The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom, shows structural similarity to mammalian coagulation factor V

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Running title: Factor V in snake venom

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Abstract

Pseutarin C is a group C prothrombin activator from the venom of the eastern brown snake *Pseudonaja textilis*. It is a multi-subunit protein complex consisting of catalytic and nonenzymatic subunits similar to coagulation factor Xa and factor Va, respectively. Here we describe the complete sequence of the nonenzymatic subunit. Based on the partial amino acid sequence of the nonenzymatic subunit degenerate primers were designed. Using a “walking” strategy based on sequentially designed primers, we determined the complete cDNA sequence of the nonenzymatic subunit. The cDNA encodes a protein of 1461 amino acid residues, which includes a 30-residue signal peptide, a mature protein of 1430 amino acid residues and a stop codon. cDNA blot analysis showed a single transcript of ~ 4.6 kb. The deduced amino acid sequence shows ~50% identity to mammalian factor V and by homology has a similar domain structure consisting of domains A1-A2-B-A3-C1-C2. Interestingly, the B domain of pseutarin C is shorter than that of mammalian FV. Although, most of the proteolytic activation sites are conserved, two of the three proteolytic sites cleaved by activated protein C are mutated and thus activated protein C is not able to inactivate this procoagulant toxin. The predicted post-translational modifications, including disulfide bonds, N-glycosylation, phosphorylation and sulfation, in pseutarin C are significantly different compared to bovine factor V. Thus our data demonstrates that the nonenzymatic subunit of group C prothrombin activators is structurally similar to mammalian FV.

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INTRODUCTION

Blood coagulation involves the sequential activation of the plasma zymogens finally culminating in the formation of a fibrin clot. Prothrombin activation, a crucial reaction in the coagulation cascade, occurs when the serine protease FXa cleaves prothrombin at two peptide bonds, Arg\textsubscript{271}-Thr\textsubscript{272} and Arg\textsubscript{320}-Ile\textsubscript{321} leading to the formation of thrombin\textsuperscript{1}. Prothrombin activation by FXa alone occurs at a very slow rate. For activation at a physiologically relevant rate, FXa must form the “prothrombinase complex” with the nonenzymatic cofactor factor Va (FVa), calcium ions and phospholipid membranes\textsuperscript{1}. Phospholipids stimulate prothrombin activation by lowering the $K_m$ for prothrombin\textsuperscript{2,4} and provide a surface on which FXa, FVa and prothrombin bind in presence of Ca\textsuperscript{2+} leading to a productive enzyme-substrate complex. FVa acts as a receptor for FXa by the virtue of its ability to bind to phospholipid membranes. It enhances the ability of FXa to activate prothrombin by increasing the $V_{max}$ by $\sim$700-fold for thrombin formation\textsuperscript{2,5,6}. Hence, the formation of the prothrombinase complex results in $10^5$-fold acceleration in the rate of prothrombin activation as compared to that by FXa alone\textsuperscript{2-4}.

In addition to its physiological activator FXa, prothrombin is activated by several exogenous factors including those from snake venom\textsuperscript{7,8}. These snake venom prothrombin activators are classified into four groups based on their cofactor requirements\textsuperscript{9}. Among these, group C and D prothrombin activators are serine proteinases. Group C prothrombin activators are large (~250-kDa) protein complexes with multiple subunits. These activators cleave prothrombin to mature thrombin and their prothrombin converting activity is
enhanced by Ca\(^{2+}\) ions and phospholipids but not by FVa. They have been isolated from *Oxyuranus scutellatus* (oscutarin C) and *Pseudonaja textilis* (pseutarin C) venoms\(^{10-13}\). Group D activators are two-chain serine proteinases\(^{14-16}\). They convert prothrombin to mature thrombin and their activity is greatly enhanced by FVa, phospholipids and Ca\(^{2+}\). Functionally, they resemble blood coagulation FXa. Hence, we initiated the study of these group C and D activators as it will significantly contribute in understanding the formation of the prothrombinase complex and FXa-FVa mediated prothrombin activation.

We recently determined the complete amino acid sequence of group D prothrombin activators trocarin D (*Tropidechis carinatus*) and hopsarin D (*Hoplocephalus stephensi*)\(^{15,16}\). Both are glycoproteins and are homologous (62% - 70% similarity) to FXa with identical domain architecture. The light chain consists of an N-terminal Gla-domain containing eleven gamma-carboxyglutamic acid (Gla) residues, followed by two epidermal growth factor-like domains; the heavy chain is a serine proteinase. Thus group D prothrombin activators are true structural and functional homologues of coagulation FXa.

Recently, we have shown that pseutarin C, a group C activator from the venom of the eastern brown snake *Pseudonaja textilis*, is a multimeric protein complex consisting of an catalytic and a nonenzymatic subunits similar to mammalian FXa-FVa complex\(^{12,13}\). In this paper, we present the complete cDNA sequence and the translated amino acid sequence of the nonenzymatic subunit of pseutarin C and its structural similarity to mammalian FV. These studies indicate that the nonenzymatic subunit of pseutarin C has identical
domain architecture like mammalian FV (A1-A2-B-A3-C1-C2). However, the nonenzymatic subunit differs significantly in its potential post-translational modifications and the size of B domain. This is the first sequence of a FV–like protein from a non-hepatic and non-mammalian origin.

MATERIALS AND METHODS

Materials. *Pseudonaja textilis* venom and venom gland were obtained from Venom Supplies (Tanunda, Australia). The RNA isolation kit, one-step RT-PCR, Qiaquick gel extraction and plasmid miniprep kits were purchased from Qiagen (Valencia, CA, USA). pDrive cloning kit was obtained from Qiagen (Valencia, CA, USA). 5’ and 3’ RACE kits were obtained from Invitrogen (Carlsbad, CA, USA). DIG nucleic acid labeling and detection kits were from Roche Diagnostics Corporation (Indianapolis, IN, USA). The ABI PRISM BigDye terminator cycle sequencing ready reaction kit was purchased from Perkin Elmer (Foster City, CA, USA). Oligonucleotides were custom synthesized from GENSET (Singapore Biotech, Singapore). Bovine prothrombin, factor Xa, factor Va and activated protein C were purchased from Haematologic Technologies (Essex Junction, VT, USA). S-2238 was purchased from Chromogenix (Mölndal, Sweden). All other chemicals and reagents were of the purest grade available.

METHODS

RNA isolation, reverse transcription - polymerase chain reaction (RT-PCR)

Total RNA was isolated from the venom gland using the Qiagen RNA isolation kit according to the manufacturer’s instructions. One-step RT-PCR
was carried out in a final volume of 25 µl using 100 ng of total RNA and degenerate primers were designed based on the peptide sequences\textsuperscript{12} [sense primers – KIVYRE (IP1: 36-41); WEYFIA (IP2: 323-328); RPYSIYV (IP3: 414-420); AAKTTF (N-ter: 821-826) and antisense primers – RPYSLHA (A2R: 877-883); FPAING (A3R: 1015-1020)]. The RT-PCR conditions were: reverse transcription at 50°C for 30 min, followed by inactivation of reverse transcriptase and activation of Taq polymerase at 95°C for 15 min, PCR was 35 cycles consisting of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, followed by final extension at 72 °C for 10 min. PCR-products obtained were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR product was purified according to the manufacturer’s instructions using the Qiagen PCR purification kit.

\textit{Cloning of pseutarin FV cDNA}

In order to determine the complete sequence of the pseutarin C nonenzymatic subunit, primer corresponding to A1 domain from 604 to 623 (A1fwd) [5’-AATGCAATGGGTTCCACAAAA-3’] and the 3’-adapter primer [5’-GGCCACGCGTCGACTAGTAC(T) 17-3’] were used in PCR, the product obtained (~4 kb) was purified, ligated with pDrive vector (Qiagen, Valencia, CA, USA) and transformed into competent \textit{E. coli} cells (JM109) by the heat shock method. The transformants were selected on LB-amp plates supplemented with IPTG and X-gal and the inserts sequenced as described below.
cDNA blot analysis

 cDNA blotting was carried out according to the protocol of Jaakola et al. Briefly, total RNA (~2 µg) isolated from the venom gland was reverse-transcribed at 42°C for 50 min in a 20 µl reaction volume containing MMLV-RT (Moloney Murine Leukemia Virus - Reverse Transcriptase) (Invitrogen, Carlsbad, CA, USA) and adapter primer [5’-GGCCACGCGTCGACTAGTAC(T) 17-3’]. The cDNA was run on a 1% agarose gel at 22 V for 10 h following which the gel was stained with ethidium bromide. The gel was then blotted onto the nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) overnight in 20X SSC (Standard Saline Citrate). After cross-linking, the membrane was hybridized with a 924 bp PCR fragment (region 1854–2778 of pseutarin C), which was labeled with digoxigenin-dUTP. Hybridization was performed overnight at 50°C. The blot was treated with anti-DIG antibody conjugated to alkaline phosphatase and detected using the DIG nucleic acid detection kit according to the manufacturer’s instructions.

In situ hybridization

 The slides containing the venom gland sections were treated with proteinase K (1 µg/ml in Phosphate buffered saline) for 10 min at 37°C, following which the slides were washed with PBS at room temperature. Pre-hybridization was carried out using DIG pre-hybridization solution for 2 h at 42°C. The DIG-labeled probe, which covered bases 1854–2778 of pseutarin C was denatured by heating at 85°C for 15 min and quickly chilled on ice before adding it to the hybridization solution. Hybridization was carried out at
42°C for 16 h. Detection of the DIG labeled probe was carried out according to manufacturer’s instructions.

3’ – Rapid Amplification of cDNA End

cDNA was synthesized from ~1.5 µg of total RNA using Superscript II reverse transcriptase (Invitrogen, CA, USA) and 3’-RACE adapter primer [5’-GGCCACGCGTCGACTAGTAC(T)17-3’] as described earlier. Following RT, the reaction was treated with RNase H (2 U) for 30 min at 37°C. The RT product (2 µl) was used in first round PCR (94° C, 1 min; 50° C, 1 min; 68° C, 1 min; 35 cycles, followed by final extension at 68° C for 10 min) with a psuetarin C nonenzymatic subunit forward primer corresponding to region 3273-3291 [5’-GAGGGAAAGGAAGATAAT-3’] and an antisense 3’-RACE abridged universal amplification primer [5’-GGCCACGCGTCGACTAGTAC-3’]. The PCR product obtained was visualized using ethidium bromide staining following agarose gel electrophoresis (1%). Sequencing of the PCR product was performed as described below.

5’-rapid amplification of cDNA end

The 5’-rapid amplification of the cDNA ends was carried out essentially according to the manufacture’s instructions (Invitrogen, Carlsbad, CA, USA). For the synthesis of the cDNA the following gene specific primer GSP (A2 domain 1646-1667) [5’-CAGTCTTTCGACTGTTTGATGA-3’] was used. cDNA (5 µl) was used as a template for PCR along with 5’-abridged anchor primer [5’-GGCCACGCGTCGACTAGTACGCGGIGGGIIGGG-3’] and an antisense GSP2 (A2 domain 1318 -1337) [5’-TAAGGTCGACTGGCCAGATT-
3' and amplification carried out as described earlier. The PCR product was visualized on an ethidium bromide stained agarose gel [1%]. PCR product was then purified and sequenced.

**DNA sequencing**

DNA sequencing was carried out using the ABI PRISM 377 automated DNA sequencer. All sequencing reactions were carried out using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit according to the manufacturer’s instructions. All the PCR products and the pseutarin C nonenzymatic subunit clone were sequenced at least four times in both directions.

**Preparation of peptide digest and mass spectrometry**

Pseutarin C from *Pseudonaja textilis* venom was purified to homogeneity by gel filtration on Superdex 75 column followed by hydroxyapatite column chromatography as described earlier. Purified pseutarin C was subjected to SDS-PAGE (4-20% gradient gel) under reducing conditions according to the method of Laemmli. Following electrophoresis the gel was stained with Coomassie blue. Individual bands were cut out and in gel digested with trypsin. The sequences of the peptides were obtained by electrospray ionization MS/MS using a Q-TOF (Micromass, Manchester, UK).

**Sequence analysis**

Sequence analysis and domain search was carried out using the BLASTX program and Conserved Domain Database respectively at the
National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). Sequence alignments were carried out using the GeneDoc program. Signal peptide and N-glycosylation site prediction was carried out using the PSORT and NetNGlyc prediction site respectively at the Expasy web site (www.expasy.ch).

**Activated Protein C (APC) resistance assay**

Pseutarin C (8 nM) was incubated in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM CaCl$_2$ and 0.5 mg/ml bovine serum albumin. APC (28, 84, 240 and 480 nM) was added and the reaction mixture was incubated for 30 min at room temperature (~25°C). Prothrombin activation was initiated by addition of prothrombin to a final concentration of 2.8 µM. At 20 min, 2.5 µl of the reaction mixture was withdrawn and diluted with 187.5 µl 50 mM Tris-HCl, pH 7.5 containing 100 mM NaCl, 25 mM EDTA solution (stop buffer) in a 96-well microtitre plate. The amount of thrombin formed was determined by the hydrolysis of thrombin-specific chromogenic substrate S-2238 (10 µl; final concentration 100 µM) measured at 405 nm. Measurements were made in triplicates using a 96-well microtiter plates on a Ceres UV 900C ELISA plate reader.

For the control assay bovine FXa (42 nM) was dissolved in 50 mM Tris-HCl buffer pH 7.5, containing 100 mM NaCl, 5 mM CaCl$_2$, 0.5 mg/ml BSA. Bovine FVa (2 nM) was added and the reaction mixture was incubated for 15 min at room temperature, following which different concentrations of APC (28, 84, 240 and 480 nM) were added and incubated further for 30 min.
prothrombin was subsequently added to a final concentration of 2.8 µM and the thrombin formation was measured as described above.

RESULTS AND DISCUSSION

cDNA sequence of pseutarin C nonenzymatic subunit

In an earlier study, we had determined the amino terminal sequences of several internal peptides of the nonenzymatic subunit of pseutarin C\textsuperscript{12}. Degenerate primers were designed based on these peptide sequences. The complete cDNA sequence of pseutarin C nonenzymatic subunit was determined by RT-PCR followed by 5'- and 3'- RACE. The first set of RT-PCR was carried out using degenerate primers (see Methods for details). Using the primer combinations of IP2/A2R we got PCR products of size 2 kb and 700 bp, with IP3/A2R a 2 kb fragment was obtained and for Nter/A2R we got a 600 bp product (data not shown). The PCR products were either gel-eluted or purified directly and their sequences were determined. The deduced amino acid sequences, showed similarity (~60%) to the A2 and A3 domains of mammalian coagulation FV\textsuperscript{19-21}. Based on this sequence information a new sense primer corresponding to A1 domain 604-623 [5'-AATGCAAATGGTTCACAAAA-3'] was designed. This primer was used along with the 3'- RACE adapter primer in order to amplify an almost full-length pseutarin C nonenzymatic subunit cDNA. A ~4 kb PCR product (data not shown) was ligated to pDRIVE vector to obtain the clone PFV-A9. This clone was further analyzed by restriction enzyme digestion (data not shown). The clone, PFV-A9 was then used to determine the sequence of the nonenzymatic subunit of pseutarin C. The sequence of the 5’-end of pseutarin C
nonenzymatic subunit was determined by 5’- RACE. On the basis of the sequence data generated from the clone PFV-A9 and 5’- RACE the complete cDNA sequence of the pseutarin C nonenzymatic subunit was assembled and the amino acid sequence deduced (GenBank number - AY168281). The open reading frame of pseutarin C nonenzymatic subunit is 4383 bp, it encodes a protein of 1461 amino acid, which includes a 30 – amino acid signal peptide and a stop codon (Figure 1).

The amino acid sequences of some of the peptides (19 of a total of 28 peptides sequenced) determined by Edman degradation\textsuperscript{12} matched with the deduced amino acid sequence. However, we observed a few differences, which are summarized in Table 1. Such differences may indicate the presence of more than one isoform of this protein. Edman degradation of some of the peptides showed heterogeneity. For example, peptide Hc101-104 (LIQY) showed both ‘Q’ and ‘I’ at the third position while peptide Hc352-357 showed ‘H’ and ‘F’ at position four. The existence of multiple isoforms is well known in snake venom proteins. In addition, the crude venom used for the purification of pseutarin C was pooled from different snakes. It is also well established that the composition of the venom varies with age, diet, geographical location and the season of the year\textsuperscript{22}. Both these possibilities could explain the observed minor differences between the peptide sequences and the deduced amino acid sequence.

In order to further confirm the deduced amino acid sequence, we also determined the sequence of few of the peptides of both heavy chain and light chain by MS/MS using Q-TOF. The sequences of the peptides obtained by
electrospray ionization MS/MS sequencing are shown in Figure 1. While most of the peptide sequences obtained by MS/MS were identical to the deduced amino acid sequence (17 of the 20 peptides sequenced), we observed the presence of isoforms as in the case of Edman degradation. For example, the sequence of the peptide Hc487-493 obtained by MS/MS was “WTVLDTPFPTAR”, however the deduced amino acid sequence is “WTVLDTDEPTVK”. Thus data from mass spectra and Edman degradation strongly support the deduced amino acid sequence of the nonenzymatic subunit.

**cDNA blot analysis**

cDNA blot analysis was used to estimate the size and abundance of the pseutarin C nonenzymatic mRNA. A 924 bp gene-specific probe (region 1854 – 2778 of pseutarin C) was used to probe the cDNA blot. A single band of ~4.6 kb was observed (Figure 2) which closely corresponded to the open reading frame (Figure 1) and confirmed that the sequence of the nonenzymatic subunit determined is the complete sequence.

In situ hybridization

The snake venom gland consists of mainly two kinds of cells, tall columnar epithelial cells and horizontal spindle-shaped cells, the principal cells being the columnar epithelial cells. *In situ* hybridization was carried out to identify the localization of pseutarin C nonenzymatic subunit mRNA in the *P. textilis* venom gland. Hybridization of the sections with the DIG labeled gene-specific probe (region 1854 – 2778 of pseutarin C) indicated localization of the
pseutarin C nonenzymatic subunit mRNA to columnar epithelial cells (Figure 3). Thus these studies indicate that pseutarin C mRNA is synthesized in the venom gland confirming the biosynthesis of pseutarin C nonenzymatic subunit in the venom gland.

**Sequence homology of pseutarin C with mammalian factor V proteins**

The nonenzymatic subunit of pseutarin C shows significant sequence similarity to mammalian FV. It has similar domain architecture to mammalian FV\(^{19-21}\), consisting of domains A1-A2-B-A3-C1-C2. Alignment of the translated amino acid sequence of pseutarin C nonenzymatic subunit with other mammalian FV sequences shows that it shares overall sequence identity of \(~50\%\) with the functionally important A and C domains (Figure 4). However, the B domain is significantly shorter.

Like the mammalian FV proteins, the A1 and the A2 domains of the nonenzymatic subunit of pseutarin C show similarity to the A domains of ceruloplasmin, a major transport protein of Cu\(^{2+}\) in the blood\(^{23}\). While both A1 and A2 domains have the Cu-oxidase domain (residues 226-332; 551-683 respectively), interestingly the A3 domain does not contain the Cu-oxidase domain; this is unlike mammalian FV proteins wherein all the three A domains have the Cu-oxidase domain\(^{19-21}\). Although Mann et al.\(^{24}\) have shown the presence of a Cu\(^{2+}\) ion in mammalian FV, the significance of this is not clear.

The B domain is a large poorly conserved segment in mammalian FV. It is released upon activation of FV by thrombin as two activation fragments, 150 kDa and 71 kDa\(^{25}\). The B domain of pseutarin C is small, consisting of only 127 amino acids as opposed to 827 residues in bovine FV (Figure 4).
The size of the transcript obtained by cDNA blot analysis confirmed the small size of B domain. In addition, it was also confirmed by RT-PCR where in a 924 bp band was obtained when the region spanning 1854 – 2778 of pseutarin C nonenzymatic subunit was amplified (data not shown). The connecting region of mammalian FV (human) is unusual in that it contains 2 tandem repeats of 17 amino acids and 31 tandem repeats of 9 amino acids\textsuperscript{19,20}. The B domain of pseutarin C nonenzymatic subunit lacks these repeats. The function of the B domain in mammalian FV is not clear. The poor conservation of this domain across the species (~35% identity) indicates that it may be a spacer segment. Further, FV-lacking B domain shows markedly increased procoagulant activity\textsuperscript{26}. The C-terminal region of the B domain has been implicated to play an important role in APC-cofactor activity\textsuperscript{27,28}.

The C1 and C2 domains of pseutarin C show ~50% identity to those of the mammalian FV proteins (Figure 4). The C domains are related to the discoidin family of proteins. The slime mold protein discoidin I, is a galactose-binding lectin\textsuperscript{29}, which is essential for cell adhesion in \textit{Dictyostelium discoideum}. The main function of the C domains (particularly the C2 domain) is to bind to the phospholipid membranes\textsuperscript{30}.

\textit{Proteolytic regulation of cofactor activity}\n
Comparison of sequences at the thrombin and FXa cleavage sites of factor V:

Coagulation FV is a single chain glycoprotein, which has little or no intrinsic procoagulant activity\textsuperscript{19-21}. It is converted to active FVa by proteolytic cleavage mediated by either thrombin or FXa\textsuperscript{31-37}. They cleave at the peptidyl bonds of
Arg709, Arg1018, and Arg1545 leading to the release of the B domain and generation of FVa. Cleavage at Arg709 and Arg1545 is important for full FVa cofactor activity and sufficient for the complete release of B domain, cleavage at Arg1018 enhances the rate of cleavage at Arg1545\textsuperscript{37}. Of the three-thrombin cleavage sites, the nonenzymatic subunit of pseutarin C has only two conserved sites (Figure 5A), the third cleavage site Arg1018, which is expected to be within the B domain, is missing. Since cleavage at Arg709 and Arg1545 is sufficient to release the B domain, absence of third cleavage site may not be of physiological relevance. Thus the size of the less important B domain is efficiently reduced in the snake while retaining the critical residues required for the activation of the nonenzymatic subunit.

Earlier studies\textsuperscript{10} have shown that the nonenzymatic subunit of oscurarin C (from \textit{Oxyuranus scutellatus}) enhances the prothrombin activation mediated by the FXa-like catalytic subunit indicating that the nonenzymatic subunit exists in the active form. Further, based on our previous studies we know that the amino terminal sequencing of the protein bands of pseutarin C obtained on a reducing SDS-PAGE correspond to A1 (heavy chain; 100 kDa), A3 (light chain; 65 kDa), A2 (52 kDa) and A1 (40 kDa) domains of nonenzymatic subunit and the heavy chain of enzymatic subunit (30 kDa), respectively\textsuperscript{12}. The amino terminal of the light chain indicates the cleavage at Arg1545, whereas the size of the heavy chain and the A2 domain indicates the (possible) cleavage at Arg709. In addition, we have not found a single peptide from the B domain during sequencing of the internal peptides. Thus the B domain is apparently absent in the pseutarin C complex.
In addition to thrombin cleavage sites, FXa also cleaves mammalian FV at position Arg348. This additional cleavage site is found in pseutarin C nonenzymatic subunit. During N-terminal sequencing of pseutarin C subunits and their fragments, we observed that there is proteolytic cleavage at position Arg346 located within the A2 domain (Figure 5B). Since the active form of the nonenzymatic subunit exists in the venom as a complex with the catalytic subunit, which is similar to mammalian FXa, it is possible that this and other proteolytic cleavages may be due to the catalytic subunit of pseutarin C. At high FXa concentrations, it cleaves FVa at position Arg1765 on the light chain. This site, however, is missing in pseutarin C (Figure 5B).

In addition to the above cleavage sites, pseutarin C nonenzymatic subunit has two additional cleavage sites at position Leu57-Ser58 in the A1 domain and Arg925-Ser926 in the A3 domain. However, the proteinase(s) that is responsible for these cleavages are yet to be identified.

**Comparison of sequences at the APC cleavage sites of factor V:** Since FVa plays a critical role in blood coagulation, it is not surprising that down regulation of FVa by activated protein C (APC) is an effective way to maintain haemostatic balance. APC, which is a vitamin-K dependent serine proteinase, cleaves FVa at positions Arg306, Arg505, and Arg662 in the heavy chain leading to its inactivation. Initial cleavage of FVa occurs at Arg505, followed by cleavage at Arg306 and Arg662. Cleavage of the FVa molecule at Arg505 and Arg662 results only in the partial loss of activity and it can still bind to FXa. However, the cleavage at Arg306 results in the release of the A2 domain and results in the complete loss of FVa cofactor.
activity\textsuperscript{39}. Interestingly, two of these three sites, including the crucial Arg306, are mutated in the nonenzymatic subunit of pseutarin C (Figure 5C). Further, it is known that binding of FXa to FVa protects it from APC inactivation\textsuperscript{41} and since the nonenzymatic subunit of pseutarin C exists as a complex with the FXa-like subunit in the venom preventing it from being exposed to APC.

To determine the importance of these mutations, we examined the proteolytic inactivation of pseutarin C by APC. Treatment of pseutarin C with APC for 30 min did not result in any loss of the cofactor activity of the nonenzymatic subunit of pseutarin C (Figure 6). In contrast, more than 90\% of the cofactor activity of bovine FVa was lost. Hence pseutarin C activity is not susceptible to inactivation by APC. Thus APC resistance gives an added advantage to pseutarin C in its role as a toxin.

\textit{Post-translational modifications}

\textbf{Disulphide linkage:} Disulphide bonds play an important role in determining the overall 3-dimensional structure and stability of the protein. Comparison of the disulphide bond pattern between bovine FVa\textsuperscript{42,43} and pseutarin C nonenzymatic subunit (proposed based on structural similarity) is depicted in Figure 7 (A and B). In mammalian FVa the A1 and A2 domains are identical in their disulphide-bonding pattern, both have a “α” loop (26 amino acids) followed by a “β” loop of 82 amino acids (Figure 7A). The A2 domain in addition has two free cysteine residues. However, the A3 domain has only a “α” loop. Similar disulphide bonding pattern exists in factor VIII (FVIII) and also ceruloplasmin in A1, A2 and A3 domains. Further the sequence identity within the “α” and “β” loops of the A1, A2 and A3 domain between mammalian
FV and FVIII is high (~75%). Interestingly, in pseutarin C nonenzymatic subunit the pattern of disulphide linkage appears to be significantly different. While the A1 domain has the “α” and “β” loops, the A2 and A3 domains have only the “α” loop (Figure 7B). The amino acids within in the “α” loops of the A1, A2 and A3 domains share 78%, 63% and 82% identity to the “α” loops in mammalian FV respectively. In addition, there are three free cysteine residues (two in A2 and one in A3 domains); Cys1032 in A3 domain and Cys570 in A2 domain of pseutarin C and Cys566 in bovine A2 domain appear to be in conserved position.

C1 and C2 domains in bovine FV have one conserved disulfide bond forming a “γ” loop (Figure 7A). Both these “γ” loops contain a single free cysteine residue. Pseutarin C also contains cysteine residues that could form “γ” loops in its C domains. However, it does not have the free cysteine residues.

**Glycosylation:** Glycosylation is known to play an important role in the stability as well as the folding of proteins. Mammalian FV and FVa both contain potential glycosylation sites. Most of the glycosylation sites (18 of the 29) in bovine FV are present in the B domain (Figure 7C); the heavy chain has 7 potential N-glycosylation sites (3 in the A1 domain and 4 in the A2 domain) and the light chain has 4 sites (2 in the A3 and 1 each in the C1 and C2 domains). Since the B domain of pseutarin C nonenzymatic subunit is small, most of the glycosylation sites are deleted. It has only 11 potential N-glycosylation sites (Figure 7D). During sequencing of the internal peptides, we determined the complete sequence of two peptides (381-386 “YLDNFS”;}
1395-1399 "DNSTW") by Edman degradation indicating that these potential sites are not glycosylated. Of the remaining nine potential N-glycosylation sites, only three are conserved and the rest are novel potential N-glycosylation sites (Figure 7D). The heavy chain has six potential N-glycosylation sites of which three are in the A1 domain and three in the A2 domain. The light chain of pseutarin C nonenzymatic subunit has three potential N-glycosylation sites, two in the A3 and one in the C1 domain.

Altering either the carbohydrate moiety or the glycosylation site can either enhance or cause loss of function of the protein. For example, the α-neurotoxins from snake venoms are targeted against nicotinic acetylcholine receptors (nAChR)44. However, these neurotoxins fail to bind to snake’s own nAChR receptor although the critical residues in the binding site are conserved in these receptors. Studies by Takacs et al.45 have shown the presence of the unique N-glycosylation signal in the ligand-binding domain of N. haje, prevents the binding of the α-neurotoxins. Nevertheless, the presence of this carbohydrate moiety does not alter the binding and response of acetylcholine45. By this strategy the snake has adapted well to resist the action of its own venom. Since the snake might have a similar, if not identical, coagulation FV present in its plasma where it performs the role of a haemostatic factor, it would be interesting to examine the role of these unique glycosylation in pseutarin C nonenzymatic subunit.

Phosphorylation and sulfation sites: Mammalian FV is both sulfated and phosphorylated46-49. Phosphoamino acid analyses combined with peptide mapping techniques indicate the presence of a phosphoserine (Ser692) at the
C-terminal end of the FVa heavy chain. It is speculated that phosphorylation may be important for regulating the inactivation of FVa by APC. This phosphorylation site (Ser692) is absent in pseutarin C nonenzymatic subunit. As mentioned above, the APC cleavage sites are not conserved in pseutarin C. The absence of Ser692 may make pseutarin C a less preferred substrate of APC. Although several other potential phosphorylation sites are present in pseutarin C nonenzymatic subunit, it is not clear if any of these sites are indeed phosphorylated.

Sulfation is known to occur in both the mammalian FV heavy chain and in the activation peptide, with the greatest amount of sulfate added to the activation peptide. There are six potential sulfation sites in human FV, residues 696, 698, 1494, 1510, 1515 and 1565 (data not shown). None of these sites are present in pseutarin C nonenzymatic subunit. It is speculated that sulfation may be important for regulating the activation of FV by thrombin. However, in the case of pseutarin C, the nonenzymatic subunit may not be activated by thrombin (or equivalent proteinase), as such proteinases have not been found in P. textilis venom.

**Pseutarin as a venom toxin**

Blood coagulation factors, including FV, are constitutively expressed in the liver and they play a critical role in the haemostatic function. Pseutarin C (and the other snake venom prothrombin activators) are expressed in an unusual tissue, the venom gland. These prothrombin activators are present in activated form in large quantities in the venom. Both group C (pseutarin C) and group D (trocarin D) activators are lethal when injected into mice.
indicating that these prothrombin activators play the role of toxin in the venom. Since snake would also need haemostatic functions for its survival, it is not far fetched to hypothesize that similar, if not identical, factor might be present in its plasma where it functions like a haemostatic factor. Thus, two closely related proteins play distinctly different roles - as a toxin in the venom and as a haemostatic factor when present in the plasma.

In conclusion, this is the first sequence of a FV-like protein from a non-hepatic and non-mammalian source. This nonenzymatic subunit of pseutarin C shows identical domain architecture to mammalian FV consisting of domains A1-A1-B-A3-C1-C2. Unique post-translational modifications and with its resistance to APC, make it highly suitable for its role as toxin. The complete sequence of this protein will help us understand structure-function relationships of FVa in prothrombin activation.

ACKNOWLEDGMENTS

The authors wish to acknowledge P. Srinivasa Rao, Department of Biological Sciences, National University of Singapore for his helpful discussions during cloning.
REFERENCES


FIGURE LEGENDS

Figure 1: Complete cDNA sequence of pseutarin C nonenzymatic subunit. Sequence was determined using the clone PFV-A9, the sequence of the 5' and the 3'-untranslated regions was determined by rapid amplification of cDNA ends (RACE). The deduced amino acid sequence is shown below the nucleotide sequence. The predicted signal peptide is shown in italics. Sequences that were obtained by Edman degradation are underlined; sequences of peptides obtained by Q-TOF are double underlined. The beginning and the end of each domain is marked by an arrow.

Figure 2: cDNA blot analysis of *P. textilis* cDNA library. (A) Marker (B) cDNA blot probed with pseutarin C nonenzymatic subunit gene specific probe (See text for details).

Figure 3: *In situ* hybridization. (A) Haematoxylin and Eosin staining. *P. textilis* venom gland showing acini’s (a) lined with single layer of epithelium. (B) Localization of nonenzymatic subunit of pseutarin C mRNA in *P. textilis* venom gland by *in situ* hybridization using digoxigenin-labeled purified PCR product as probe. Arrows indicate gene specific staining. The mRNA of pseutarin C is localized to the columnar epithelial cells.

Figure 4: Sequence alignment of pseutarin C nonenzymatic subunit with mammalian FV. The deduced amino acid sequence of pseutarin C nonenzymatic subunit (AY168281) is compared with bovine FV (Q28107), human FV (P12259) and mouse FV (T42764) sequences. Sequence alignment was done using the GeneDoc program. Gaps (-) have been inserted for optimal alignment. Identical amino acid residues are shaded in black. In B domain amino acid residues identical in all three mammalian sequences are shaded in grey.

Figure 5: Proteolytic cleavage sites (A) Thrombin cleavage site. Of the three-thrombin cleavage sites only two are present (Arg709 and Arg1545) in pseutarin C nonenzymatic subunit, but they are sufficient to release the activation peptide (B domain) (See text for details). (B) FXa cleavage site. Although similar site is found in pseutarin C nonenzymatic subunit, a proteolytic cleavage is found at
Arg346 (dotted arrow) in native pseutarin C. Additional FXa cleavage site on the light chain (Arg1765) is absent in pseutarin C (See text for details). Numbering of the amino acids is based on human FVa sequence. (C) APC cleavage site. Two of the three APC cleavage sites are absent in pseutarin C. Arrows indicate the site of cleavage in bovine FV. (See text for details)

**Figure 6: APC resistance assay.** Varying concentrations of APC was added either to pseutarin C (8 nM) or bovine FXa-FVa (FXa 42 nM; FVa 2 nM) complex diluted in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 5 mM CaCl₂ and 0.5 mg/ml BSA. The reaction mixture was incubated for 30 min at room temperature. Prothrombin was added to a final concentration of 2.8 μM and thrombin formed was assayed using thrombin specific chromogenic substrate S-2238 as described in the methods section. Each point represents an average of two independent experiments each carried out in triplicates.

**Figure 7: Post-translational modifications in bovine V and pseutarin C nonenzymatic subunit.** The disulphide pattern in bovine FVa (A) and pseutarin C nonenzymatic subunit (B) are shown. The “α”, “β” and “γ” loops are labeled according to Xue et al. Free cysteines are shown as -SH. Both bovine FV and pseutarin C nonenzymatic subunit are glycosylated. The potential N-glycosylation sites in bovine FV (C) and pseutarin C nonenzymatic subunit (D) are shown. The open circles represent potential novel N-glycosylation sites in pseutarin C nonenzymatic subunit compared to bovine FV.
GGGGGGGCAGGCAGAACTGACTTCCGTGTATCTTTCAGCACATTAGCATCA
1  ATGGGAAGATACAGTGTGAGCCCTGTCCCCAAATGTCTTCTACTGATGTTCCTGGGTTGG 60
21  S G L K Y Y Q V N A A Q L R E Y H I A A 40
61  TCAGGGCTGAATATTACCATGCCGAGTCCGCTACAGCGGGAGTACCATATAGCTGCT
121  M  G  R  Y  S  V  S  P  V  P  K  C  L  L  M  F  L  G  W 20
181  GATCTTACGTTTAAAAAATCTTCTATAGAAATATGAACTAGATTTCAAACAAGAGGAG
241  S  G  L  K  Y  Y  Q  V  N  A  A  Q  L  R  E  Y  H  I  A  A 40
121  CAGCTGGAAGACTGGGATTACAACCCCCAACCTGAGGAGCTATCCAGATTACGATGCC
181  Q  L  E  D  W  D  Y  N  P  Q  P  E  E  L  S  R  L  S  E  S 60
241  GATCTTACGTTTAAAAAATCTTCTATAGAAATATGAACTAGATTTCAAACAAGAGGAG
301  CTCATAATTTATTTCCAAGAATTTTGCTACTCTAGCTGAGATTATCTCATGAGAGTG
361  GATCTTACGTTTAAAAAATCTTCTATAGAAATATGAACTAGATTTCAAACAAGAGGAG 420
121  V Y N K W S E G S S S Y S D G T S D V E R 140
421  CTGAATGCAAATGGTTCACAAAAATTCTTCAACAGAGAATATGTGCTGATGTTTTCTGTG
201  L  N  A  N  G  S  Q  K  F  F  N  R  E  Y  V  L  M  F  S 220
481  ATGGGCGCAAAAGAAGCTGATCCTCCGTCAGCAGAGATGGATATCTTCTCTGGGTCTG
1021  AAGTTATCCCTAGAAGACATGATGGATATCTTCTCTGGGTCTG
341  K L S F R E L N K I R N W E Y F I A E 360
Figure 1
Figure 2
A3 Domain

PFV  : TINS QIEHI TTAAEV KL S I P LGSQ-VRSRAAR7T-----P P H A K QTTSSQ N R GIGP I ARYXV TIV CTFN YLTLARQ R E S S E C D----KKE
BTV  : SNT- QIEHI TTAAEV KL S I P LGSQDD YD YV Y----PEDTVY K R Y A K YLTLARQ R E S S E C D----KKE
HIV  : SNN- QIEHI TTAAEV KL S I P LGSQDD YD YV Y----PEDTVY K R Y A K YLTLARQ R E S S E C D----KKE
MIV  : GHS- QIEHI TTAAEV KL S I P LGSQDD YD YV Y----PEDTVY K R Y A K YLTLARQ R E S S E C D----KKE

BTV  : D N A IQ PN KT YTYVW HATT RSGP ENPGSA C RA WAYYS A VN P E K DIHSGLIGP L LIC R KG TLDKET N MPV D M REFVL L FM V FDE K KSWY YD K KPTRSWRRASSEVKNS H K F H AING MI Y N L P GL R M
HIV  : D N A VQ PN SS YTYVW HATE RSGP ESPGSA C RA WAYYS A VN P E K DIHSGLIGP L LIC Q KG ILHKDS N MPV D M REFVL L FM T FDE K KSWY YE K KSRSSWRLTSSEMKKS H K F H AING MI Y S L P GL K M
MIV  : D D A VQ PN SS YTYVW HATK RSGP ENPGSA C RA WAYYS A VN V E R DIHSGLIGP L LIC R KG TLHMER N LPM D M REFVL L FM V FDE K KSWY YE K SKGSRRIESPEEK-NA H K F Y AING MI Y N L P GL R M

PFV  : Y KD E N V HW HLLN M GG PK DIHVV N FHGQT FTEEGREDN QLGV L PLLPG T F ASIK MK P SK I G T WLL E TEVGE N Q ER GMQ AL F TVI D K
BTV  : Y EQ E W V RL HLLN L GG SR DIHVV H FHGQT LLENGTQQH QLGV W PLLPG S F KTLE MK A SK P G W WLL D TEVGE I Q RA GMQ TP F LIV D R
HIV  : Y EQ E W V RL HLLN I GG SQ DIHVV H FHGQT LLENGNKQH QLGV W PLLPG S F KTLE MK A SK P G W WLL N TEVGE N Q VA GMQ TP F LIM D R
MIV  : Y EQ E W V RL HLLN M GG SR DIHVV H FHGQT LLDNRTKQH QLGV W PLLPG S F KTLE MK A SK P G W WLL D TEVGE N Q VA GMQ TP F LII D K

C1 Domain

PFV  : D CK L PMGL AS G I I Q DSQI S AS GHVGY WEP K LARLNN T G K YNAW SII K KEHE---H PWIQ I D L Q RQ V VI TGIQTQG TVQL L QHSY T V E YF V T YS EDGQ NW IT F K G RHSETQ M H F E GNSD GT T VK E

C2 Domain


PFV  : D GH V K H FF K PPI L SRFIRIIPKTWNQ Y IALRIELFGC EVF
BTV  : R GH V K N FF N PPI I SRFIRIIPKTWNQ S IALRLELFGC DMY
HIV  : K GH V K N FF N PPI I SRFIRVIPKTWNQ S ITLRLELFGC DIY
MIV  : K GH M K N FF N PPI I SRFIRIIPKTWNQ S IALRLELFGC DIY

Figure 4
A. Thrombin cleavage sites

PFV : GLRSFK  //  PFV : YLRTIN
BFV : GLRSFR  //  BFV : YLRSNT
HFV : GIRSFR  //  HFV : YLRSNN
MFV : GIRSFR  //  MFV : YLRGHG
P FV: GIRSFK  //  P FV: YLRSNN

B. FXa cleavage sites

PFV : RRYKAQ  //  PFV : EEKLIG
BFV : KKYRSL  //  BFV : RRASSE
HFV : KKYRSQ  //  HFV : RLTSSE
MFV : KIYRSQ  //  MFV : IESPEE
P FV: KKYRSL  //  P FV: RLTSSE

C. Protein C cleavage sites

PFV : PDTLTR  //  PFV : SVKGVQ
BFV : KTRNPK  //  BFV : DRRGIQ
HFV : KTRNLK  //  HFV : DRRGIQ
MFV : KTRSPK  //  MFV : DQRGVQ
P FV: KTRKPK  //  P FV: DKRGIQ

PFV : DANYDD
BFV : CIRNDD
HFV : CIPDDD
MFV : CNRDYD
P FV: CIRDDD

Figure 5
Figure 6

The graph illustrates the effect of APC concentration on FVa activity (%). The x-axis represents nM APC, with values ranging from 0 to 500 nM. The y-axis represents FVa activity (%), ranging from 0 to 110%. Two sets of data points are shown: black circles for BFXa-FVa and white circles for Pseutarin C. The data points are plotted with error bars indicating variability. The graph shows a decrease in FVa activity as the concentration of APC increases.
A. Mammalian Factor Va

B. Pseutarin C nonenzymatic subunit

C. Bovine factor V

D. Pseutarin C nonenzymatic subunit

Figure 7
### TABLE 1

N terminal sequences of pseutarin C peptides

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<tr>
<th>Location</th>
<th>Edman degradation</th>
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<tr>
<td>Hc79–82</td>
<td>EKPQ</td>
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<td>GLIGP</td>
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The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (Pseudonaja textilis) venom, shows structural similarity to mammalian coagulation factor V

Veena S Rao, Sanjay Swarup and R Manjunatha Kini