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Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved

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Abstract

Snake venom glands synthesize a variety of serine proteinases capable of affecting the haemostatic system. They act on macromolecular substrates of the coagulation, fibrinolytic, and kallikrein–kinin systems, and on platelets to cause an imbalance of the haemostatic system of the prey. In this review we describe their biochemical/biophysical characteristics, biological activities as well as aspects of their evolution and structure-activity relationship.
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1. Introduction

Snake venom serine proteinases (SVSPs) are among the best-characterized venom enzymes affecting the haemostatic system. They act on a variety of components of the coagulation cascade, on the fibrinolytic and kallikrein–kinin systems and on cells to cause an imbalance of the haemostatic system of the prey (Seegers and Ouyang, 1979; Markland, 1997; Pirkle, 1998). Serine proteinases belong to the trypsin family S1 of clan SA, the largest family of peptidases (Halfon and Craik, 1998). Despite the high degree of mutual sequence identity, SVSPs are quite specific toward a given macromolecular substrate. Although they show primary substrate specificity similar to that of trypsin, their stringent macromolecular substrate specificity contrasts with the less specific activity of trypsin. They are present in venoms of the families Viperidae, Crotalidae, Elapidae and Colubridae. Snake venom enzymes characterized as serine proteinases are defined by a common catalytic mechanism that includes a highly reactive serine residue that plays a key role in the formation of a transient acyl-enzyme complex, which is stabilized by the presence of histidine and aspartic acid residues within the active site (Barrett and Rawlings, 1995). Hence, they are all sensitive to the serine-modifying reagents phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP). Most SVSPs are likely to be glycoproteins showing a variable number of *N*- or *O*-glycosylation sites in sequence positions that differ from one SVSP to the other. They contain twelve cysteine residues, ten of which form five

Abbreviations ε-ACA; ε-aminocaproic acid; ADP; adenosine diphosphate; ATIII; antithrombin III; BPTI; basic (bovine) pancreatic trypsin inhibitor; DAPI; 4',6-diamidino-2-phenylindole; DFP; diisopropylfluorophosphate; Fbg; fibrinogen; FP; fibrinopeptide; FRE; fibrinogen recognition exosite; Mab; monoclonal antibody; NPGB; *p*-nitrophenyl *p*-guanidinobenzoate; PAI-1; plasminogen activator inhibitor 1; PAR; protease activated receptor; PMSF; phenylmethylsulfonyl fluoride; PPACK; D-Phe-Pro-Arg-CH2Cl; SBTI; soybean trypsin inhibitor; (c)SVSP; (coagulating) snake venom serine proteinase; TLCK; tosyl lysyl chloromethylketone; t-PA; tissue-type plasminogen activator; u-PA; urokinase plasminogen activator; TPCK; tosyl phenylalanyl chloromethylketone; UTR; untranslated region.

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disulfide bonds, based on the homology with trypsin (Itoh et al., 1987); the remaining two cysteines form a unique and conserved bridge among SVSPs, involving Cys245e (chymotrypsinogen numbering), found in the C-terminal extension (Parry et al., 1998). This review will focus on the different activities displayed by SVSPs and will describe their peptide and macromolecular substrates, inhibitors, methods of purification, preparation of recombinant proteins and studies on 3D structure and its relationship to activity. Elsewhere in this special issue are described SVSPs that activate prothrombin (classified in the groups C and D of snake venom prothrombin activators), by R.M. Kini, SVSPs with fibrin(ogen)olytic activity, by S. Swenson and F.S. Markland Jr., and the use of SVSPs in the development of diagnostic reagents and in the treatment of haemostatic disorders, by N. Marsh and V. Williams.

2. A historical perspective

The disturbances in blood clotting are among the most dramatic effects of envenomation by viperid and crotalid snakes. Among the venom components, serine proteinases were shown to be directly involved in proteolytic events, causing disturbances on hemostasis observed upon envenomation. The literature contains much evidence of the attraction exerted by snake venom components upon investigators of the fields of hemostasis and thrombosis, who used them in practical laboratory tests and in theoretical and clinical studies. In the development of the general understanding of blood coagulation, SVSPs proved to be very useful agents in clarifying some basic concepts.

The clotting of blood by snake venoms was recognized by Fontana in the late 1700s (Markland, 1998). He observed that the injection of a viper venom in the jugular vein of rabbits caused rapid blood-coagulation followed by death. In early investigations, Eagle (1937) used snake venoms to obtain data that were used as a basis for emphasizing that blood coagulation is primarily caused by proteolytic enzymes. Moreover, it was first shown by Rocha e Silva, et al. (1949) that the venom of *Bothrops jararaca* contained a bradykinin-releasing enzyme. In the 1950s, investigators had already evidence for the ability of snake venoms enzymes to accelerate blood coagulation and attempts were made to isolate the enzymes responsible for this effect. Studying the venom of *B. jararaca*, Henriques and colleagues (Henriques, et al., 1960) first reported on the partial purification of a blood-clotting factor, and in Raudonat and Rocha e Silva 1962, showed the separation of the bradykinin-releasing enzyme from the clotting factor in *B. jararaca* venom. In the following decades SVSPs have been extensively studied and various homogeneous preparations were obtained and characterized. Currently, more than fifty amino acid sequences of SVSPs are available in the databanks.

3. Purification from crude venoms

SVSPs can be easily purified from crude venoms by a variety of chromatographic methods. Most procedures start with a separation based on molecular size followed by either ion-exchange or affinity-binding (benzamidine- or arginine-Sepharose) chromatography. Although less frequently, the use of reversed-phase chromatography has also been reported (Hung and Chiou, 1994; Serrano et al., 1998; Amiconi et al., 2000; Wang et al., 2001). Both acidic and basic serine proteinases have been isolated from snake venoms. Basic serine proteinases usually display direct platelet-aggregating activity while acidic proteins are ascribed a variety of proteolytic activities on substrates related to hemostasis. Various SVSP preparations show electrophoretically distinct forms with slightly different molecular masses and p*I*s due to the variations in their amino acid sequences and glycosylation levels (Tokunaga et al., 1988; Silveira et al., 1989; Burkhart et al., 1992; Aguiar et al., 1996; Giovanni-De-Simone et al., 1997; Serrano et al., 1998; 2000; Samel et al., 2002). The isolation procedure can be followed either by measuring amidolytic or esterolytic activities or by assays using protein substrates or cells. A number of chromogenic or fluorogenic peptide substrates contain arginine or lysine at the P1 position (Schechter and Berger, 1967). These substrates have been used to measure the amidolytic activity of SVSPs (Kirby et al., 1979; Pirkle et al., 1986; Lollar et al., 1987; Farid and Tu, 1989; Bakker et al., 1993; Serrano et al., 1993, 2000; Hahn et al., 1996; Braud et al., 2002; Samel et al., 2002; Dekhil et al., 2003). The hydrolytic activity of SVSPs toward these peptide substrates is strongly influenced by the peptide moiety adjacent to the scissile bond. Assays to detect the specific proteolytic activities of SVSPs on macromolecular substrates include the determination of coagulant activity on plasma or purified fibrinogen (Stocker et al., 1982), the kinin-releasing activity using purified kininogen, or synthetic kininogen analogs or plasma (Komori and Sugihara, 1988; Serrano et al., 1998), the factor V activation assay (Kahn and Hemker, 1972), the protein-C activation assay (Orthner et al., 1988), the induction of platelet-aggregation on platelet rich plasma or washed platelet suspensions (Kirby et al., 1979; Schmaier and Colman, 1980), and the activation of plasminogen (Zhang et al., 1995).

Isolated, homogeneous SVSPs are one-chain proteins that show a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions with molecular mass varying between 26 and 67 kDa, depending on their carbohydrate content. One exception is brevinase, a heterogeneous two-chain enzyme with fibrinolytic activity, isolated from the venom of *A. blomhoffii brevicaudus* (Lee et al., 1999) in which disulfide bridges link the two polypeptide chains (16.5 and 17 kDa). However, a cDNA encoding brevinase was cloned

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