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Research paper

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

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A B S T R A C T

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

Abbreviations: LC-MS/MS, liquid chromatography-mass spectroscopy/mass spectroscopy; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectroscopy; NSA, Non Swiss Albino; PLA_2, Phospholipase A_2; FPLC, fast protein liquid chromatography; RP-HPLC, reversed-phased high performance liquid chromatography; EGF, equilibrium gel filtration; RVV, Russell's Viper venom; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; AEBSF, 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride; DTT, dithiothreitol; TPCK, tosyl phenylalanyl chloromethyl ketone; TLCK, N-α-tosyl-L-lysine chloromethyl ketone hydrochloride; EDTA, ethylenediaminetetraacetic acid; *p*BPB, *p*-bromophenacyl bromide; VEGF, vascular endothelial growth factor; CD, circular dichroism; DLS, dynamic light scattering; UV, ultraviolet; BSA, bovine serum albumin.

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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

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