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Research paper

Structural and biochemical characterisation of VaF1, a P-IIIa fibrinogenolytic metalloproteinase from *Vipera ammodytes ammodytes* venom

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ABSTRACT

A high molecular mass metalloproteinase with α-fibrinogenolytic activity, termed VaF1, was purified from nose-horned viper (*Vipera ammodytes ammodytes*) venom. Subcutaneous injection of 9 μg of VaF1 did not induce bleeding in rats. Nevertheless, *in vitro* it degraded collagen IV, nidogen and fibronectin, components of the extracellular matrix, although with low efficacy and narrow specificity. VaF1 would be expected to exert anti-coagulant action, due to its hydrolysis of fibrinogen, factor X, prothrombin and plasminogen, plasma proteins involved in blood coagulation. The enzyme is a single-chain glycoprotein with a molecular mass of 49.7 kDa, as determined by mass spectrometry, and multiple isoelectric points centred at pH 5.8. The complete amino acid sequence of the precursor of VaF1 was deduced by cloning and sequencing its cDNA. Composed of metalloproteinase, disintegrin-like and cysteine-rich domains, VaF1 is a typical P-IIIa subclass snake venom metalloproteinase. Although it possesses a collagen-binding sequence in its disintegrin-like domain, VaF1 displayed no effect on collagen-induced platelet aggregation *in vitro*. Two consensus *N*-glycosylation sites are present in the sequence of VaF1, however, the extent of its glycosylation is low, only 5.2% of the total molecular mass. Interestingly, in standard experimental conditions VaF1 is not recognised by antiserum against the whole venom, so it can contribute to post-serotherapy complications, such as ineffective blood coagulation, in the envenomed patient.

1. Introduction

Snake venom (SV) is a mixture of various pharmacologically active proteins and peptides that affect different physiological systems, mostly nervous, muscular, and cardiovascular. Many of these components are highly specific for a variety of molecular targets and thus constitute potential therapeutic and diagnostic agents [1–4]. Viperid snake venoms are particularly rich in proteins that disturb the haemostatic system, acting as pro-coagulants that activate coagulation factor X (FX), prothrombin and fibrinogen, or as anti-coagulants, lysing fibrin(ogen), inhibiting coagulation factors and interfering with platelet aggregation [4]. Of these proteins,

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin type-1 motif; BM, basal membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Cys-rich, cysteine-rich; dis-like, disintegrin-like; ECM, extracellular matrix; FX, factor X; GP, platelet glycoprotein; MES, 2-(*N*-morpholino)ethanesulfonic acid; MP, metalloproteinase; PNGF, peptide *N*-glycosidase F; SP, serine protease; SV, snake venom; SVMP, snake venom metalloproteinase; *Vaa*, *Vipera ammodytes ammodytes*; VaF1, *Vipera ammodytes* fibrinogenase 1; VaH, *Vipera ammodytes* hemorrhagin.

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metalloproteinases (MPs) have the broadest spectrum of activities, a consequence of their modular structure. Snake venom MPs (SVMPs) are Zn^{2+}-dependent enzymes that are synthesised in venom glands as multidomain, inactive zymogens and subsequently activated by the cysteine-switch (Cys-switch) mechanism, common to SV- and matrix MPs [5,6]. According to their domain organisation and post-translational processing, three major classes of SVMPs exist [7]. In the P-I class, the pro-domain is followed by a catalytic MP domain. Two motifs characterise the catalytic site in the MP domain, the Zn^{2+}-binding motif (HEXXHXXGXXH) and the methionine-turn (C-I-M-S-X) [8]. P-II SVMPs are composed of the MP domain and a disintegrin domain, and serve as precursors of disintegrins. In the P-III class SVMPs, the MP domain is followed by a disintegrin-like (dis-like) and a cysteine-rich (Cys-rich) domain. Depending on the type of maturation of the P-II and P-III class SVMPs, which can be dimerisation, proteolytic processing and inter-molecular disulphide bond formation, these molecules are further categorised into sub-classes (P-IIa–e, P-IIIa–d). SVMPs are structurally and evolutionarily related to physiologically and pathologically important mammalian ADAM (A Disintegrin And Metalloproteinase) proteins [9]. Together they belong to the reprolysin family of the M12B clan of zinc metalloproteinases (MEROPS classification: http://merops.sanger.ac.uk/cgi-bin/famsum?family=M12B).

Degradation of plasma fibrinogen is one of the most pronounced clinical effects following snake bites and is caused by metallo- and serine proteinases. SVMPs are directly acting fibrin(ogen)olytic enzymes, *i.e.*, they do not require additional factors from the blood for their activity. They cleave preferentially the Aα- or Bβ-chain of fibrin(ogen) and, accordingly, are referred to as α- or β-fibrin(ogen) ases [10]. SVMPs are not inhibited by human serum proteinase inhibitors (serpins), which makes them very useful in the development of diagnostic tests and therapeutic agents [11]. For example, this characteristic is one of the main advantages of SVMP-based anti-thrombotics over the anti-thrombotics currently used in the clinic. The latter affect coagulation indirectly by activation of the endogenous fibrin(ogen)olytic system (*i.e.* activating plasminogen) and are readily inhibited by serpins.

Since haemorrhage and necrosis are the most pronounced clinical effects in humans following envenomation by nose-horned viper (*Vipera ammodytes ammodytes*, *Vaa*) venom, the proteolytic components of this venom have been studied intensively. All four potent *Vaa* hemorrhagins characterised so far, VaH1, VaH3, VaH4 and ammodytagin, have pronounced fibrinogenolytic activity [12–15]. Non-hemorrhagic ammodytase acts as a potent fibrin(ogen)ase [16], while FX activators, VAFXA-I and VAFXA-II, express low fibrinogenolytic activity [17]. A serine protease (SP) with fibrino(geno)lytic activity, VaSP1, has recently been purified from *Vaa* venom [18]. We report here the purification and extensive biochemical characterisation, including complete primary structure determination, of a new α-fibrinogenolytic MP, VaF1 (*V. ammodytes* fibrinogenase 1). This molecule is not hemorrhagic. In standard experimental conditions it is not recognised by *Vaa* venom antiserum, suggesting its participation in delayed pathological effects following serotherapy of envenomed patients, possibly as an anti-coagulant.

2. Materials and methods

Crude *Vaa* venom was obtained from the Institute of Immunology, Inc., Croatia. All other chemicals were of analytical or sequencing grade.

2.1. Isolation of VaF1

VaF1 was isolated in the course of purification of hemorrhagic MP VaH3 as described [14]. In the last step of VaH3 purification, FPLC on a Mono S column (binding buffer: 20 mM MES, 2 mM $CaCl_2$, 5% (w/v) glycerol, pH 6.5; linear gradient elution from 0 to 1 M NaCl in binding buffer), VaF1 was eluted at 200 mM NaCl. This fraction was tested for fibrinogenolytic and hemorrhagic activities. It was concentrated and dialysed against 20 mM Tris/HCl buffer, 2 mM $CaCl_2$, 5% (w/v) glycerol, pH 8.5 and applied onto an FPLC Mono Q column equilibrated in the same buffer. Bound proteins were eluted with a 30 min linear gradient of 1 M NaCl in the equilibration buffer at a flow rate of 1 ml/min. Proteins were monitored by absorbance at 280 nm throughout the isolation procedure.

2.2. SDS-polyacrylamide gel electrophoresis and isoelectric focussing

SDS-PAGE on 12.5%, 10% or 7.5% (w/v) polyacrylamide gels under reducing and non-reducing conditions [19] was used to analyse the isolated protein and cleavage products of different proteins hydrolysed by VaF1. Molecular mass standards used were from Fermentas (Lithuania).

IEF was performed on a Phast System (Amersham Pharmacia Biotech, Sweden) using a PhastGel isoelectric focussing (IEF) 3–9 slab-gel (0.35 × 43 × 50 mm) according to the manufacturer's instructions. pI (3.5–9.3) standards used were from the same manufacturer. Proteins were visualised by Coomassie Brilliant Blue R250 staining.

2.3. N-Deglycosylation

4 μg of dry VaF1 were incubated with 1% (w/v) SDS at 100 °C for 5 min and mixed with 1.5% (w/v) CHAPS in 50 mM Na_2HPO_4, pH 7.5. Half the sample was mixed with 3 U of peptide *N*-glycosidase F (PNGF, Roche, Germany); the other half served as control. After overnight incubation at 37 °C both samples were analysed by SDS-PAGE on 12.5% gel under reducing conditions.

2.4. Edman sequencing and mass spectrometry

N-terminal amino acid sequencing of proteins and peptides was performed on a Procise 492A Automated Sequencing System (Applied Biosystems, USA).

The molecular mass of natural VaF1 was determined by MALDI-TOF mass spectrometry (MS) as described in Ref. [15].

2 μg of VaF1 purified by HPLC and vacuum dried was reconstituted in 10 μl of 6 M urea in 50 mM NH_4HCO_3. It was reduced by adding 0.25 μl of 100 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 for 1 h at 37 °C. The mixture was then diluted with 10 μl of 50 mM NH_4HCO_3 and alkylated with 0.8 μl of 250 mM iodoacetamide in 50 mM NH_4HCO_3 for 45 min at room temperature in the dark. The alkylation was stopped by adding 2.5 μl of DTT, and 50 mM NH_4HCO_3 then added to 90 μl, followed by 10 μl (0.05 μg) of trypsin in 50 mM NH_4HCO_3. Hydrolysis proceeded overnight at 37 °C. The reaction was stopped by adding formic acid to 0.1% (v/v) final concentration. The sample was desalted and concentrated using Vivapure C18 micro spin columns (Sartorius Stedim Biotech, Germany) according to the manufacturer's instructions, except that formic acid was used instead of trifluoroacetic acid. The protein hydrolysate was analysed on an ion trap mass spectrometer 1200 series HPLC-Chip-LC/MSD Trap XCT Ultra (Agilent Technologies, Germany). MS and tandem MS (MS/MS) spectra were searched against the *Vaa* venom gland cDNA transcripts library and the non-redundant NCBI database using the Spectrum Mill (Agilent Technologies, CA, USA) and Mascot software (Matrix Science, Ltd., UK).