Research paper

Structural and functional characterization of complex formation between two Kunitz-type serine protease inhibitors from Russell's Viper venom

Ashis K. Mukherjee [a, c, *], Sumita Dutta [a], Bhargab Kalita [a], Deepak K. Jha [b], Pritam Deb [b], Stephen P. Mackessy [c]

[a] Microbial Biotechnology and Protein Research Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India
[b] Advanced Functional Material Laboratory, Department of Physics, Tezpur University, Tezpur 784028, Assam, India
[c] School of Biological Sciences, University of Northern Colorado, Greeley, CO 80639-0017, USA

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ABSTRACT

Snake venom Kunitz-type serine protease inhibitors (KSPIs) exhibit various biological functions including anticoagulant activity. This study elucidates the occurrence and subunit stoichiometry of a putative complex formed between two KSPIs (Rusvikunin and Rusvikunin-II) purified from the native Rusvikunin complex of Pakistan Russell's Viper (*Daboia russelii russelii*) venom (RVV). The protein components of the Rusvikunin complex were identified by LC-MS/MS analysis. The non-covalent interaction between two major components of the complex (Rusvikunin and Rusvikunin-II) at 1:2 stoichiometric ratio to form a stable complex was demonstrated by biophysical techniques such as spectrofluorometric, classical gel-filtration, equilibrium gel-filtration, circular dichroism (CD), dynamic light scattering (DLS), RP-HPLC and SDS-PAGE analyses. CD measurement showed that interaction between Rusvikunin and Rusvikunin-II did not change their overall secondary structure; however, the protein complex exhibited enhanced hydrodynamic diameter and anticoagulant activity as compared to the individual components of the complex. This study may lay the foundation for understanding the basis of protein complexes in snake venoms and their role in pathophysiology of snakebite.

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1. Introduction

Russell's Viper (RV; *Daboia russelii russelii*) bites are responsible for a heavy toll on human life in southeast Asian countries, and RV is considered as a category I medically important snake in India [1]. Several biological functions of snake venom components are initiated and executed by biochemical interactions between components in the form of a protein complex [2—7]. The formation of protein complexes in snake venom may eliminate non-specific binding, in addition to enhancing binding to the pharmacological target molecule(s), thus enhancing the toxicity of components of the protein complex [3,4].

Our studies have shown that Rusvikunin, a 6.9 kDa Kunitz-type serine protease inhibitor isolated from the venom of *D. r. russelii* of Pakistan origin [8], forms a basic complex (Rusvikunin complex) with a 7.1 kDa peptide, Rusvikunin-II, from the same venom [9]. It has been demonstrated that the pharmacological properties of Rusvikunin complex are more pronounced as compared with individual components of this complex, and its natural biological role likely involves subduing agile mammalian prey [9]. However, this

complex was insufficiently characterized in structural terms, and little evidence was presented to support a structural association of the components of the complex. Furthermore, our studies also suggested that the Rusvikunin complex contributes to the overall toxicity of RV bites [9], thus warranting further characterization. Therefore, in this study we identify the protein components of the Rusvikunin complex by LC-MS/MS analysis. Furthermore, spectrofluorometric, classical gel-filtration, equilibrium gel-filtration, circular dichroism, dynamic light scattering, RP-HPLC, and SDS-PAGE analyses (under reducing and non-reducing conditions) were performed to examine non-covalent interactions, if any, among the components of this complex. Results show that the stoichiometry of the interaction of Rusvikunin with Rusvikunin-II is at a ratio of 1:2, and this interaction resulted in augmentation of anticoagulant activity.

2. Materials and methods

Rusvikunin complex was isolated from Russell's Viper (*D. r. russelii*) venom by using previously described procedures [8,9]. Proteomics grade trypsin was procured from Promega, USA. All other chemicals were of analytical grade and obtained from Sigma-Aldrich, USA.

2.1. LC-MS/MS analysis of the Rusvikunin complex

For peptide mass fingerprinting (PMF) analysis of trypsin digested peptide fragments of the Rusvikunin complex using LC-MS/MS, our previously described procedures were followed [10,11]. Briefly, 40 μg of the lyophilized sample (Rusvikunin complex), after reduction and alkylation, was subjected to in-solution digestion with trypsin overnight at 37 °C. The digested peptides were dried, reconstituted in 15 μl of the 0.1% (v/v) formic acid and were subjected to RP-nanoHPLC-MS/MS analysis. The ion source was ESI (nano-spray), fragmentation modes were collision induced dissociation (y and b ions), MS scan mode was FT-ICR/Orbitrap, and MS/MS scan mode was linear ion trap. The tryptic fragments were identified using PEAKS 7.0 search engine against Viperidae snake venom online databases. For the purpose of identification, only peptides and proteins showing -10lgP score (PEAKS score) of ≥ 30.3 and ≥ 20, respectively, were considered. Furthermore, at least one high confidence peptide (unique peptide) of the complex was set as a prerequisite to identify the protein(s)/peptide(s).

2.2. Assay of enzyme activity and anticoagulant property

The following chromogenic substrates (final concentration 0.2 mM) were examined for amidolytic activity following a previously described procedure [12]: N-Benzoyl-Pro-Phe-Arg-*p*-nitroanilide hydrochloride (substrate for plasma kallikrein), *N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (substrate for trypsin), D-Val-Leu-Lys-*p*-nitroanilide dihydrochloride (substrate for plasmin), N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide acetate salt (substrate for factor Xa), N-(*p*-Tosyl)-Gly-Pro-Arg-*p*-nitroanilide acetate and N-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide hydrochloride (substrates for thrombin), and N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (substrate for chymotrypsin).

Proteolytic activity against bovine serum albumin, bovine serum gamma globulin, human plasma fibrinogen (fraction I), and fibrin was determined by biochemical assay [12,13]. The reaction mixture was incubated for 6 h at 37 °C. One unit (U) of protease activity was defined as 1.0 μg of tyrosine equivalent liberated per min by the enzyme. Fibrinogen degradation products were separated by 12.5% SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and destained in methanol: acetic acid: water (40:10:50) to visualize the proteins and degradation fragments in the gel. Fibrinogen clotting activity was assessed using a BBL-Fibrinosystem fibrometer [9].

Esterolytic activity was assayed by a spectrophotometric method as described previously using Nα-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) and Nα-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates [12]. One unit of TAME or BAEE-esterase activity was defined as an increase in absorbance of 0.01 at 244 or 254 nm, respectively during the first 5 min of the reaction at 37 °C. For every experiment, a control was run in parallel where enzyme was replaced by an equivalent volume of buffer. Activity was expressed as units of TAME or BAEE activity/mg protein.

PLA$_2$ activity was assayed using a sPLA$_2$ assay kit (Cayman Chemical). The reaction was initiated by adding 0.05 μg/ml Rusvikunin complex or Rusvikunin (in 10 μl volume) to a reaction mixture containing 10 μl of 10 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) in 0.4 M Tris-HCl, pH 8.0 and 200 μl of 1.66 mM 1,2-dithio analog of diheptanoyl phosphatidylcholine, and the final volume was brought to 225 μl with assay buffer (25 mM Tris-HCl, pH 7.5, containing 10 mM CaCl$_2$, 100 mM KCl, and 0.3 mM Triton X-100). The absorbance was read every min at 414 nm using a microplate reader (Multiskan GO, ThermoScientific, USA) and at least five time points were obtained. As a positive control, bee venom PLA$_2$ (supplied with the kit) was used instead of RVV-PLA$_2$. From the linear portion of the curve, ΔA_{414}/min was determined and enzyme activity was calculated following the instructions given in the kit. One unit of PLA$_2$ activity was defined as enzyme catalyzed hydrolysis of one μmol of diheptanoyldithio-PC per min at 25 °C. To determine the dose-dependent PLA$_2$ activity, the reaction mixture was incubated with graded concentrations (0.05–0.5 μg/ml) of Rusvikunin complex and the enzyme activity at each concentration was calculated as stated above.

For enzyme (protease, PLA$_2$) inhibition assay, 1.0 μg Rusvikunin complex was incubated with one of the following inhibitors (final concentration): benzamidine-HCl (0.5–5.0 mM), aprotinin (100 μM), dithiothreitol (5–10 mM), diNa-EDTA (5–10 mM), heparin(100 IU/ml), soybean trypsin inhibitor (100–150 μg), α_2-macroglobulin (100 μg), antithrombin-III (100 μg), TPCK (100 μM), TLCK (100 μM), iodoacetamide (5 mM), *p*-bromophenacyl bromide (5 mM), and 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (5 mM) for 30 min at 37 °C. The remaining enzyme (protease or PLA$_2$) activity after treatment with inhibitors was expressed as percent activity remaining relative to the enzyme activity in the absence of inhibitors [12–14].

Preparation of platelet-poor plasma (PPP) and the assay of recalcification time of PPP in the presence of graded concentrations (1.5–15 μg/ml in 20 mM Tris-HCl, pH 7.4) of Rusvikunin, Rusvikunin-II or Rusvikunin complex were conducted as described previously [9]. A control was run in parallel where PPP was incubated with the Tris buffer only. One unit of anticoagulant activity was defined as an increase of 1 s of clotting time of PPP (treated) compared with clotting time of control PPP (incubated with buffer only) [9,15].

2.3. Determination of interactions among the components of the Rusvikunin complex by spectrofluorometric analysis

The protein-protein interaction was studied by spectrofluorometric titration [10,11] using a fluorescence spectrometer (LS 55, Perkin Elmer, Palo Alto, CA). The interaction between Rusvikunin and Rusvikunin-II was determined by incubating a fixed concentration of Rusvikunin-II (400 nM) with different concentrations of Rusvikunin (40–800 nM) for 10 min at ~23 °C [4]. The fluorescence spectra were measured as above and dissociation constant (*Kd*) for

the binding of Rusvikunin with Rusvikunin-II was determined by the one site-specific binding model (Eq. (1)).

Before determining the fluorescence spectrum, a fixed concentration (600 nM) of Rusvikunin-II (the major protein component of the Rusvikunin complex) was incubated with different concentrations (6-36 mM) of serine protease RV-FVP$_\alpha$ [10] or anticoagulant PLA$_2$ (RVAPLA$_2$) enzyme [14] previously purified from the same RVV for 10 min at ~23 °C [10,14]. In another set of experiments, Rusvikunin-II, RV-FVP$_\alpha$ and RVAPLA$_2$ were mixed in different molar ratios and incubated for 10 min at ~23 °C before the measurement of fluorescence spectrum. The interaction between RV-FVP$_\alpha$ and RVAPLA$_2$ at 1:1, 1:2, and 2:1 (mol:mol) ratio was then determined spectrofluorometrically. In every experiment, the spectrum of individual protein was also determined.

The fluorescence spectra were acquired at an excitation wavelength of 280 nm, excitation and emission slits set at 5 nm at room temperature (~23 °C). The emission spectra were recorded from 300 to 400 nm. As a control, the fluorescence spectrum of individual protein was also determined and compared with the relative intensity of the fluorescence spectra due to interactions between Rusvikunin-II with protease or PLA$_2$ enzyme. Wavelength shifts were analyzed by taking the mean at two-thirds heights of spectra. For determining the dissociation constant (Kd) for the binding of Rusvikunin-II with RV-FVP$_\alpha$, the following one-site binding model was considered:

$$\Delta F = \frac{\Delta F_{max} \times C}{K_d + C} \qquad (1)$$

where $\triangle F$ is the change in fluorescence intensity of Rusvikunin-II in the presence of RV-FVP$_\alpha$, $\triangle Fmax$ is the maximum change in fluorescence intensity of Rusvikunin-II when saturated with RV-FVP$_\alpha$, and C is the concentration of RV-FVP$_\alpha$. The dissociation constant (Kd) for the binding of RV-FVP$_\alpha$ to Rusvikunin-II was calculated using the Graph Pad Prism 6.03 software (Graph Pad Software, CA, USA).

2.4. Determination of interaction between Rusvikunin and Rusvikunin-II by classical gel-filtration and equilibrium gel-filtration chromatography followed by RP-HPLC analysis of GF peaks

The stoichiometry of interaction between Rusvikunin and Rusvikunin-II in solution was determined by performing a series of equilibrium gel-filtration chromatography experiments [16, with slight modification]. Briefly, a Shodex KW-803 HPLC gel-filtration column (8 mm × 300 mm) coupled to a Dionex 3000 Ultimate UHPLC (Thermo Scientific, USA) was equilibrated with 50 mM sodium phosphate buffer, pH 7.0 containing 150 mM NaCl (buffer A). The column was injected with Rusvikunin (7.5 nM) or Rusvikunin-II (7.5 nM) and the elution of individual protein at a flow rate of 1.0 ml/min was monitored at 215 nm. The gel-filtration column was washed with five volumes of buffer A and then it was equilibrated with two volumes of buffer B (buffer A containing 7 nM Rusvikunin II). The column was injected with different concentrations (1.4-35.0 nM) of Rusvikunin, and the elution of proteins with buffer B at a flow rate of 1.0 ml/min at 4 °C was monitored at 215 nm. The gel-filtration column was calibrated with the following molecular markers-aprotinin (6.5 kDa), cytochrome C (12 kDa), carbonic anhydrase (32 kDa), and albumin (66 kDa). The molecular mass of protein complexes eluted from the gel-filtration column was determined from the above calibration curve.

The major protein peaks eluted from the gel-filtration column were lyophilized and then injected onto an Acclaim 300 C$_{18}$ RP-HPLC column (2.1 × 150 mm, 3 μm) pre-equilibrated with 95% solvent A (Milli Q H$_2$O with 0.1% TFA) and 5% solvent B (90% acetonitrile with 0.1% TFA). Bound proteins were eluted with a gradient of 5-100% solvent B from 2 to 16 min at a flow rate of 0.5 ml/min at room temperature (~23 °C), and protein elution was monitored at 215 nm. In a separate set of experiments, the RP-HPLC elution profiles of different concentrations (1-20 nM) of Rusvikunin and Rusvikunin-II were also determined as above. From a standard curve of absorbance at 215 nm vs protein (Rusvikunin or Rusvikunin-II) concentrations, the concentration of Rusvikunin or Rusvikunin-II present in a particular gel-filtration peak was determined. The actual concentration of Rusvikunin-II eluted from RP-HPLC column was determined by subtracting the concentration of Rusvikunin-II (7 nM) present in buffer B used for equilibrium gel filtration.

The stability of complex formed between Rusvikunin and Rusvikunin-II was further confirmed by classical gel-filtration chromatography. Purified Rusvikunin and Rusvikunin-II were incubated at a 1:2 (mol:mol) ratio at 4 °C overnight and then subjected to gel-filtration chromatography on a Shodex KW-803 column coupled to Dionex 3000 Ultimate UHPLC system. The pre-equilibration and elution of protein(s) were done with buffer A at a flow rate of 1.0 ml/min and protein elution was monitored at 215 nm. The protein peaks were collected and subjected to RP-UHPLC analysis as stated above.

2.5. Far-UV circular dichroism (CD) spectroscopic study

The Rusvikunin and Rusvikunin II (dissolved in 50 mM sodium phosphate buffer, pH 7.0 containing 150 mM NaCl) were incubated at 1:1 and 1:2 molar ratio for 2 h at 37 °C, allowing for complex formation. The far UV-CD spectra (190-250 nm) of Rusvikunin, Rusvikunin-II and their complexes were monitored on a JASCO J-815 spectropolarimeter (Tokyo, Japan) at room temperature (25 °C) in a quartz cuvette with a path length of 1 cm [8,9]. The scan speed of the spectra was maintained at 100 nm/min. Four scans were recorded for each spectrum, their values were averaged and baseline was subtracted. The CD spectra were expressed in ellipticity (θ, in millidegrees).

2.6. Dynamic light scattering (DLS) experiment to study complex formation

To study the complex formation between Rusvikunin and Rusvikunin II, dynamic light scattering experiments were performed at 37 °C using a NanoPlus, Zeta Potential and Nano Particle Analyzer (Particulate Systems, USA). Rusvikunin, Rusvikunin-II and Rusvikunin: Rusvikunin-II (mixed in 1:2 molar ratio and then incubated for 2 h at 37 °C) were prepared in 20 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.0. The solution was filtered with a 0.2 μM syringe filter before measuring the protein size by DLS analysis. A diode laser of 660 nm with an output power of 70 mW was used as a light source. The measurement angles for the size determination of the particles were 160° and 15° at 37 °C. The number of acquisitions was maintained at 70 for each run. The hydrodynamic radius (R$_H$) of each molecule was obtained from the measured translational diffusion coefficient (D$_T$) using the Stokes-Einstein relation:

$$D_T = k_B T / 6\pi \eta R_H \qquad (2)$$

In the above equation, k$_B$ is the Boltzmann constant, T is the temperature in Kelvin, and η is the viscosity of the solvent. The particle size and size distribution were obtained using the regularized CONTIN method [17].

3. Results and discussion

Fractionation of Rusvikunin complex (see Supplementary Fig. S1) on a RP- C_{18} HPLC column resulted in separation into two protein peaks - one large peak, named Rusvikunin-II [9], and a small peak, named Rusvikunin [8] (see also supplementary Fig. S2). In addition to the Rusvikunins, small amounts of other proteins/peptides, may have co-eluted with the Rusvikunin complex or are non-covalently bound components of the Rusvikunin complex, was also observed by RP-HPLC fractionation of this RVV complex (Supplementary Fig. S2). It was noted that RP-HPLC fractionation of the Rusvikunin complex isolated from different batches of RVV showed identical results, suggesting that the same proteins/peptides of RVV are always associated with Rusvikunin and Rusvikunin-II [8,9] to form a RVV protein complex, termed Rusvikunin complex.

3.1. Peptide mass fingerprinting analysis of native Rusvikunin complex purified from RVV

The filtered results of LC-MS/MS analysis to identify the proteins/peptides associated with the native Rusvikunin complex is shown in Table 1. To eliminate the false identification of RVV proteins, the false discovery rate (FDR) curve was set at 0.8%. The PMF analysis indicates that in addition to containing Kunitz-type protease inhibitors and basic protease inhibitors isolated from Viperidae snake venom samples (average mass in the range of 6850−9287 Da), Rusvikunin complex also consists of a phospholipase A_2 (mass 13626 Da), a serine protease (25518 Da), and a precursor of snake venom vascular endothelial growth factor toxin (16278 Da). By PMF analysis, one tryptic peptide sequence of the Rusvikunin complex showed highest sequence homology to a serine protease (gi|297593780) purified from *Echis carinatus sochureki* venom (Table 1). The N-terminal sequence (VIGGAECNIN) of this serine protease (mature peptide) showed 90% sequence homology to the partial N-terminal sequence of fibrinogenolytic serine protease isoenzymes (RV-FVP$_\alpha$) purified from the same RVV [10] suggesting Rusvikunin complex contains the above serine protease(s). Furthermore, one of the tryptic peptides with sequence NLFQFAEMIVK of the Rusvikunin complex identifies the presence of a PLA$_2$ enzyme in Rusvikunin complex (Table 1). This tryptic peptide also demonstrated 100% sequence identity with the N-terminal sequence of an anticoagulant PLA$_2$ (RVAPLA$_2$) purified from the same RVV [14] demonstrating Rusvikunin complex contains an extremely low amount of RVAPLA$_2$. The alignments of MS-MS derived peptide sequences with the identified proteins are shown in supplementary Table SI.

3.2. Enzymes assay confirms the presence of serine protease and PLA$_2$ enzymes in the Rusvikunin complex

The LC-MS/MS analysis indicated the presence of fibrinogenolytic and PLA$_2$ enzymes in the Rusvikunin complex. Therefore, Rusvikunin complex was assayed for fibrinogenolytic and PLA$_2$ activity to confirm their non-covalent association in this toxic complex of RVV. Similarly to RVV serine protease isoenzymes, Rusvikunin-complex also did not show fibrinogen clotting activity [13]. Assay of esterolytic activity showed that the Rusvikunin complex lacked TAME-esterase activity, but it hydrolyzed BAEE with a specific activity of 3683 ± 19.1 units/mg protein (mean ± S.D.). Rusvikunin complex did not show appreciable amidolytic activity against any of the tested chromogenic substrates (data not shown). Rusvikunin complex showed fibrinogenolytic activity; however, Rusvikunin and Rusvikunin-II did not demonstrate fibrinogenolytic activity (Fig. 1A). The dose-dependent fibrinogenolytic activity demonstrated by Rusvikunin complex (Fig. 1B) was similar to that shown by fibrinogenolytic serine protease isoenzymes purified from RVV [13]. The fibrinogen degradation pattern showed that the Aα-chain of fibrinogen was degraded within 2 h of incubation with the Rusvikunin complex, whereas degradation of the Bβ-chain progressively increased with time, and complete degradation was observed only after 8 h of incubation at 37 °C (Fig. 1B). The γ-chain of fibrinogen remained intact after 8 h of Rusvikunin complex treatment (Fig. 1B). This result is also in accordance with the fibrinogen degradation pattern demonstrated by fibrinogenolytic serine protease isoenzymes purified from RVV [13]. The RP-HPLC analysis of fibrinogen degradation by Rusvikunin complex suggested that the protease present in this complex did not release fibrinopeptide A or fibrinopeptide B from fibrinogen (data not shown). This was confirmed by MALDI-TOF MS analysis of fibrinogen degradation products, and no peptide of 1537 Da (FPA) or 1553 Da (FPB) was detected.

Pre-incubation of Rusvikunin complex with fibrinogen solution for 30 and 60 min delayed the fibrinogen clotting activity of thrombin by ~36.7% and 66.0%, respectively. This result indicates that defibrinogenation activity of protease(s) present in Rusvikunin complex also contributes to progressively incoagulable blood, a characteristic pathophysiological feature of RV envenomation [1]. Rusvikunin complex at a concentration of 5 μg/ml did not show protease activity toward azocasein, bovine serum albumin, bovine serum globulin or fibrin.

The serine protease inhibitors benzamidine-HCl and AEBSF at a concentration of 5 mM significantly inhibited the fibrinogenolytic activity of the Rusvikunin complex (supplementary Table SII). The disulfide bond reducing agent DTT (5 mM), TPCK, and

Table 1

The LC-MS.MS analysis of the Rusvikunin complex. The proteins/peptides showing −10lgP value ≥ 30.3 and at least one high confident peptide were the prerequisites to identify the protein(s) of interest. The proteins matches were searched in NCBI using PEAKS 7.0 search engine against the Viperidae family and *D. r. russelii* snake venom database.

Accession	−10lgP	Coverage (%)	MS-MS derived peptides	PTM	Avg. Mass (Da)	Description of protein/peptide	Snake species	Protein family
gi|68708	123.21	17	2	N	6850	Venom basic proteinase inhibitor II	*D. r. russellii*	KSPI
gi|125041	123.21	17	2	N	6850	Protease inhibitor 2	*D. siamensis*	KSPI
gi|87130862	123.21	12	2	N	9287	Kunitz protease inhibitor-I	*D. r. russellii*	KSPI
gi|239977245	118.15	12	2	N	9318	Kunitz-type serine protease inhibitor B1	*D. siamensis*	KSPI
gi|157834128	66.43	9	1	N	13626	Chain A, Anticoagulant class II phospholipase A_2	*D. r. russellii*	PLA$_2$
gi|297593780	61.98	3	1	N	25518	Serine protease	*Echis carinatus sochureki*	SVSP
gi|87130864	51.27	13	1	N	9683	Kunitz protease inhibitor-II	*D. r. russellii*	KSPI
gi|327478537	47.99	6	1	N	16278	Snake venom vascular endothelial growth factor toxin VR-1	*D. r. russellii*	VEGF

In the above Table, −10lgP denotes the protein confidence score; coverage means the percentage of the protein sequence covered by supporting peptides; PTM shows the identified modifications, if any; Avg. Mass represents protein mass calculated using average mass.
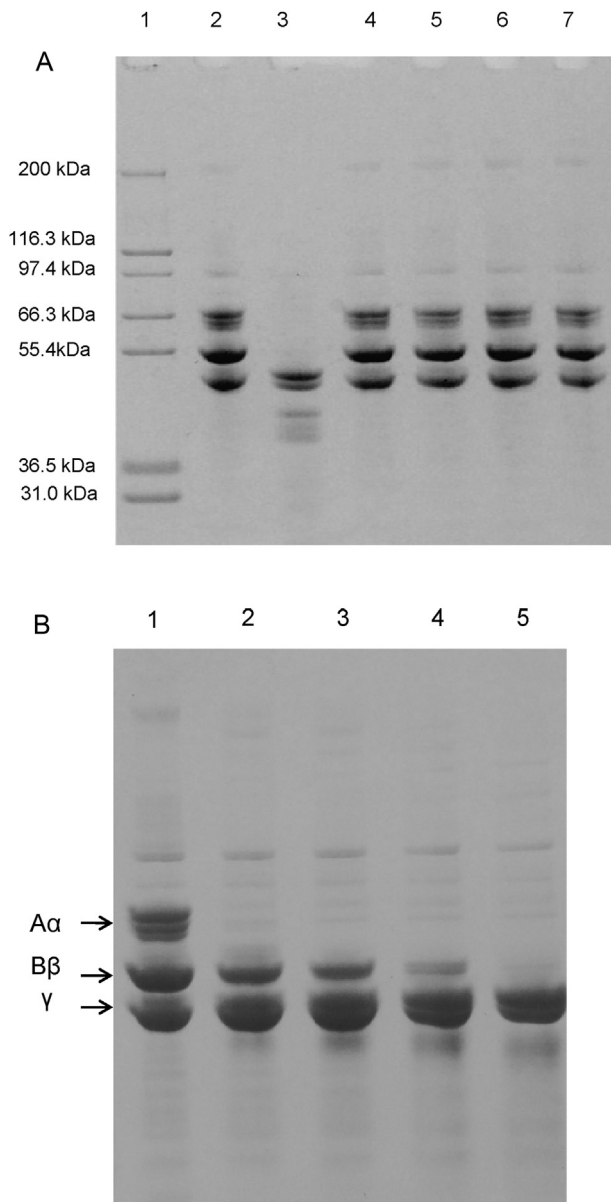
Fig. 1. A. Fibrinogenolytic activity displayed by the Rusvikunin complex and its two major components-Rusvikunin and Rusvikunin-II. Lane 1, molecular markers; lane 2, fibrinogen (undigested, 7.5 μg); lane 3, fibrinogen treated with the Rusvikunin complex (2.5 μg/ml); lanes 4–5, fibrinogen treated with Rusvikunin (2.5 and 5.0 μg/ml, respectively); lanes 6–7, fibrinogen treated with Rusvikunin-II (2.5 and 5.0 μg/ml, respectively). The proteins were incubated with fibrinogen for 8 h at 37 °C, pH 7.4. **B**. Kinetics of fibrinogenolytic activity shown by the Rusvikunin complex (5 μg) at 37 °C, pH 7.4. Lane 1, fibrinogen (undigested, 10 μg); lanes 2–5, fibrinogen (10 μg) treated with the Rusvikunin complex (5 μg/ml) for 2,4,6 and 8 h, respectively at 37 °C, pH 7.4.

α_2—macroglobulin also inhibited the fibrinogenolytic activity of Rusvikunin complex (supplementary Table SII). Interestingly, in the presence of EDTA, the Rusvikunin complex showed enhanced proteolytic activity (supplementary Table SII). The results of biochemical studies as well as PMF analysis confirm that fibrinogenolytic serine protease isoenzyme(s) [13] present as minor components of the Rusvikunin complex could not be detected otherwise.

The LC-MS/MS analysis result shows the presence of PLA$_2$ enzyme in the Rusvikunin complex; therefore, PLA$_2$ enzyme activity of this protein complex as well as Rusvikunin and

Rusvikunin-II was also assayed. Only the Rusvikunin complex showed PLA$_2$ activity. The dose-dependent catalytic activity of Rusvikunin complex on thio-PC is shown in supplementary Fig. S3. The Km and $Vmax$ values for thio-PC hydrolysis by PLA$_2$ were determined at 1.7 mM and 11.3 mmol min^{-1} mg^{-1}, respectively. The histidine modifier (PLA$_2$ inhibitor) pBPB (2 mM) inhibited the thio-PC hydrolytic activity of Rusvikunin complex to 88% of its original PLA$_2$ activity (activity in absence of inhibitor). The results of this study substantiate the presence of a PLA$_2$ enzyme [14] in the Rusvikunin complex. In sharp contrast, Rusvikunin or Rusvikunin-II did not exhibit PLA$_2$ activity.

3.3. Spectrofluorometric analysis to determine the interactions among the components of Rusvikunin complex

The venom of snakes contain many enzymes and non-enzymatic proteins (toxins) that act individually or with coordinated synergism to induce various toxic effects in experimental animals and victims [3,4,9,18]. The Rusvikunin complex exhibited potent biological activity as well as *in vivo* toxicity in mice [8,9]; nevertheless, Rusvikunins (Rusvikunin and Rusvikunin-II), RVA-PLA$_2$, and fibrinogenolytic serine protease isoenzymes at a dose of 5 mg/kg were non-toxic to NSA mice and they did not induce any behavioral changes/adverse effects in treated animals [8,9,13,14]. It may therefore be anticipated that interactions among the above components may potentiate their biological activity.

Fluorescence spectroscopy is considered as one of the reliable tools to study protein-protein interactions due to its great selectivity and sensitivity [4,19]. Furthermore, this technique has been proven to be useful in determining the stoichiometry of binding [20]. The three aromatic amino acids *viz.*, tyrosine, tryptophan and phenylalanine, which have distinct absorption and emission wavelengths, contribute to intrinsic fluorescence of a protein. However, the tryptophan residue is excited at 280 nm and shows emission in 340–345 nm range, and it has stronger fluorescence and higher quantum yield compared to tyrosine and phenylalanine residues. Depending on the nature of the protein molecules, their interaction may result in either a decrease in yield owing to quenching of fluorescence of tyrosine and/or phenylalanine residues by a nearby tryptophan residue [20] or it can lead to an increase in fluorescence intensity of the protein complex compared to the individual components of the complex [14,21].

The interaction of different concentrations of Rusvikunin with a fixed concentration of Rusvikunin-II (400 nM) resulted in a progressive decrease in the fluorescence intensity peak at ~342 nm, (Fig. 2A). These data suggested an interaction between the above Kunitz type serine protease inhibitors from RVV. The lowest fluorescence intensity was observed at a concentration of 400 nM Rusvikunin, and beyond this concentration no further significant change in fluorescent intensity (ΔF) was observed (Fig. 2A). The spectrofluorometric data suggested that complex formation between Rusvikunin and Rusvikunin-II started at a 1:1 stoichiometric ratio, with a Kd value of 280.8 nM ± 7.9 nM (mean ± SD, n = 3) (Fig. 2B). These data also imply a strong interaction between Rusvikunin and Rusvikunin-II (at nanomolar concentrations) to form a complex (Fig. 2B).

The protease enzyme present in Rusvikunin complex was not detected by SDS-PAGE analysis; nevertheless, biochemical analysis suggested the association of a trace quantity of protease in the Rusvikunin complex preparation. The LC-MS/MS analysis identified this protease as previously purified RV-FVP$_\alpha$ from RVV [13]. The interaction of different doses of RV-FVP$_\alpha$ (6–36 nM) with a fixed concentration of Rusvikunin II (600 nM) resulted in a steady increase in the fluorescence intensity, suggesting the binding between these two proteins (Fig. 3A). The fluorescence changes were
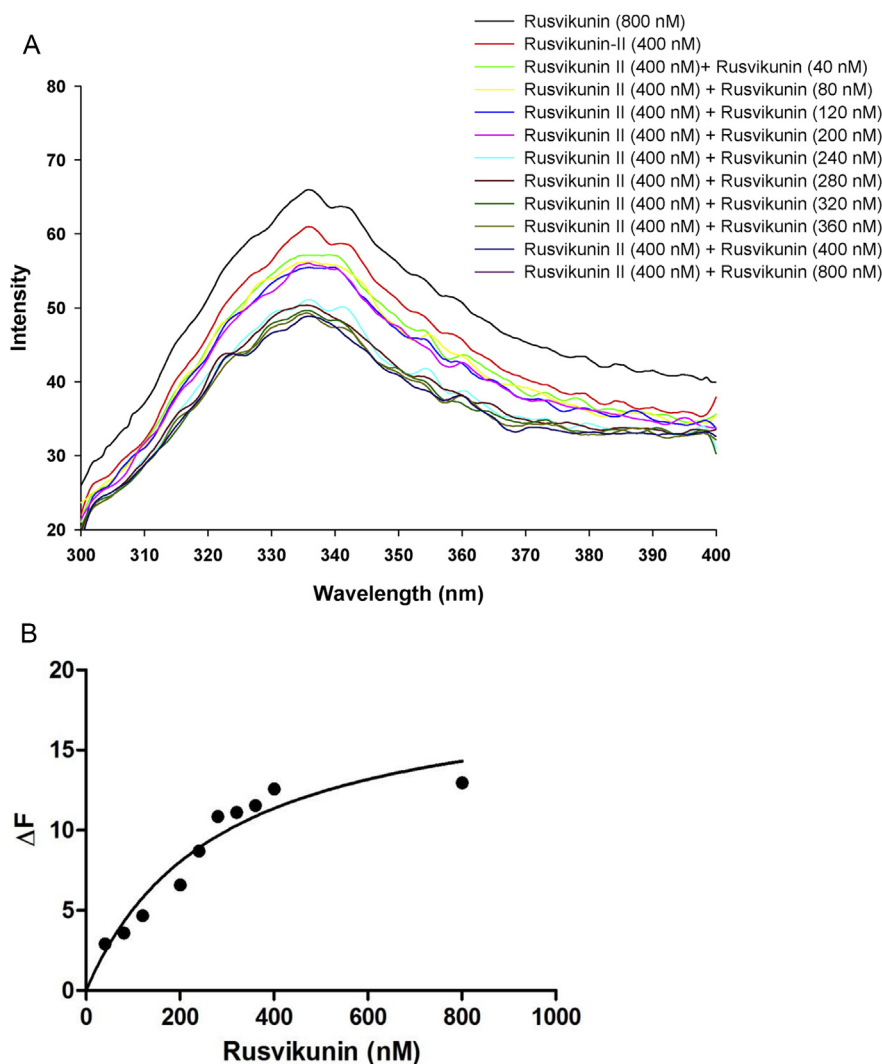
Fig. 2. Spectrofluorometric measurement of interactions among components of the Rusvikunin complex. **A**. Interaction between Rusvikunin and Rusvikunin-II. **B**. OneSite-Specific Binding curve showing the change in the maximum fluorescence intensity (λ_{max}) of 400 nM of Rusvikunin II in presence of different concentrations (40–800 nM) of Rusvikunin. The fluorescence spectra were obtained at an excitation wavelength 280 nm and the emission spectra was recorded from 300 to 400 nm range.

fitted in OneSite-Specific binding isotherm, which yielded a *Kd* value of 13.2 ± 1.3 nM (mean ± SD, n = 3) suggesting a possible interaction between RV-FVP$_\alpha$ and Rusvikunin II at nanomolar concentration to form a complex (Fig. 3B). The interaction of RV-FVP$_\alpha$ with RVAPLA$_2$ (1:2 protein:protein ratio) showed a small increase in fluorescence intensity (data not shown) which may be due to non-specific interaction between this protease and PLA$_2$ from RVV. Furthermore, addition of different concentrations of RVAPLA$_2$ to Rusvikunin-II-RV-FVP$_\alpha$ complex did not result in a significant increase in the fluorescence intensity of the combined Rusvikunin-II-RV-FVP$_\alpha$-RVAPLA$_2$ proteins (Fig. 3C) compared to the fluorescence intensity exhibited by Rusvikunin-II-RV-FVP$_\alpha$ complex (Fig. 3A). However, Risch et al. [22] have shown the simultaneous migration of PLA$_2$ and protease enzymes in *D. r. siamensis* venom by 2D gel-electrophoresis analysis indicating their interaction *in vivo*. Nevertheless, the spectrofluorometric data and biochemical analysis presented in this study indicate that a trace quantity of PLA$_2$ may always be co-eluted along with Rusvikunin complex due to a strong ionic interaction and may not be a real component of the Rusvikunin complex.

Snake venom-type vascular endothelial growth factors (VEGF-

Fs) are diversified in their structures resulting in the acquisition of specific and potent functions unobserved in related proteins isolated from other viperid snake venoms [23]. No direct evidence has shown lethality or toxicity of VEGF-Fs in mice, and we have yet to purify VEGF-Fs from RVV; therefore; its potential interaction and stoichiometry of interaction with Rusvikunin or Rusvikunin-II could not be determined.

3.4. Classical and equilibrium gel-filtration chromatography followed by RP-HPLC analysis of GF complexes show interaction between Rusvikunin and Rusvikunin-II to form a stable complex

Equilibrium gel-filtration chromatography is one of the efficient and classical methods to demonstrate protein-protein interactions as well as to determine the stoichiometry of interacting proteins to form a stable complex [16,24]. In this technique, gel-filtration is performed under equilibrium conditions by including one of the components of protein complex in the running buffer [16,24].

The individual proteins (Rusvikunin or Rusvikunin-II) eluted from the gel-filtration column at 12.3 min. However, injection of different concentrations (1.4–35.0 nM) of Rusvikunin in the gel-
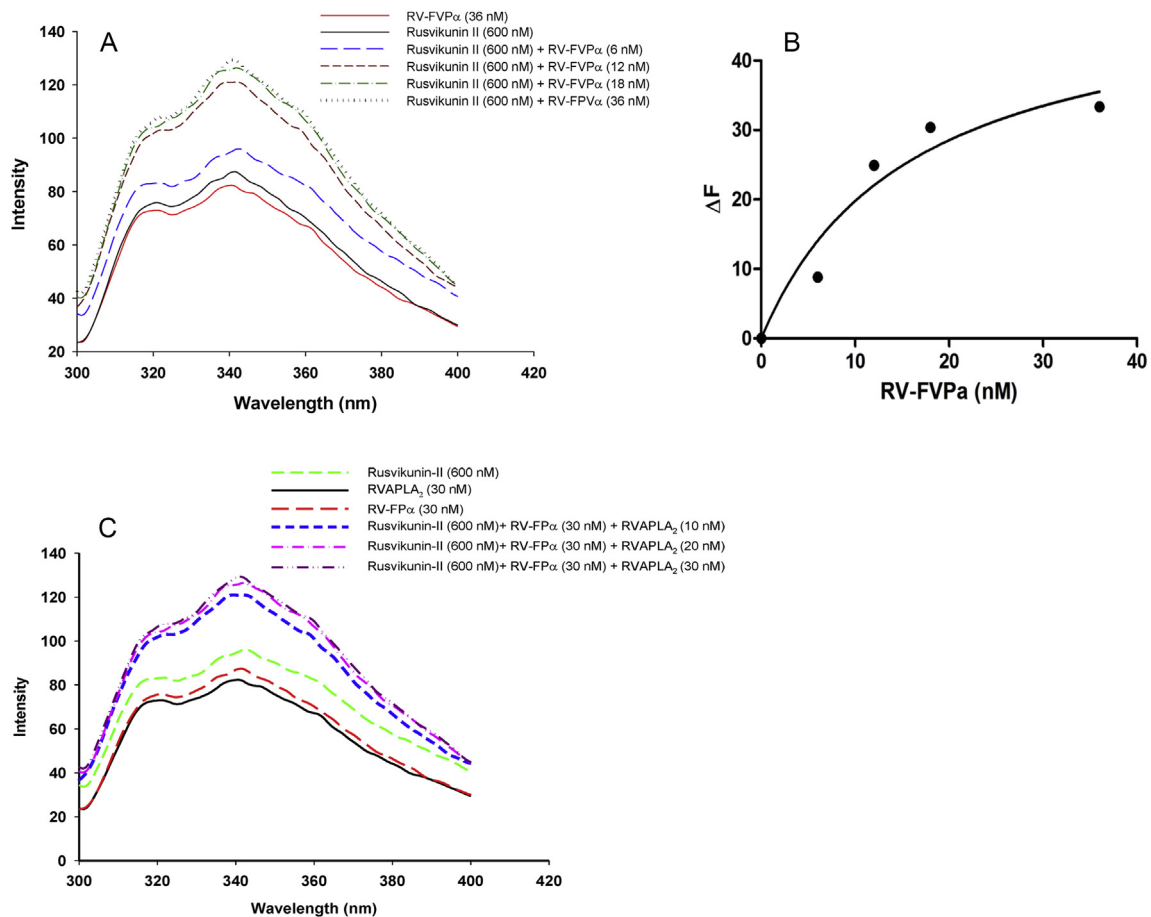
Fig. 3. A. Spectrofluorometric interaction between Rusvikunin-II and RV-FVPα. **B**. OneSite-Specific Binding curve showing the change in the maximum fluorescence intensity (λ_{max}) of 600 nM of Rusvikunin II in presence of different concentrations (6−36 nM) of RV-FVPα. **C**. Determination of interaction among Rusvikunin-II, RV-FVPα and RVAPLA$_2$. The fluorescence spectra were obtained at an excitation wavelength 280 nm and the emission spectra was recorded from 300 to 400 nm range.

filtration column which was equilibrated with buffer containing Rusvikunin-II resulted in appearance of three major protein peaks with retention times of 11.7 min, 12.1 min and 12.3 min with corresponding molecular mass of ~21 kDa, ~14 kDa and ~7 kDa, respectively (Fig. 4A). The intensity of these equilibrium gel-filtration peaks was dependent on the injected Rusvikunin: Rusvikunin-II ratio (Fig. 4A). The highest intensity of the two major peaks eluted from the GF column with a retention time of 11.7 min and 12.1 min was displayed when Rusvikunin and Rusvikunin-II were injected in a 1:2 (mol: mol) ratio.

The RP-UHPLC separation of ~21 kDa protein complexes eluted at 11.7 min from the equilibrium gel-filtration column confirmed their solution complex formation between Rusvikunin and Rusvikunin-II in a 1:2 molar ratio (Fig. 4B). The RP-UHPLC analysis of ~14 kDa protein complex (eluted at 12.1 min from gel-filtration column) showed dimer formation of Rusvikunin-II in solution (Fig. 4C) suggesting self-aggregation of Rusvikunin-II which is in accordance to our previous observation [9]. The mass spectroscopic analysis of gel-filtration peak at 12.3 min confirmed the elution of free Rusvikunin.

The equilibrium gel-filtration analysis suggested that the above Kunitz-type protease inhibitors from RVV interact at a 1:2 ratio to form a complex. Therefore, the stability of Rusvikunin-Rusvikunin-II protein complex was also ascertained by classical gel-filtration chromatography. As shown in Fig. 4D, gel-filtration of previously purified Rusvikunin and Rusvikunin-II proteins incubated in 1:2 ratio for overnight resulted in elution of proteins in a major, sharp peak with a retention time of 11.7 min. Appearance of three

additional small peaks with retention time of 5.8 min, 9.7 min, and 11.2 min was also observed (Fig. 4D). The gel-filtration data was substantiated by SDS-PAGE analysis of Rusvikunin: Rusvikunin-II proteins incubated in a 1:2 ratio (Fig. 4E). Analysis of this protein complex under reduced SDS-PAGE demonstrated a single protein band of ~7.0 kDa albeit the non-reduced protein complex showed a broad band in the range of ~21−60 kDa (Fig. 4E). These data support the complex formation between Rusvikunin and Rusvikunin-II in 1:2 molar ratio. However, in solution this 21 kDa complex is further self-aggregated to form complexes of higher molecular masses [9]. The RP-UHPLC analysis of the major protein peak eluted at 11.7 min (molecular mass ~21 kDa) from the GF column confirmed that Rusvikunin and Rusvikunin-II interact in 1:2 stoichiometric ratio to form a stable protein complex (Fig. 4B).

In order to verify the *in vivo* complex formation between Rusvikunin and Rusvikunin-II, the gel-filtration peaks of crude RVV [see Fig. 1A of Ref. [12]] containing venom proteins in the mass range of 14−21 kDa (determined by native SDS-PAGE, data not shown) were analyzed by LC-MS/MS. Our study substantiates the presence of Rusvikunin and Rusvikunin-II in addition to PLA$_2$ and serine protease enzymes in this peak (Mukherjee A. K., unpublished observation).

3.5. CD analysis of conformational changes during Rusvikunin complex formation

CD spectroscopy is an effective biophysical method to understand the molecular conformation of proteins in solution.
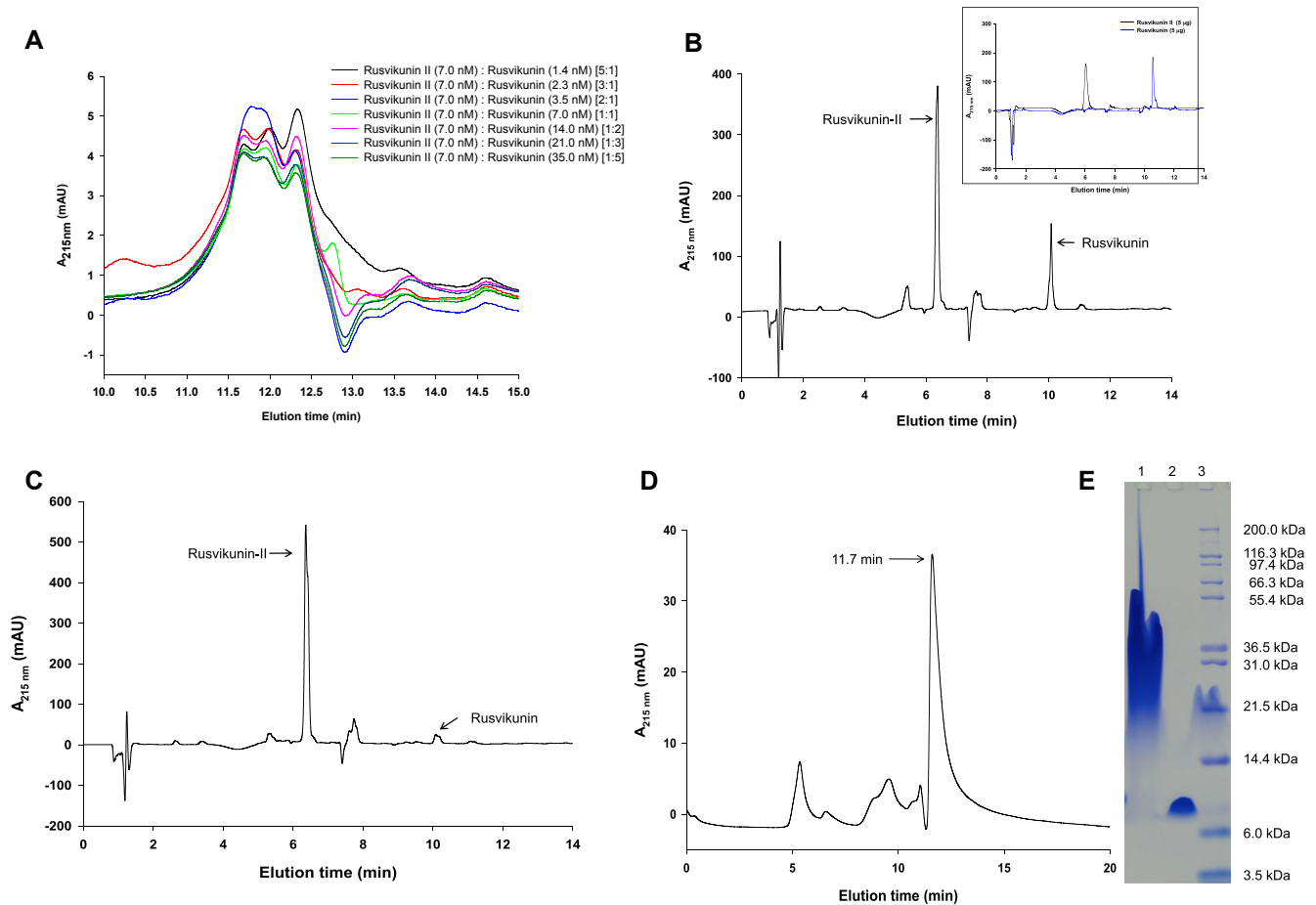
Fig. 4. A. Gel-filtration equilibrium analysis to determine the in solution interaction between Rusvikunin and Rusvikunin-II. The Shodex KW-803 HPLC gel-filtration column was equilibrated with 50 mM Na-phosphate buffer containing 150 mM NaCl, pH 7.0 and Rusvikunin-II (7.0 nM). Different concentrations (1.4–35.0 nM) of Rusvikunin were injected into the column and in solution complex formation was monitored at 215 nm. **B**. RP-UHPLC analysis of gel-filtration equilibrium peak eluted at 11.7 min on an Acclaim 300 C_{18} RP-HPLC column (2.1 × 150 mm, 3 μm) pre-equilibrated with solvent A (Milli Q H_2O with 0.1% TFA). The elution of Rusvikunin and Rusvikunin-II is shown with arrow. Inset: Retention time of Rusvikunin or Rusvikunin-II from the same RP-UHPLC column under identical chromatographic conditions. **C**. RP-UHPLC analysis of gel-filtration equilibrium peak eluted at 12.1 min on an Acclaim 300 C_{18} RP-HPLC column. **D**. Classical gel-filtration analysis of *in vitro* incubated Rusvikunin: Rusvikunin-II protein in 1:2 ratio (mol:mol) for 24 h at 4 °C. The protein complex was fractionated through Shodex KW-803 HPLC gel-filtration column and elution of proteins was monitored at 215 nm. Elution of the major peak (11.7 min) is indicated by an arrow. **E**.12.5% SDS-PAGE analysis of Rusvikunin: Rusvikunin-II proteins incubated in 1:2 ratio for overnight. Lanes 1 and 2: Non-reduced and reduced Rusvikunin: Rusvikunin II complex (8.0 μg), respectively; Lane 3: Protein molecular markers.

Additionally, this technique is quite efficient for studying protein-protein interactions in solution [25,26], and measurement of CD in the far UV region (178–260 nm) can provide valuable information on changes in the conformations of proteins when they interact to form a complex [26]. The CD spectra of Rusvikunin and Rusvikunin-II demonstrated predominance of β-sheet structure (~60%) which is in accordance to our previous observations [8,9]. Interaction of Rusvikunin with Rusvikunin-II at 1:1 and at 1:2 molar ratio did not show any significant change in the secondary structure of interacting proteins (Fig. 5). These data support the earlier observations that protein-protein interactions may result in only minor perturbations in protein structure and does not lead to overall changes in secondary structures of interacting proteins [25,27]. However, a slight change in the signal intensity of interacting proteins at 203 nm was observed in this order: Rusvikunin ≈ Rusvikunin-II < Rusvikunin:Rusvikunin-II (1:1) < Rusvikunin:Rusvikunin-II (1:2) (Fig. 5). Therefore, CD spectroscopic study has provided convincing evidence of interaction to form a stable complex between Rusvikunin and Rusvikunin-II at a 1:2 M ratio [25,27].
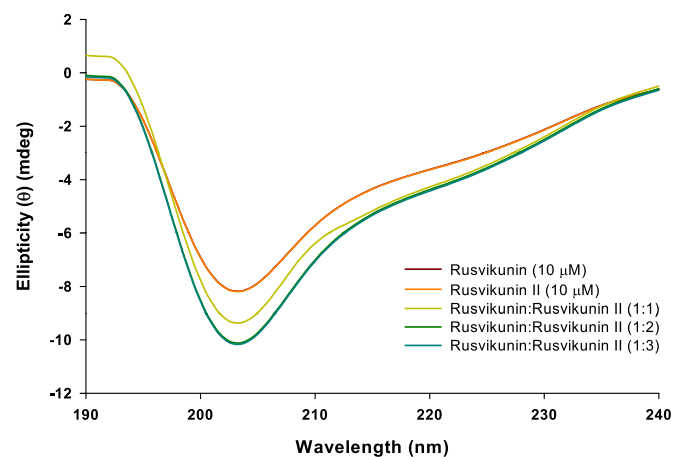

Fig. 5. Far-UV CD spectroscopic analysis of Rusvikunin, Rusvikunin-II and interaction between these two proteins to form a complex. The experimental procedure is described in the text. Data represent average of four scans.

3.6. Determination of interaction between Rusvikunins in solution by dynamic light scattering study

Dynamic light scattering (DLS) has been used to study protein-protein interactions in solutions to determine the hydrodynamic diameter (D_H) or radius (R_H) of interacting protein molecules and their complexes [25,28,29]. DLS measures the time-dependent fluctuations in the intensity of scattered light that arise due to Brownian motion of the protein molecules. A variety of physical factors such as the ionic strength and/or pH of the buffer or medium, viscosity of the medium, and temperature of the system are the key determinants of D_H of a protein molecule [25,28,30]. To verify the accuracy of the DLS study to determine the protein size, 10 μM of BSA solution was used as a positive control. The D_H of the BSA molecule was determined at 5.9 nm (data not shown), which is in close proximity with the reported R_H of BSA [31,32]. The hydrodynamic diameter of in vitro constituted Rusvikunin complex or native Rusvikunin complex did not change after storage for 14 days at 4 °C suggesting stability of this protein complex (data not shown).

Rusvikunin and Rusvikunin II showed a unimodal distribution with their corresponding hydrodynamic diameters of 12.25 nm and 11.8 nm, respectively (Fig. 6). Banerjee et al. [25] have also determined the hydrodynamic diameters of two 6.8 kDa protein molecules (Hemextin A and Hemextin B) in presence of 150 mM NaCl at 11.35 and 9.89, respectively. The Rusvikunin and Rusvikunin II incubated at a molar ratio of 1:2 for 2 h at 37 °C showed an increased hydrodynamic diameter (D_H = 16.1 nm) (Fig. 6) indicating complex formation between the interacting proteins [25,28,29]. However, the hydrodynamic diameter obtained for in vitro constituted Rusvikunin complex was slightly less as compared to the hydrodynamic diameter shown by the native Rusvikunin complex (D_H = 19.15 nm) isolated from RVV (Fig. 6). This difference in complex size can be attributed to the fact that the native Rusvikunin complex contains traces of a serine protease (RV-FVPα), a phospholipase A_2 enzyme (RVAPLA$_2$) and VEGF, which may add to the size of the native Rusvikunin complex (Fig. 6).

It is noteworthy that the hydrodynamic diameters of Rusvikunin molecules and their complex were found to be significantly higher as compared to other protein molecules of nearly identical mass [33]. This increase in size may be attributed to presence of 150 mM NaCl in the buffer [34,35]. Furthermore, lyophilization also causes
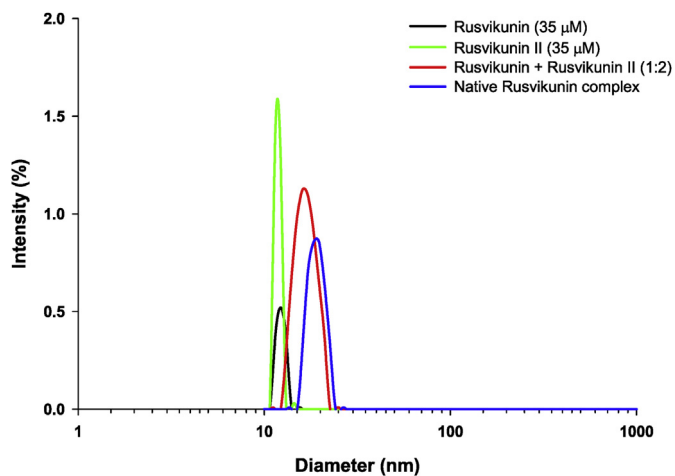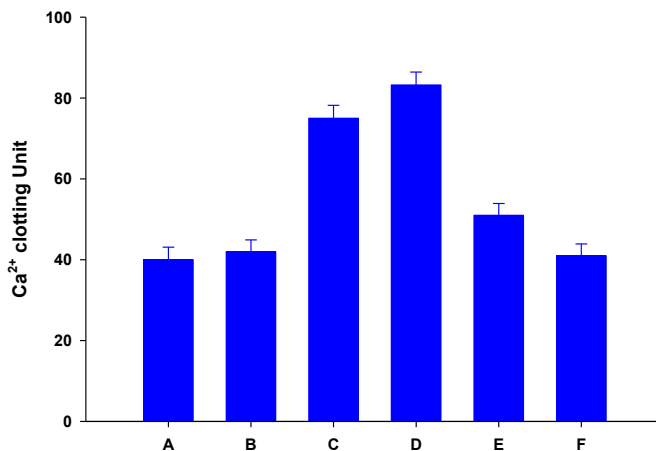
Fig. 7. Comparison of anticoagulant activity (calcium clotting time) of goat PPP by 5.0 μg/ml of- (A) Rusvikunin, (B) Rusvikunin II, (C) native Rusvikunin complex, (D) equilibrium gel-filtration (EGF) 11.7 min eluted peak, (E) EGF 12.1 min eluted peak, and (F) EGF 12.3 min eluted peak. The calcium clotting time of the control plasma was determined at 87.1 ± 2.2 s. Values are mean ± SD of three determinations. Significance of difference with respect to Ca-clotting time of control plasma, *p < 0.05.

protein aggregation that result in an increase in size [36].

3.7. A comparison of anticoagulant activity of gel-filtration peaks, native Rusvikunin complex, Rusvikunin, and Rusvikunin-II

Rusvikunin, Rusvikunin-II, in vitro constituted Rusvikunin complex and the native Rusvikunin complex demonstrated dose-dependent anticoagulant activity; however, the anticoagulant potency of Rusvikunin complex was found to be higher (Fig. 7). The anticoagulant potency was observed in the following order: EGF peak (retention time 11.7 min) > native Rusvikunin complex (previously purified from RVV) > EGF peak (retention time 12.1 min) ≫ EGF peak (retention time 12.3 min) ≈ Rusvikunin-II ≈ Rusvikunin (Fig. 7). The most common symptom of RV envenomation is interference in the hemostatic system of victims; it appears that complex formation between Rusvikunin and Rusvikunin-II, predominantly in a 1: 2 molar ratio, augments the biological activity (blood anticoagulation) of the components of RVV [9].

Notably, a 44.6 kDa protein complex (Reprotoxin) isolated from venom of RV of India consists of a PLA$_2$, protease and a trypsin inhibitor; however, Reprotoxin showed only PLA$_2$ activity [2]. On the contrary, Rusvikunin complex displayed PLA$_2$, protease as well as serine protease inhibition activities. Furthermore, the anticoagulant potency of Rusvikunin complex was significantly higher than that exhibited by Reprotoxin [2]. These combined data suggest that Reprotoxin and Rusvikunin complex are distinctly different complexes of venom of RV from two distinct geographical locations although these complexes serve a common function for the producing Russell's Viper.

4. Conclusion

The Rusvikunin complex furnishes a typical example of protein complementation, which has been defined as the reinstatement of biological activity by non-covalent interactions of different proteins/polypeptides of venom. Nevertheless, the results of this study endorse that the non-toxic Kunitz-type protease inhibitors (Rusvikunin and Rusvikunin-II) from RVV non-covalently interact at 1:2 stochiometric ratio to form a protein complex (Rusvikunin complex) that leads to augment their anticoagulant (biological) activity.

Fig. 6. Determination of hydrodynamic diameter of Rusvikunins, in vitro constituted Rusvikunin: Rusvikunin-II (1:2), and native Rusvikunin complexes by DLS study. The proteins were dissolved in 20 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.0. The native Rusvikunin complex was isolated from Russell's Viper venom.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2016.08.005.

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