices).

2.11. Purification of CX3 (cytotoxin-3)

Dry *Naja kauthia* venom (40 mg) was suspended in 1 ml of 20 mM K-phosphate, pH 7.4, at 25 °C for 30 min, and tissue debris were discarded by centrifugation at 1000 × *g* for 10 min at 4 °C. The clear supernatant was subjected to CM-sephadex C-50 chromatography (250 mm × 15 mm) pre-equilibrated with the same buffer at 4 °C. After removal of the unabsorbed fractions, bound materials were eluted using a linear gradient of 0–0.5 M NaCl in the same buffer. Elution of proteins from the column was continued using the final buffer. SDS-PAGE indicated that the last peak contained CX3 with a high Mw protein contamination. This fraction was pooled, and dialyzed against 20 mM K-phosphate, pH 7.4, and loaded on Sephadex G-75 column (30 cm × 1.2 cm) equilibrated with the same buffer. Flow rate was 12 ml/h and fraction size was 2 ml. All steps were carried out at 4 °C. The column was pre-calibrated with the following markers: BSA (66 kDa, elution volume V_e, 12 ml), ovalbumin (45 kDa, 17 ml), lysozyme (14 kDa, 32 ml), and aprotinin (6.5 kDa, 40 ml) where a linear dependency of log M_w *versus* V_e was observed. Mw of purified CX3 was derived from the calibration curve that corresponded to the earlier report [19].

2.12. PLA$_2$ assay

PLA$_2$ activity of RVV-7 or CX3 was followed as described recently using scattering mode of a spectrofluorimeter and micelle of DM-PC as substrates [20]. About 10 mg of DM-PC was dissolved in 50 μl of absolute ethanol and was diluted to 1 ml by water to yield a uniform suspension of micelle that served as a stock. For each experiment, approximately 0.295 μmole of DM-PC in 20 mM K-phosphate, pH 7.4 containing 8 mM $CaCl_2$ and variable concentrations of RVV-7 or CX3 was added in a 3 ml quartz cuvette. Immediately after mixing, the reaction was followed by measuring the decrease in scattering intensity at 650 nm at 37 °C for 10 min. To calculate kinetic parameters, 100% completion of the reaction was assumed from the drop in scattering intensity that remained constant for at least 5 min during hydrolysis. The rate of hydrolysis was measured from the time zone where the change of the scattering intensity with time was linear.

2.13. Protease assay

Proteolytic activities of trypsin, chymotrypsin, bromelain, collagenase and papain were estimated using azocasine or azoal-

bumin as substrate. In brief, variable amounts of proteases was incubated with 0.25 ml of 1% azocasein in 0.1 M Na-phosphate, pH 7.5, containing 0.005 M of EDTA and 0.05 M β-mercaptoethanol at 37 °C for 1 h in 1 ml. Proteolysis was terminated by the addition of 0.5 ml of 10% TCA. The precipitate was allowed to settle for 30 min at ambient temperature and was removed by centrifugation at 900 × *g* for 15 min. The supernatant (0.5 ml) was added to 0.5 ml of 0.5 N NaOH, and its absorbance at 440 nm was read [21]. An assay mixture without enzyme served as control. Kinetics of proteolysis by trypsin was carried out with azocasine between 33.3 and 80 μM holding trypsin at 2.0 nM.

2.14. Esterase assay

Esterolytic activity of trypsin was followed using TAME as substrate [22]. Briefly, trypsin (5 μg) was added to 0.38 mg of TAME in 1 ml of 0.1 M Tris–HCl, pH 8.8. Continuous change in absorbance with time was monitored at 247 nm. Spontaneous hydrolysis of TAME under identical conditions without trypsin served as control.

2.15. Protease treatment

RVV-7 was treated with the above-mentioned proteases (10:1, w/w) in 0.1 M Na-phosphate, pH 7.5, at 37 °C for 5 h. Aliquots were withdrawn at different intervals of time and reactions were arrested by freezing the content at −20 °C. Proteolytic pattern was viewed by 15% SDS-PAGE analysis.

2.16. Amino acid modifications

Histidine residues were modified by treating RVV-7 (0.5 mg/ml) or CX3 (0.4 mg/ml) with 0.25 mM diethylpyrocarbonate (DEPC) in 20 mM K-phosphate, pH 7.4, at 30 °C for 30 min. The number of residues modified was calculated using $\varepsilon_{242\,nm} = 3200\,M^{-1}\,cm^{-1}$ for the product. This modification was reversed by treatment with 0.01 M hydroxylamine, HCl [23]. RVV-7 (0.5 mg/ml in 0.1 M Na-borate, pH 9.8) was allowed to react with 0.025 ml of 0.1% 2,4,6-trinitrobenzene sulphonic acid (TNBS) in water for 3 h at 30 °C for modification of lysine residues. The number of residues modified was calculated using $\varepsilon_{367\,nm} = 11{,}000\,M^{-1}\,cm^{-1}$ for released trinitrophenyl amino group [24]. Arginine residues were modified by incubating 0.05 mg of RVV-7 in 1 ml of 0.2 M Na-borate, pH 9.8, with 40 mM of 1,2-cyclohexanedione for 3 h at 37 °C [25].

2.17. Tumor growth

Exponentially growing B16F10 cells were harvested by trypsinization and suspended at 5×10^5 cells/ml in DMEM. These cells were injected subcutaneously in the left thigh of adult male BLJ6 mice that served as positive controls. In separate culture plates, B16F10 cells were treated with 30 μg/ml of RVV-7 for 7 days with daily change of media. The experimental set of mice was injected with these pretreated cells. Mice injected with 50 μl of DMEM served as negative controls. After 21 days the animals were sacrificed by cervical dislocation and the development of tumor was noted.

2.18. Optical instruments

For PLA_2 assay, a Hitachi F-4500 spectrofluorimeter attached with a constant temperature circulating water bath (Polyscience, USA) was employed. Excitation and emission wavelengths and corresponding slit widths were set at 650 nm and 2.5 nm, respectively. A uniform distribution of size of DM-PC particles in aqueous phase was ensured from scattering intensity after repeated dispensing the stock with 100-fold dilution in 20 mM K-phosphate, pH 7.4. All spectrophotometric measurements were done with a Biochrom S2000 diode array spectrophotometer (UK).

2.19. Statistical analysis

Data are expressed as either means or mean ± SEM from a minimum of three experiments, unless otherwise indicated. Statistical analyses were performed with Student's *t*-test, using $p < 0.01$ as a criterion of significance.

3. Results

3.1. Purification of RVV-7

So far, several toxins of RVV were purified using CM-sephadex C-50 chromatography at the initial step where the crude venom was separated into several fractions [5,11,12]. SDS-PAGE revealed the presence of a component of 6–7 kDa of comparable intensities in all these fractions (unpublished observation). Wide distribution of this low Mw component raised the possibility of its association with different fractions and thus, the ion-exchange step was omitted. Taking the advantage of its unusual small size, attempts were made for its separation using Centricon Ym-10 but that resulted in poor yield, possibly either because of its self-association or interaction with other proteins as indicated in CM-Sephadex chromatography [11]. To facilitate dissociation under a moderately denaturing condition, RVV was treated with 4 M urea followed by filtration through Centricon Ym-30. Selection of membrane was based on the assumption that unfolding of all proteins occurred to the same extent without affecting their relative distribution according to size. Here use of Centricon Ym-10 was inappropriate as the apparent Mw of the 6–7 kDa components were likely to be close to 10 kDa after partial unfolding. The filtrate was concentrated and dialyzed to remove urea that appeared as single band in electrophoresis (described later). During purification, sp. activity of RVV-7 as PLA_2 was reduced by 3-fold. This was likely because of removal of other PLA_2 present in the venom (Table 1).

3.2. Purity and molecular weight

A basic protease inhibitor of 6.8 kDa is known to be present in RVV [26]. Since the present protocol for purification of RVV-7 depends primarily on size exclusion, it was of significance

Table 1
Purification of RVV-7

Fractions	Total protein (mg)	Total activity (nmoles/min)	Specific activity (nmoles/min/ mgPLA$_2$)
Crude RVV	60	208000	3470 ± 16
Centricon filtration followed by dialysis	4.5	5400	1210 ± 17

Values are mean ± SEM, $n = 6$.

to be sure that the preparation was free from the above mentioned inhibitor. Homogeneity of RVV-7 has been demonstrated by 15% SDS-PAGE, 10% PAGE containing 8 M urea at pH 4.2 or 8.8, IEF between pH 3–10 and 4–40% gradient PAGE (Fig. 1A–D). In all cases, a single band was observed. Overloading or under loading of RVV-7 did not reveal additional or closely spaced bands in the electro grams. Approximate Mw and p*I* of RVV-7 appeared to be 6.5–7.0 kDa and 9.3 kDa, respectively.

Mass spectrometric analysis of RVV-7 revealed one dominant peak of 7201 Da (M^+, 100% abundance) together with one of 7224 Da probably as ($M + Na^+$, 15%) (Fig. 2). This spectrum confirmed absence of 6.8 kDa protein in the preparation. Purity of the freshly prepared sample was also evaluated from RP– and SE–HPLC as described later (Figs. 3 and 4).

3.3. Aggregation

Peptides and similar molecules form aggregates upon standing, altering their physical and biological properties. RP- and SE-HPLC have been employed to verify aggregation profiles of RVV-7. While a fresh preparation appeared as a single peak of retention time (R_t) 16.64 ± 0.08 min in RP–HPLC, the same sample could be resolved into two peaks of R_t 16.64 ± .08 min and 20.43 ± 0.04 min under identical conditions once it was stored at 30 °C for 3 days (Fig. 3A and B). It appears that the generated component was of higher hydrophobicity than the monomer.

Similarly, RVV-7 when freshly purified, appeared as a single peak from Protein Pak-60 SE-HPLC having $R_t = 10.89 \pm 0.12$ min that corresponded closely to that of aprotinin (Mw 6.2 kDa, $R_t = 11.0 \pm 0.05$ min). When the same sample was stored at 30 °C for 3 days, another peak of $R_t = 8.6 \pm 0.06$ min appeared that corresponded closely to that of cytochrome *C* (Mw 12.5 kDa, $R_t = 8.4 \pm 0.05$ min) (Fig. 4A–C). Thus, formation of a dimer was suggestive. RP– and SE–HPLC profiles collectively indicate purity of RVV-7 and its tendency to form a dimer without generation of an array of multimers.

3.4. Amino acid sequence

Derived amino acid sequence of 20 residues from N-terminal of RVV-7 was LECNKLVDIAYITXPAGKNL. Analysis with EXPASY protein sequence homology search (BLAST) showed a maximum homology of 85% with a cytotoxic component from *Naja melanoleuca* venom that belongs to type IA cytotoxin sub-

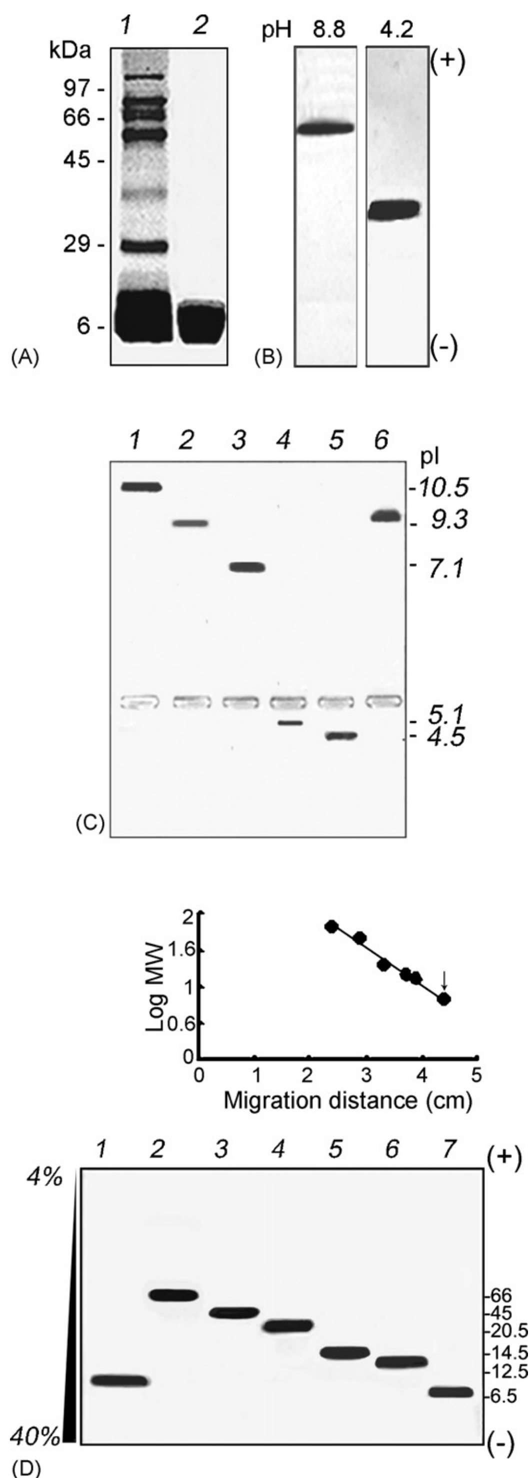

Fig. 1. PAGE profiles of purified RVV-7. (A) 15% SDS-PAGE; lane 1, Crude RVV; lane 2, RVV-7. Position of Mw markers has been indicated at the left. (B) 10% PAGE in presence of 8 M urea at pH 8.8 and 4.2 as indicated. (C) Isoelectric focusing (IEF) of RVV-7 under a linear gradient of pH 3–10; lane 1–5, IEF markers; lysozyme, trypsinogen, myoglobin, α-lactalbumin, and STI, respectively. Lane 6, RVV-7. p*I* of the markers has been indicated at the right. (D) Relative migration of RVV-7 (lane 1) in 4–40% gradient PAGE at pH 4.2 with respect to Mw markers: BSA, ovulbumin, soyabean trypsin inhibitor, lysozyme, cytochrome C and aprotinin (lane 2–7). Respective Mw has been marked at right. Inset: dependence of log Mw *vs.* migration of the markers. The arrow indicates the position of RVV-7 in the plot. Signs in right panel of B and D indicate electrode arrangement.

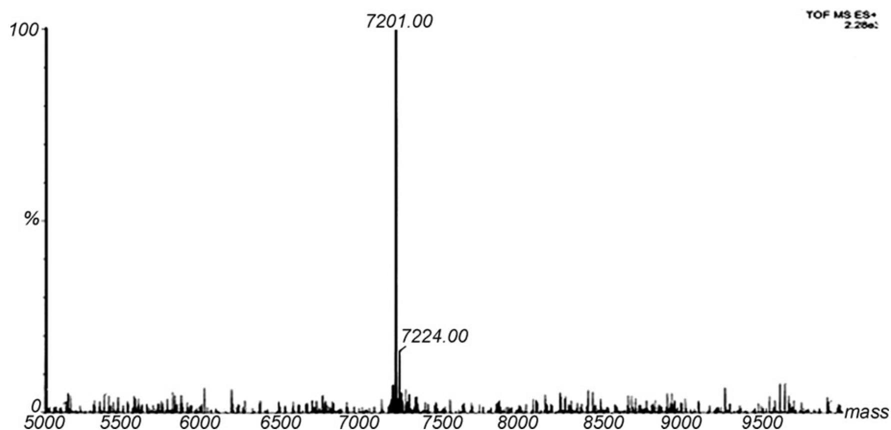

Fig. 2. ESI–MS spectrum of RVV-7 after deconvolution of the original spectrum with maximum entropy minimization—3 algorithm.

family (Fig. 5). Multiple sequence alignment showed that RVV-7 has at least 65% sequence similarity with several low Mw cytotoxins from *Naja sp*. The N-terminal leucine residue is conserved for all the cytotoxins. The conserved regions within this short span of N-terminal 20 residues, which have been demonstrated by conserved sequence search program of EXPASY, are as follows: L–[EK]–C–N–K–L–[IV]–[DP]–[IL]–A–[HY]–X–T–C–P–[AE]–G–K–N–L. This higher degree of similarity with cytotoxic proteins indicated probable cytotoxic property of RVV-7.

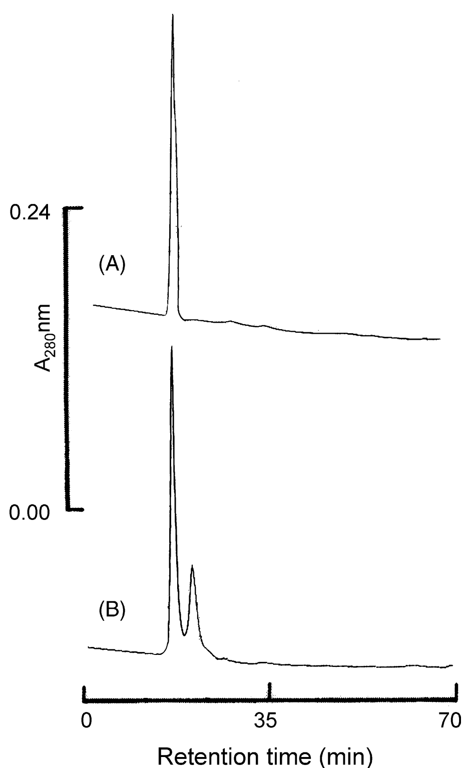

Fig. 3. Reverse-phase HPLC profile of purified RVV-7: (A) freshly purified sample; (B) same sample after 3 days of storage at 30 °C. Fractions were eluted only at 0–5% water-acetonitrile gradient between 10 and 30 min. Nothing appeared as unabsorbed fraction during initial washing for 10 min or 5–100% water-acetonitrile gradient. Description of the experiment has been provided in the text.

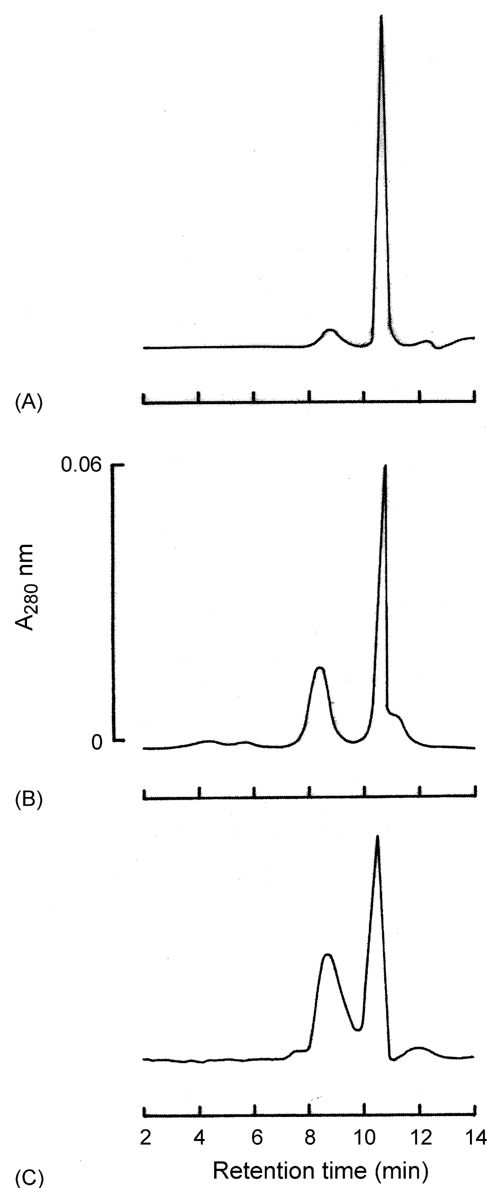

Fig. 4. Size-exclusion HPLC profile of purified RVV-7: (A) freshly purified sample; (B) after storage at 30 °C for 3 days; and (C) after storage under same conditions for 6 days.

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RVV - 7      LECNKLVDIAYITCPAGKNL------------------------------
CX1_NAJME    LECNKLVPIAHKTCPAGKNLCYQMYMVSKSTIPVKRGCIDVCPKSSLLV
CX1_NAJOX    LKCNKLVPIAYKTCPEGKNLCYKMFMMSDLTIPVKRGCIDVCPKNSLLV
CX9_NAJHA    LECNKLVPIAHKTCPEGKNLCYKMFMVSTSTVPVKRGCIDVCPKDSALV
CX1_NAJSG    LKCNKLVPLAYKTCPAGKNLCYKMYMVANKKVPVKRGCIDVCPKKSLLV
CX4_NAJMO    LKCNKLIPIAYKTCPEGKNLCYKMMLASKKMVPVKRGCINVCPKNSALV
CX5_NAJKA    LKCNKLIPLAYKTCPAGKNLCYKMFMVAAPKVPVKRGCIDACPKNSLLV
CX3_NAJNA    LKCNKLIPLAYKTCPAGKNLCYKMFMVSNKTVPVKRGCIDVCPKNSLVL
CX3_NAJKA    LKCNKLIPLAYKTCPAGKNLCYKMFMVSNKTVPVKRGCIDACPKNSLLV
CX2_NAJKA    LKCNKLIPLAYKTCPAGKNLCYKMFMVSNKTVPVKRGCIDVCPKNSLLV
CX1_NAJNA    LKCNKLIPLAYKTCPAGKNLCYKMYMVSNKTVPVKRGCIDVCPKNSLVL
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		Accesion No.	Organism	Function
RVV - 7	----------			
CX1_NAJME	YVCCNTDRCN	sp|P01448|	*N melanoleuca*	Cytotoxic
CX1_NAJOX	YVCCNTDRCN	sp|P01451|	*N oxiana*	Cytotoxic
CX9_NAJHA	YVCCNTDRCN	sp|P01454|	*N haje annulifera*	Cytotoxic
CX1_NAJSG	YECCNTDRCN	sp|P83345|	*N sagittifera*	Cardiotoxic
CX4_NAJMO	YVCCSTDRCN	sp|P01452|	*N mossambica*	Cytotoxic
CX5_NAJKA	YVCCNTDRCN	sp|P24779|	*N kauthia*	Cytotoxic
CX3_NAJNA	YVCCNTDRCN	sp|P24780|	*N naja*	Cytotoxic
CX3_NAJKA	YVCCNTDRCN	sp|P01446|	*N kauthia*	Cytotoxic
CX2_NAJKA	YVCCNTDRCN	sp|P01445|	*N kauthia*	Cytotoxic
CX1_NAJNA	YECCNTDRCN	sp|P01447|	*N naja*	Cytotoxic

Fig. 5. Sequence alignment of RVV-7, up to 20th residue, with whole sequences of 10 toxins that scored a minimum of 44 in EXPASY sequence similarity search (BLAST). The sequences were aligned using CLUSTAL W (1.82) program of EXPASY. ⋆ indicates identical residues, : indicates different residues, unmarked residues differ only in RVV-7.

3.5. Purifcation of CX3 from Naja kauthia venom

CX3 from *N. kauthia* venom being one of the low Mw cytotoxins that have sequence homology with RVV-7 was purified to homogeneity for comparative studies. In the first step using CM-sephadex C-50, CX3 appeared as an unresolved component after application of salt gradient (Fig. 6A). Pooled fractions were concentrated and applied to Sephadex G-75 chromatography where two major peaks were resolved (Fig. 6B). The first fraction appeared as a single band of 6 kDa, a Mw similar to that obtained from size exclusion column. Finally, CX3 was identified by ESI mass spectrometric analysis that matched with its reported Mw of 6708 [19].

3.6. Cytotoxic activity

Cytotoxic effect of RVV-7 on B16F10 melanoma cells has been illustrated in Fig. 7. It shows that RVV-7 up to 10^{-7} M was ineffective to the cells, while at 10^{-6} M, it killed about 20% of the cell population. A sharp drop in viability was observed at 10^{-5} M of RVV-7. The concentration of RVV-7 that killed 50% of cells (EC_{50}) was found to be $2.56 \pm 0.51 \times 10^{-6}$ M.

3.7. PLA_2 activity

Two PLA_2s viz. VRV-PL-VI and VRV-PL-VIIIa from RVV have been reported [27,28]. Strong PLA_2 activity of RVV, as mentioned in Table 1, leaves the possibility of additional PLA_2s in it. RVV-7 showed strong PLA_2 activity using DM-PC aggregates as substrate. Linear dependence of hydrolysis rate with enzyme and substrate concentration was consistently observed between 2 and 15 μM of RVV-7 and 0.148 and 0.885 μM of DM-PC (Fig. 8A). Observed kinetic lag remained inversely but linearly proportional to the enzyme and substrate concentration. These features were at par with the crude venom activity [20]. Specific activity was found to be 1210 ± 17 nmoles/min/mg of enzyme against 3470 ± 16 nmoles/min/mg for crude venom (Table 1). The sp. activity of RVV-7 being lower than that of crude RVV, it is likely that some PLA_2 of higher sp. activity have been removed during its purification.

3.8. Correlation between cytotoxic and PLA_2 activities

PLA_2s, a well studied class of enzymes, show no significant sequence homology with RVV-7 except for very short stretches (data not shown). On the other hand, N-terminal sequence homologues like low Mw cytotoxins from *Naja* sp. were not reported to have PLA_2 activities. CX3 from *N. kauthia* showing 65% sequence similarity with RVV-7 was selected as a representative of the cytotoxins and evaluated for its PLA_2 activity. Fig. 8B shows that CX3 was indeed associated with PLA_2 activity. The rate of hydrolysis with 0.295 μM of DM-PC was found to be linear with 1.5–7.5 μM of CX3. Inverse linear relation between kinetic lag and enzyme concentration was also observed. Sp. activity of the enzyme was higher (3703 ± 18.04 nmoles/min/mg of enzyme) than that of RVV-7.

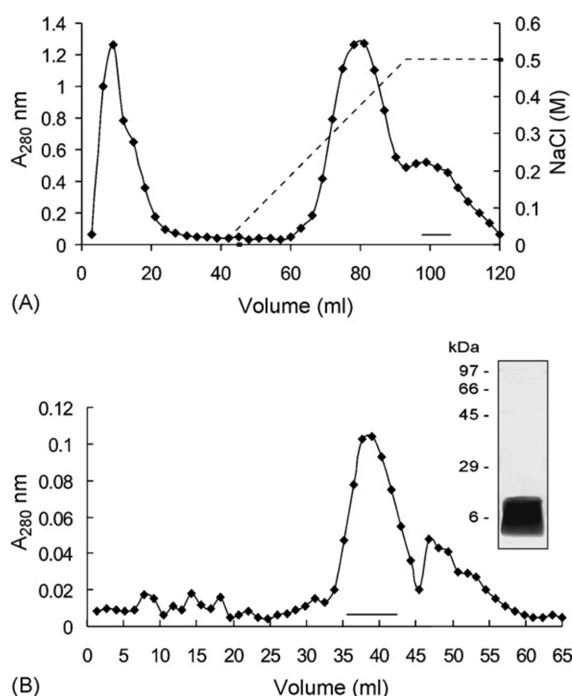

Fig. 6. Chromatographic separation of CX3 from *Naja kauthia* venom. (A) Venom dissolved in 20 mM K-phosphate, pH 7.4 was loaded on CM Sephadex C-50 column. Unabsorbed fractions were eluted with the same buffer. The dashed line indicates NaCl concentration applied as gradient. Elution of proteins were monitored at 280 nm. SDS-PAGE indicated that the fractions marked contains proteins of 6 kDa and were pooled, dialyzed against the same buffer and concentrated by dilysis against poly ethylene glycol. (B) Sephadex G-75 gel filtration profile of the pooled fraction. SDS-PAGE indicated that the fractions marked contains proteins of 6 kDa and were pooled. Inset: SDS-PAGE profile of the pooled fraction. Position of Mw markers have been indicated.

The active site His_{48} is conserved in all venom PLA_2s [29] and is the principle component of the catalytic diad [30]. Therefore, effect of modification of histidine residue/s of RVV-7 by DEPC was evaluated. From spectrophotometric measurement, it was observed that 0.85 ± 0.08 mol of histidine/mole of RVV-7 was modified. This single histidine modification led to concomitant loss of $99.2 \pm 0.4\%$ PLA_2 activity and $92.52 \pm 3.5\%$

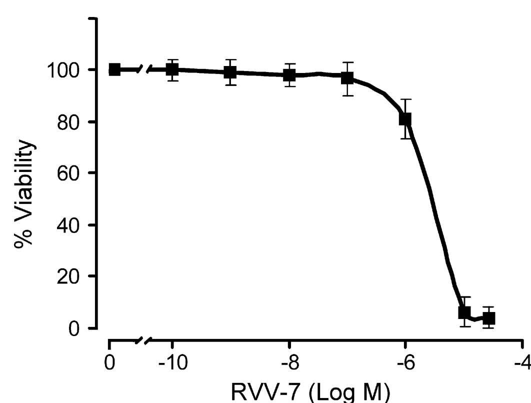

Fig. 7. Cytotoxic response of B16F10 culture to RVV-7. Cells were exposed to indicate concentrations of RVV-7 for 24 h. Cell viability was assayed using MTT. Results are expressed as percentage viability relative to untreated controls. The horizontal bars represent mean ± SEM for triplicate cultures.

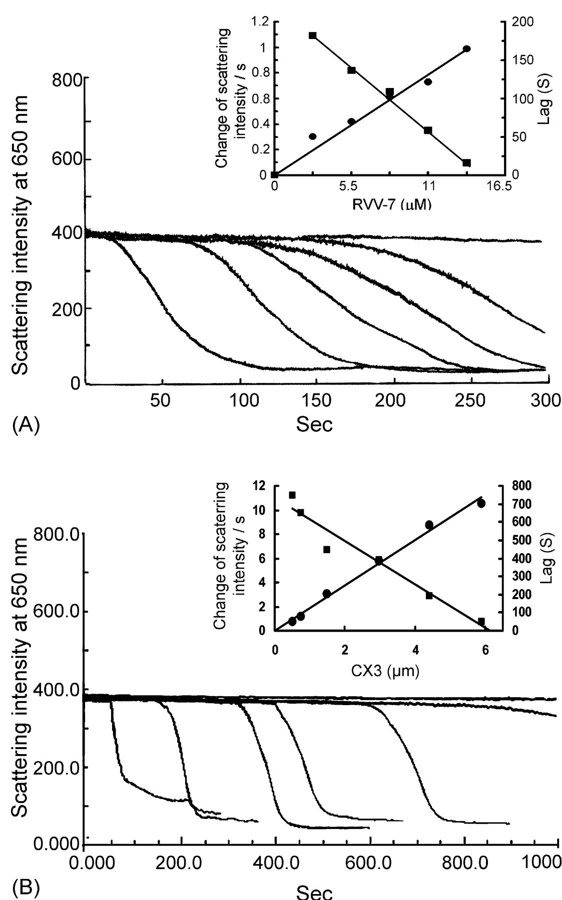

Fig. 8. Phospholipase A_2 assay of RVV-7 and CX3 through kinetic measurement of DM-PC hydrolysis by light scattering at pH 7.4 in presence of 8 mM $CaCl_2$. The horizontal linear portion of the curve shows initial lag of reaction. Rate of reaction was measured from linear decrease of scattering intensity. The continuous straight spectrum shows stability of DM-PC in absence of enzyme. (A) Kinetics of DM-PC hydrolysis by different concentrations (0–80 μM) of RVV-7. Lowest lag indicates the reaction of highest RVV-7 concentration; (B) phospholipase A_2 activity of different concentrations (0–6 μM) of CX3. Insets: plot of reaction rate (●) and initial lag (■) *vs.* protein concentration.

of cytotoxic activity. Incubation of the inactive enzyme with 0.024 N hydroxylamine, HCl reversed back ∼70% of the PLA_2 activity indicating that the modification was directed at histidine and not at tyrosine. PLA_2 active site of CX3 has also been found to contain histidine. Chemical modification of CX3 with DEPC resulted in $98.5 \pm 0.3\%$ loss of PLA_2 activity (result not shown). Simultaneous inactivation of PLA_2 and cytotoxic activity of RVV-7 was observed after AVS treatment. Thus, these two functional sites appear to be overlapping or very close to each other or it could be possible that cytotoxicity is the manifestation of PLA_2 activity,for example, a low degree of residual PLA_2 activity might cause cytotoxicity provided enough time is allowed.

These two sites were distinguished by thermal denaturation and withdrawal of Ca^{2+} by chelation (Table 2). Thermal exposure at 50 °C for 3 h rendered RVV-7 inactive up to 80% in terms of PLA_2 activity without affecting cytotoxicity, though this cannot explain independent sites for these activities. Enzymatic activity was dissociated from cytotoxicity by chelation of Ca^{+2} with 2 mM EGTA in both PLA_2 and cytotoxicity assay. In absence of Ca^{2+}, RVV-7 was enzymatically inactive with-

Table 2
Effects on different activities of RVV-7 upon modification

	Cytotoxicity (%)	PLA_2 activity (%)	Trypsin inhibition (%)
RVV-7[a]	100	100	100
His modification	7.5 ± 3.2*	nd	92.6 ± 0.8
AVS treatment	11.4 ± 2.8*	5.6 ± 0.6*	98.5 ± 0.4
Treatment at 50 °C for 3 h	98.3 ± 3.6	27.36 ± 0.86*	–
Ca^{+2} chelation	95.02 ± 2.58	nd	99.21 ± 0.55
Lys modification	95.92 ± 1.85	94.5 ± 0.56	1.55 ± 0.68*
Arg modification	68.27 ± 4.85**	97.08 ± 0.26	3.56 ± 0.08*

(–), Not determined; nd, not detectable; values are mean ± SEM, $n = 3$.
[a] Activity of 2 nM, 10 μM and 15 μM of RVV-7 was assumed as 100% for trypsin inhibition, PLA_2 and cytotoxic activity, respectively.
* $p < 0.001$ compared to RVV-7.
** $p < 0.01$ compared to RVV-7.

out altering cytotoxic activity (Table 2). Complete retention of cytotoxicity in a medium devoid of Ca^{2+}, where the toxin is enzymatically inactive, leads indication towards independent functional sites.

3.9. Trypsin inhibition

Protease inhibitors of low Mw are known from RVV [26]. One such trypsin inhibitor inactivates blood coagulation factor-X activator *in vitro* and was speculated to be important for stabilization of the proteases within venom gland [31]. Therefore, inhibitory property of RVV-7 on serine and cysteine proteases were verified using azocasein, azoalbumin or TAME as substrates. A sigmoidal dependence of inhibition was obtained with 2.0 nM of trypsin in the presence of 0.38–2.66 nM of RVV-7 using azocasein as substrate (Fig. 9A). It was calculated that under such conditions, 1.84 nM of RVV-7 could completely inhibit the same concentration of trypsin. Kinetic studies of trypsin inhibition showed that RVV-7 followed competitive inhibition and thus, interact at the catalytic site of trypsin (Fig. 9B). Corresponding K_i was 1.18×10^{-6} M. RVV-7 also inhibited trypsin against azoalbumin and TAME hydrolysis (data not shown). Inhibition of esterolysis indicated that RVV-7 specifically blocks active site serine residue of trypsin apart from preventing sub-site interactions with protein substrates.

It is invariably an arginine or lysine residue of a trypsin inhibitor that lures trypsin to form a dead end complex [32]. Thus, modification of surface arginine and lysine residues of such molecules is likely to affect inhibitory property. When group specific reagents modified these amino acids, it resulted in a complete loss of trypsin inhibitory property. These data indirectly support RVV-7 as a trypsin inhibitor. Trypsin inhibitory property of 20 ng of RVV-7 remained unaltered after pretreatment of 350 μl of AVS that was capable of neutralizing 22 μg of RVV-7. But it led to complete inactivation of PLA_2 and cytotoxic activities (Table 2). Surface lysine/arginine modification did not alter PLA_2 activity though partial inactivation of cytotoxicity of RVV-7 was observed. This observation indicated that trypsin inhibition site might be different from antibody interaction, PLA_2 and cytotoxicity expression sites.

RVV-7 was unable to inhibit other proteases like chymotrypsin (serine protease) and cysteine proteases like bromelain, papain and collagenase (data not shown). RVV-7, on the other hand, was found to be resistant against proteolysis by bromelain, chymotrypsin and trypsin for at least 16 h at 37 °C.

3.10. Tumor inhibition

Tumor inhibitory effect of RVV-7 was assayed on BLJ6 mice using B16F10 cells. These cells, either pretreated or untreated with RVV-7, were applied to mice and tumors were allowed

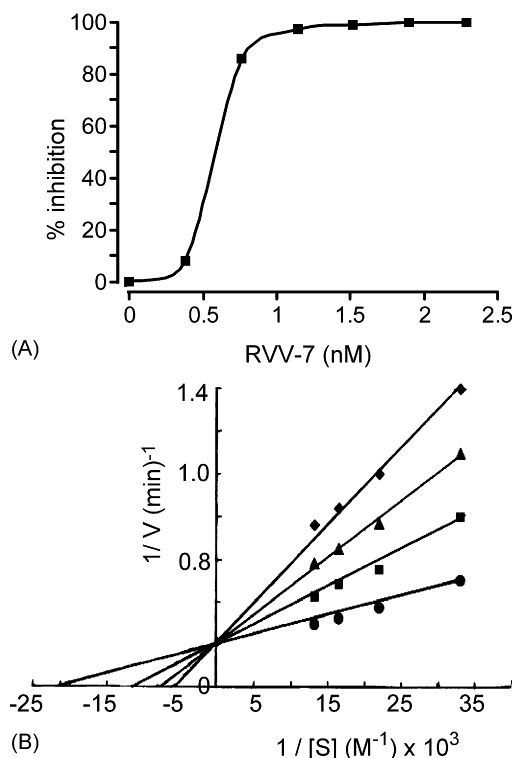

Fig. 9. Effect of RVV-7 on proteolytic activity of Trypsin on azocasein at pH 7.4. (A) Inhibition of trypsin by RVV-7. Proteolytic activity was assayed after pre-incubation of 2.05 nM of trypsin with increasing concentrations of RVV-7. Results expressed as percentage of inhibition with respect to activity of untreated trypsin. (B) Double-reciprocal plots for kinetic inhibition of trypsin by RVV-7. Assays were run in presence of 11 μM (♦), 5.5 μM (▲), 2.75 μM (■) and 0 μM (●) of RVV-7. The intercept on the $1/v$ axis in these double reciprocal plots indicates competitive inhibition. Values are means of three independent experiments.

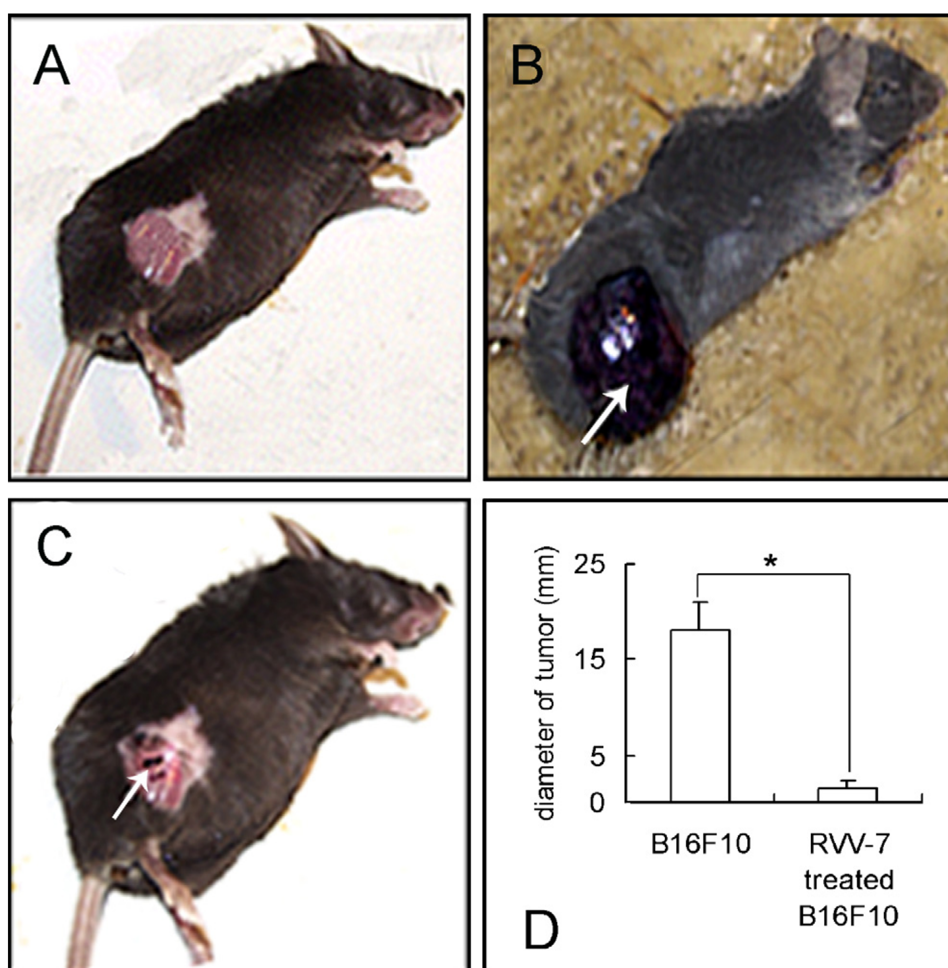

Fig. 10. Inhibition of tumor development *in vivo* by RVV-7. (A) mice injected with DMEM only used as negative control; (B) development of tumor in mice injected with B16F10 melanoma cells (10^5). This was considered as positive control; (C) reduced development of tumor in mice injected with B16F10 melanoma cells (10^5) pretreated with 30 μg/ml of RVV-7. Arrow indicates the position of tumor growth; (D) histogram represents comparative development of tumor by RVV-7 treated B16F10 and untreated B16F10 melanoma cells. Values are mean ± SEM, $^{*}p < 0.01$ of six experiments.

to grow for 21 days. Post mortem examination revealed that the cells treated with RVV-7 were able to develop tumor of 1.5 ± 0.5 mm diameter as compared with 18.4 ± 3.6 mm for the untreated group ($n = 10$). No tumor was observed in control sets devoid of B16F10 cells. Fig. 10A–C shows best illustrations of such sets and Fig. 10D summarizes the tumor inhibitory effect of RVV-7 under the experimental conditions.

4. Discussion

A basic cytotoxic PLA_2 of 7.2 kDa, named as RVV-7, has been isolated to homogeneity from Russell's viper venom of eastern Indian origin (Figs. 1 and 2). Many myotoxins or cytotoxins from viper venoms are reported to be phospholipase A_2 [33,34]. RVV-7 showed Ca^{2+} dependence and involvement of a histidine residue in lipase activity similar to other PLA_2s [35]. PLA_2s are usually dimer in solution [36] and under certain physiological conditions tend to oligomerize [37]. Freshly prepared RVV-7, however, exists as monomers that dimerize upon storage at 30 °C (Figs. 3 and 4). Its cytotoxic activity is in agreement with the homology of its N-terminal amino acid sequence with other snake venom cytotoxins (Fig. 5). Although RVV-7 have some common characters of PLA_2s reported so far, quite unexpectedly, it did not show any significant sequence homology with the known PLA_2s. The reverse possibility that venom cytotoxins might be PLA_2s became apparent when CX3 from *N. kauthia* showed PLA_2 activity similar to RVV-7.

Venom PLAs have been divided into classes I, II and III, based on their amino-acid sequence and distribution of disulphide bondings [38]. Size of these enzymes vary between 12 and15 kDa [35]. Of these, only myotoxin-I from *B asper* is 10.7 kDa and is likely to be the smallest PLA_2 known so far [39]. From the comparative crystallographic structural analysis of these venom PLA_2s, it is evident that the major structural motif is formed by the two antiparallel disulphide linked helices in conjunction with the Ca^{2+} binding loop [35]. The other motif is the β-wing that form a part of homodimer interface in PLA_2 homologues of *Bothrops asper* venom [40]. The entire length of around 143 residues from N-terminal is utilized to accommodate these two motifs along with N-terminal amphiphilic helix that

direct the substrate towards catalytic site. Assuming average Mw of amino acids to be 100, merely 72 amino acid length of RVV-7 seem to be insufficient to conform those structural motifs. Therefore, alternative structures with similar active site conformation might be the possible way to carry out phospholipid hydrolysis. Subject to its existence, a new class of sequentially homologous cytotoxins of low Mw with PLA_2 activity may emerge.

Snake venoms are often good sources of low Mw protease inhibitors [26,41]. RVV-7 interacts with trypsin at 1:1 molar ratio leading to inactivation of proteolytic and esterolytic activities. Its competitive nature of inhibition indicates that it reversibly interacts at the catalytic site of trypsin (Fig. 9). RVV-7 appears to be resistant against proteolysis as proteases of varying specificity were unable to digest it.

A group of low Mw proteins from venoms called 'disintegrins' is known to inhibit tumor growth, angiogenesis and metastasis [42]. Comparable size of RVV-7 led to search similar function in it. It was observed that pretreatment of B16F10 melanoma cells by RVV-7 at sub-lethal dose significantly inhibited growth of tumor in BLJ6 mice (Fig. 10). This anti-tumor activity although a very preliminary observation, may indicate 'disintegrin' like function of RVV-7 that antagonize platelet aggregation suppressing both metastasis and solid tumor growth [43]. Pretreatment with RVV-7 may alter cell–cell adhesion property, which in turn affects three-dimensional growth of tumor mass. However, as tumor inhibition is a multifunctional process with lots of ambiguity till date, other mechanisms may come into play as well.

Apart from their primary catalytic function, snake venom PLA_2s often display additional pharmacological activities like hemorrhage [44], myotoxicity [34] and neurotoxicity [45]. Each pharmacological effect is proposed to be due to the presence of specific sites on the PLA_2 molecule [46]. It is likely that those specific sites have high-affinity receptors in specific target tissues that mediate the pathophysiological responses [47]. Recently, existence of different sites of a PLA_2 from viper venoms has been confirmed after dissociation of enzymatic activity from toxic property by chemical modifications [48,49]. Effects of RVV-7 on cytotoxic and PLA_2 activities upon modification of surface lysine and histidine residues are suggestive of such a notion.

Proteins, either as substrate or inhibitor, interact with the catalytic site of trypsin through arginine or lysine residues. As modification of these surface residues independently abolished trypsin inhibitory activity, close proximity of these residues on RVV-7 is likely. Moreover, unaltered trypsin inhibitory property after histidine modification leads to the speculation that this site is different from PLA_2 catalytic site. On the other hand, AVS inhibited PLA_2 and cytotoxic activities do not affect inhibition of trypsin. This observation indicated that trypsin inhibitory site and epitopes might be different. Coexistence of different functional sites within a small protein like RVV-7 might accommodate complex clinical manifestations of Russell's viper ophidism. Furthermore, multifunctionality of RVV-7 along with the unreported PLA_2 activity of a well known low Mw cytotoxin (CX3) from *N. kauthia* may strengthen exploration of novel property of known proteins from the pool of snake venoms.

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