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Recombinant snake venom prothrombin activators

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Three prothrombin activators; ecarin, which was originally isolated from the venom of the saw-scaled viper *Echis carinatus*, trocarin from the rough-scaled snake *Tropidechis carinatus*, and oscutarin from the Taipan snake *Oxyuranus scutellatus,* were expressed in mammalian cells with the purpose to obtain recombinant prothrombin activators that could be used to convert prothrombin to thrombin. We have previously reported that recombinant ecarin can efficiently generate thrombin without the need for additional cofactors, but does not discriminate non-carboxylated prothrombin from biologically active γ-carboxylated prothrombin. Here we report that recombinant trocarin and oscutarin could not efficiently generate thrombin without additional protein cofactors. We confirm that both trocarin and oscutarin are similar to human coagulation factor X (FX), explaining the need for additional cofactors. Sequencing of a genomic fragment containing 7 out of the 8 exons coding for oscutarin further confirmed the similarity to human FX.

Introduction

Thrombin is a key enzyme in blood coagulation and formation of thrombin leads to activation of platelets and plasma coagulation.[1] Thrombin is formed from prothrombin by proteolytic activation in the prothrombinase complex which include coagulation factors Xa and Va.[2,3] In addition prothrombinase activation requires calcium ions and phospholipids such as those on membrane surfaces. For activation by the prothrombinase complex prothrombin must have a functional Gla-domain including 8–10 γ-carboxylated glutamic acid residues. Some patients with cancer and patients treated with vitamin K antagonists have incompletely γ-carboxylated coagulation factors and thus reduced coagulation ability.[4-7]

Apart from the natural activation pathway, prothrombin can also be activated by prothrombin activators in snake venoms.[8,9] Such prothrombin activators are known since long and belong to different classes depending on their requirements for cofactors. Several snake venom prothrombin activators are utilized in coagulation factors assays for research purposes and as diagnostic reagents.[10] However, the use of components from snake venom for assay and other purposes is not optimal as many of the snakes belong to endangered species and the prothrombin activators may show considerable variability in their prothrombin-activating properties. Depending on the source of prothrombin activator variable results are often obtained.

We were interested in developing robust and reproducible methods to quantify both incompletely and fully γ-carboxylated prothrombin and we have therefore investigated the possibilities of producing snake venom prothrombin activators by recombinant technologies. In this paper we report our conclusions from this work.

Results and Discussion

We selected two types of prothrombin activators for further studies; ecarin from the venom of the saw-scaled viper *Echis carinatus*, and, the snake venom prothrombin activators trocarin from *Tropidechis carinatus* and oscutarin from *Oxyuranus scutellatus*. According to Kini, trocarin depends on calcium and phospholipids as well as

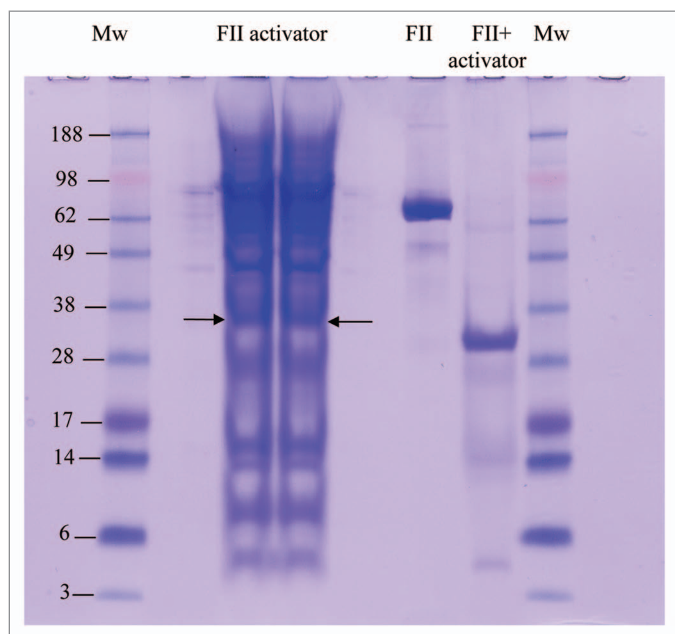

Figure 1. Prothrombin (FII) activator purchased from Venom Supplies was separated on SDS-PAGE gels. Samples from the gels were used for identification of the prothrombin-activating protein. The bands indicated with arrows were found to contain a trocarin-like protein. Several bands were analyzed both by N-terminal sequencing and by MS/MS but conclusive identification was achieved only with the bands indicated, although traces of trocarin like sequences were detected in additional bands by MS/MS. On the right side of the gel prothrombin (FII) incubated with and without prothrombin activator is shown. Incubation of prothrombin with prothrombin activator as described in Materials and Methods generated thrombin, which was confirmed by N-terminal amino acid sequencing. Molecular weight marker (Mw) is SeeBlue+2 from Invitrogen; numbers in the figure indicate the molecular weights in kilodaltons.

FVa for activity and generates thrombin, while ecarin is not dependent on any co-factors but generates meizothrombin.[8] Oscutarin was said to require calcium and phospholipids as cofactors, but not FVa.[8]

From assays with commercially available venom from *Oxyuranus scutellatus* (Coastal Taipan) as well as with purified prothrombin activator from *Oxyuranus scutellatus* we found that prothrombin was activated to thrombin without addition of FVa (**Fig. 1**). In further analysis of the prothrombin activator we identified a protein with similarity to trocarin (database accession P81428) as one of the components (**Fig. 1**), although we were not able to confirm that the protein was identical to trocarin.

Based on the trocarin and oscutarin protein sequences available we constructed synthetic sequences for expressing trocarin and oscutarin, and the trocarin and oscutarin constructs were used to obtain protein from transient expression in COS-7 cells. Supernatants from trocarin and oscutarin transfected cells did not activate prothrombin to thrombin under the same buffer and incubation conditions as we obtained thrombin with using the commercially available prothrombin activator. Since lack of thrombin formation could potentially be due to that the recombinant trocarin and oscutarin were produced in the non-activated form, we treated the trocarin and oscutarin-containing supernatants with Russell's Viper Venom X (RVV-X) as described in Materials and Methods. However, either the activation or the addition of phospholipids improved thrombin formation and we concluded that recombinant trocarin and oscutarin could not efficiently generate thrombin without additional factors. We obtained similar results with human FX-containing cell culture supernatant that was used in parallel for control purposes.

As limited sequence information was available at this time, we cloned the genomic sequences spanning the start of exon 2 to the stop codon in exon 8 from a taipan snake (**Fig. 2**). The sequences obtained from translating the exons showed to be almost identical to oscutarin (database accession FAXC_OXYSU), and highly similar to trocarin (**Fig. 3**). The genomic trocarin sequence later published by Reza et al. is also highly similar to our sequence, our sequence giving 96% identity and score 10870 to accession DQ533832.1 in a BLAST search.[11] In all the reported FXa-like snake proteases, including the one we report here, the exon-intron pattern is the same as in the human FX gene, although intron sizes differ. Both trocarin and oscutarin are similar to human coagulation factor X, and both were after activation with RVV-X able to hydrolyze a FXa chromogenic substrate. Research by Reza et al. has indeed shown that trocarin has been recruited to the venom by duplication of the snake FX gene.[12] The activity we observed with the *Oxyuranus scutellatus* prothrombin activator, which did not require addition of FVa, may be explained by either the presence of additional prothrombin activators not dependent on cofactors, or, by the presence of a FVa-like protein in the venom.

In parallel with expressing and characterizing trocarin and oscutarin, we also produced and characterized recombinant ecarin from CHO cells as was recently described.[13] Our characterization of recombinant ecarin confirmed that r-ecarin could efficiently generate thrombin without any additional co-factors.[13-15] We found that although the primary product from digesting prothrombin with ecarin is meizothrombin, the meizothrombin was very rapidly converted to thrombin, and therefore the final product is thrombin. We have characterized thrombin generated from prothrombin digested with r-ecarin, and have not been able to detect any differences from thrombin generated through the prothrombinase complex. The N-terminal amino acid sequence of the two peptide chains in ecarin-generated thrombin is the same as for ordinary thrombin, and titration with Hirudin and assays with chromogenic substrates indicate thrombin activity similar to international thrombin standards (results not shown).

As opposed to FXa and FXa-like snake venom proteases such as trocarin and

oscutarin, ecarin converts prothrombin to thrombin also if the prothrombin is not γ-carboxylated.[12]

Non-carboxylated prothrombin cannot be converted to thrombin through the coagulation cascade and therefore ecarin-based assays cannot be used to quantify biologically active prothrombin. The work summarized in this paper shows that recombinant snake venom prothrombin activators are not likely to fill this need, as multiple components would need to be produced and those components could more conveniently be substituted by the human or bovine FVa and FXa that are easily accessible. An easy to use chromogenic method to quantify biologically active prothrombin utilizing FXa and FVa is available as the Prothrombinase assay.[16-18]

Snake venom based methods are still important in coagulation factor research and in diagnostic reagents, and research in this field provides insights in the mechanisms and evolution of the coagulation system. However, both snake venoms and the coagulation system are complex and consist of multiple components. Many of the components are enzymes or zymogens potent at very low concentrations and tiny amounts of such residual components may result in obscured data. Therefore, recombinant venom components and recombinant coagulation factors are likely to be very useful in future research as well as in the development of improved diagnostic reagents.

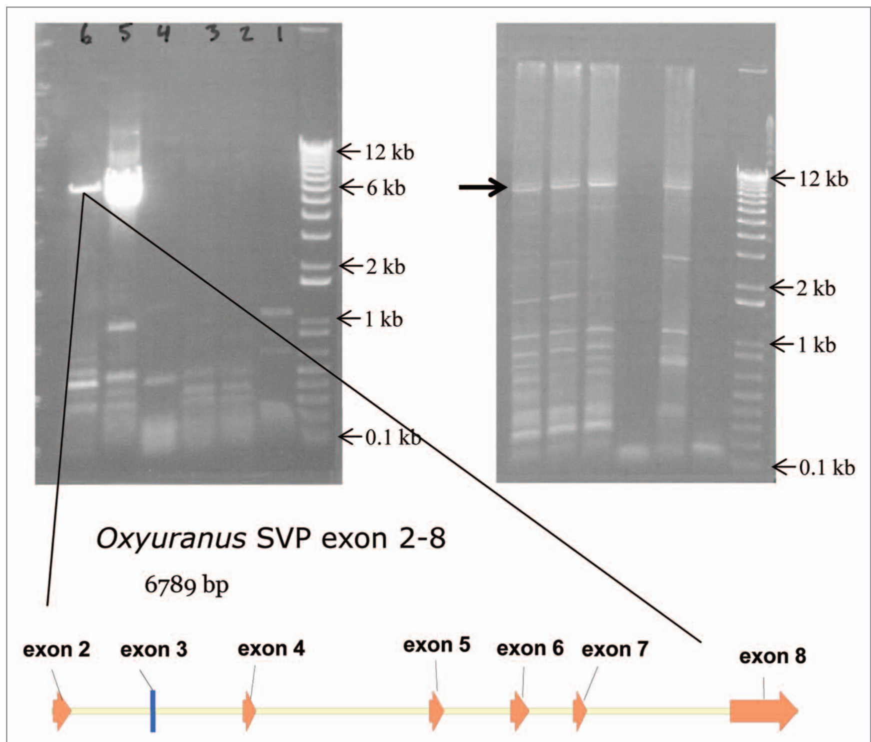

Figure 2. The two photos show agarose gels on which PCR fragments from PCR reactions with genomic DNA prepared from taipan snake tissue have been separated. The indicated band in lane 6 (left photo) contained a fragment spanning exon 2–8, which was cloned and named *Oxyuranus* SVP (SVP for snake venom protease). Lane 5 contains a PCR product spanning exon 4–8. On the right-side photo the PCR product from primers to span exon 1–2 is indicated by an arrow. The molecular weight marker is 1 kb+ ladder from Invitrogen; selected band sizes are indicated in the figure.

Materials and Methods

Material. Prothrombin activator from *Oxyuranus scutellatus* was purchased from Venom Supplies, Australia. Snake venom from *Oxyuranus scutellatus* was purchased from Sigma (article no V31299). Snake tissue from a taipan snake was a kind gift from Skansen Akvariet, Stockholm Sweden. DNA was prepared from snake tissue using a kit for hard tissue (cat. No RPN8509) from Amersham. Recombinant prothrombin was produced by AstraZeneca.

Generation of thrombin. The purchased prothrombin activators were used to generate thrombin by incubating 16 μg prothrombin with 1 μg prothrombin activator (Venom Supplies) in activation buffer consisting of 17.5% (w/vol) PEG6000, 50 mM TRIS-HCl, 100 mM NaCl and 5 mM $CaCl_2$, pH 7.4, for 20–120 min at 37°C. As a control FII was incubated under the same conditions without the addition of prothrombin activator. Formation of thrombin was confirmed by incubating diluted samples (approximately 1 μg/mL) with a 1:1 mix of 1 mM S-2238, a chromogenic thrombin substrate (Chromogenix), and measuring absorbance at 405 nm, and, by SDS-PAGE (**Fig. 1**). Correct N-terminal sequence of the thrombin formed was confirmed by N-terminal sequencing.

Identification of proteins in prothrombin activator. Samples were analyzed by SDS-PAGE (**Fig. 1**) and by N-terminal sequencing of the same samples blotted to Immobilon P membranes (Millipore). Samples from SDS-PAGE (**Fig. 1**) were also analyzed by mass spectrometry; MS (peptide mapping) and MS/MS (amino acid sequence information).

Recombinant prothrombin activators. The amino acid sequences for trocarin (database accession FAXD_TROCA) and oscutarin (database accession FAXC_OXYSU) were reverse translated and the nucleotide sequences were optimized for expression in a mammalian host. Synthesized sequences were cloned into the pCDNA 3.1 expression vector (Invitrogen). Recombinant trocarin and oscutarin were obtained from COS-7 cells by transient transfection using Lipofectamine 2000 as described by Invitrogen. COS-7 cells were cultured in DMEM F12 with 10% FBS (Invitrogen). To prevent background coagulation factor activity from FBS in the cell culture medium, the FBS was heat inactivated to remove coagulation factor activity (water bath 65°C for 30 min). During transient transfection procedures FBS was reduced to 1%. Recombinant ecarin was

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FAXC_OXYSU    (1) MAPQLLLCLILTFLWSLPEAESNVFLKSKVANRFLQRTKRANSLYEEFRSGNIERECIEERCSKEEAREVFEDDE
FAXD_TROCA    (1) MAPQLLLCLILTFLWSLPEAESNVFLKSKVANRFLQRTKRSNSLFEEIRPGNIERECIEEKCSKEEAREVFEDNE
exon 2        (1) ------------------------FLKSKVANRFLQRTKRANSLFEEFRSGNIERECIEERCSKEEAREVFEDDE

FAXC_OXYSU   (76) KTETFWNVYVDGDQCSSNPCHYRGTCKDGIGSYTCTCLSGYEGKNCERVLYKSCRVDNGNCWHFCKPVQNDIQCS
FAXD_TROCA   (76) KTETFWNVYVDGDQCSSNPCHYRGTCKDGIGSYTCTCLPNYEGKNCEKVLYQSCRVDNGNCWHFCKRVQSETQCS
exon 2       (52) K--------------------------------------------------------------------------
exon 3        (1) --ETFWNVYV-----------------------------------------------------------------
exon 4        (1) -----------GDQCSSNPCHYRGTCKDGIGSYTCTCLFGYEGKNCER---------------------------
exon 5        (1) -------------------------------------------------LYKSCRVDNGNCWHFCKPVQNDIQCS

FAXC_OXYSU  (151) CAEGYLLGEDGHSCVAGGNFSCGRNIKTRNKREASLPDFVQSQNAILLKKSDNPSPDIRIVNGMDCKLGECPWQA
FAXD_TROCA  (151) CAESYRLGVDGHSCVAEGDFSCGRNIKARNKREASLPDFVQSQKATLLKKSDNPSPDIRIVNGMDCKLGECPWQA
exon 5       (27) CAEGYLLGEDGHSCVAGG---------------------------------------------------------
exon 6        (1) -------------------FSCGRNIKTRNKREASLPDFVQSQNATLLKKSDNPSPDIRIVNGMDCKLGECPWQ-
exon 7        (1) --------------------------------------------------------------------------A

FAXC_OXYSU  (226) VLVDEKEDAFCGGTILSPIYVLTAAHCINQTKMISVVVGEINISRKNPGRLLSVDKIYVHQKFVPPKKGYEFYEK
FAXD_TROCA  (226) VLINEKGEVFCGGTILSPIHVLTAAHCINQTKSVSVIVGEIDISRKETRRLLSVDKIYVHTKFVPPN-YYYVHQN
exon 7        (2) VLVDEKEGVFCGGTILSPIYVLTAAHCINQTEKISVVV-------------------------------------
exon 8        (1) ---------------------------------------EIDKSRVETGHLLSVDKIYVHKKFVPPKKGYKFYEK

FAXC_OXYSU  (301) FDLVSYDYDIAILQMKTPIQFSENVVPACLPTADFANQVLMKQDFGIVSGFGRIFEKGPQSKTLKVLKVPYVDRH
FAXD_TROCA  (300) FDRVAYDYDIAIIRMKTPIQFSENVVPACLPTADFANEVLMKQDSGIVSGFGRIQFKQPTSNTLKVITVPYVDRH
exon 8       (37) FDLVSYDYDIAIIQMKTPIQFSENVVPACLPTADFANQVLMKQDFGIISGFGRIFEKGPKSNTLKVLKVPYVDRH

FAXC_OXYSU  (376) TCMLSSESPITPTMFCAGYDTLPRDACQGDSGGPHITAYRDTHFITGIVSWGEGCAQTGKYGVYTKVSKFILWIK
FAXD_TROCA  (375) TCMLSSDFRITQNMFCAGYDTLPQDACQGDSGGPHITAYRDTHFITGIISWGEGCARKGKYGVYTKVSKFIPWIK
exon 8      (112) TCMVSSESPITPTMFCAGYDTLPRDACQGDSGGPHITAYRDTHFITGIVSWGEGCAKKGKYGIYTKVSKFILWIK

FAXC_OXYSU  (451) RIMRQKLPSTESSTGRL
FAXD_TROCA  (450) KIMSLK-----------
exon 8      (187) RIMRQKLPSTESSTGRL
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Figure 3. Alignment of the amino acid sequences of oscutarin (FAXC_OXYSU), trocarin (FAXD_TROCA) and the translated exons 2–8 from the taipan snake. Identical residues are indicated in blue. Residues deviating from FAXC_OXYSU are highlighted in cyan. Non-conservative changes are indicated in red and conservative changes are indicated in black font. Amino acids derived from codons split between exons are not included for the translated exons.

obtained from CHO cells cultured in chemically defined medium as described by Jonebring.[13] Diluted cell culture supernatants containing the respective protein were used in further experiments. The ability of recombinant trocarin and recombinant oscutarin-containing supernatants to generate thrombin was tested by addition of 1–10 μg/mL prothrombin followed by incubation at 37°C in activation buffer as above. Generated thrombin was detected by addition of 1 mM S-2238 and measuring absorbance at 405 nm. Ecarin-containing supernatant was used as positive control.

Recombinant trocarin and oscutarin were activated by incubation of supernatants diluted in buffer stock to a final concentration of 50 mM TRIS-HCl, pH 7.4, 1% BSA (w/v), 150 mM NaCl and 50 ng/mL RVV-X. Activation and activity of trocarin and oscutarin was confirmed by adding (1:1) a 1 mM solution of the FXa chromogenic substrate S-2765 (Chromogenix) and measuring absorbance at 405 nm. Supernatant containing human FX was used as positive control. The supernatants containing activated trocarin, oscutarin or human FX were also incubated with prothrombin (approximately 1 μg/mL) and tested for formation of thrombin by addition of S-2238 to 0.5 mM in FVIII assay buffer (Chromogenix). Addition of phospholipids was done as recommended by supplier (Rossix).

Cloning and sequencing of exon 2–8. Sequences for cloning primers were obtained from mRNA sequences from *Oxyuranus* snake venom proteases available in databases. The positioning of the primers was guided by predicting exon-intron boundaries through aligning the protein sequences of trocarin and *Oxyuranus* snake venom proteases with the human FX protein sequence for which the exon-intron boundaries was known. A 6789 bp fragment containing exon 2–8 was amplified using primers EX2F (5'-TAT TCT TAA AAA GCA AAG TGG CAA ATA G-3') and EX8R (5'-TTA GAG CCG ACC AGT GCT TGA C-3'), and the Expand Long Template PCR system (Roche). A second fragment containing exon 4–8 was also amplified using primers EX4F (5'-ATG GGG ATC AGT GTT CAT CAA A-3') and EX8R. These two fragments were cloned and sequenced. Primers mexf1 (5'-ATG GCT CCT CAA CTA CTC CTC TG) and EX2R (5'-GTT TTC TCG TCA TCT TCA AAT ACC TC-3') for amplification of exon 1–2 gave a PCR product which we did not succeed in cloning (**Fig. 2**). Multiple clones of the exon 2–8 fragment were sequenced to obtain a consensus sequence. The amino

acids sequences of the translated exons are shown in **Figure 3**. The genomic sequence is available in the database under accession number JX661061.

Disclosure of Potential Conflicts of interest

The author declares that there is no conflict of interest.

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