Diagnostic Uses of Snake Venom

Neville A. Marsh
School of Life Sciences, Queensland University of Technology, Brisbane, Qld., Australia

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Snake venom toxins • Snake venom thrombin-like enzymes • Disintegrins • Haemostasis • Platelet function tests • Coagulation assays

Abstract
Snake venom toxins are invaluable for the assay of coagulation factors and for the study of haemostasis generally. Thrombin-like enzymes (SVTLE) are used for fibrinogen and fibrinogen breakdown product assays as well as detecting dysfibrinogenaemias. Since SVTLE are not inhibited by heparin, they can be used for assaying antithrombin III in samples containing heparin. Snake venom prothrombin activators are utilised in prothrombin assays, whilst Russell’s viper venom (RVV) can be used to assay clotting factors V, VII, X and lupus anticoagulants (LA). Activators from the taipan, Australian brown snake and saw-scaled viper have also been used to assay LA. Protein C (PC) and activated PC (APC) resistance can be measured by means of RVV, Protac™ (from Southern copperhead snake venom) and STA-Staclot (from Crotalus viridis helleri) whilst von Willebrand factor can be studied with Botrocetin™ (Bothrops jararaca). Finally, snake venom C-type lectins and metalloproteinase disintegrins are being used to study platelet glycoprotein receptors and show great potential for use in the routine coagulation laboratory.

Introduction
Snake venoms contain a rich variety of materials having profound effects on the haemostatic mechanism, and these have been extensively reviewed [1–4]. These materials can be classified into five groups: coagulant (thrombin-like and coagulation-activating toxins), anticoagulants (PC activators), toxins inhibiting platelet function, activators of fibrinolysis and haemorrhagins. Some of these
snake venom materials have become invaluable in laboratory diagnosis of haemostatic disorders and these practical applications are reviewed in this paper.

**Thrombin-Like Enzymes and Investigation of Fibrinogen**

Over 90 thrombin-like enzymes from 35 snake species have been recorded [5] being serine proteases with active site residues recognisable in positions H57-D102-S195. The group does not share all properties common to thrombin since enzymes typically cleave fibrinopeptide A. The most widely studied enzymes are from *Bothrops atrox* (batroxobin, reptilase), *Calloselasma rhodostoma* (ancrod) and *Agkistrodon c. contortrix* (ACTE).

Since snake venom thrombin-like enzymes (SVTLEs) are not inhibited by heparin, they can be used to test plasma samples containing this anticoagulant or to remove fibrinogen from samples containing heparin. The reptilase time is a simple alternative to the thrombin time for rapid fibrinogen assay in samples containing heparin [6] and is particularly useful in the assay of antithrombin III where plasma can be prepared free of fibrinogen [7, 8]. The presence of fibrin degradation products (FDPs), hypofibrinogenaemia and defects in fibrin polymerisation will prolong the reptilase time, thus if this prolongation is less than that of the thrombin time, then the presence of FDPs is indicated [9]. SVTLEs are useful for investigating fibrin function including magnetic birefringence studies of fibrin assembly, [10] and other aspects of the clotting mechanism [11, 12]. Batroxobin and ACTE can also be used in preparing desaa- and desbb-fibrinogen, the former for use as a fibrin stimulant in the functional assay of tissue plasminogen activator [13].

**Prothrombin: Assay and Functional Aspects**

Commercially available prothrombin activators include ecarin from saw-scaled viper (*Echis carinatus*) venom, textarin from the Australian brown snake (*Pseudonaja textilis*) and the enzyme from the taipan (*Oxyuranus s. scutellatus*) [14]. Crude taipan venom contains a high proportion of enzyme and can be used without further purification in a one-stage assay of prothrombin [15]. However, the use of these enzymes is of limited value in warfarin-treated patients since they cleave functionally abnormal types of prothrombin which are present in addition to normal prothrombin [16]. These forms include the descarboxy variety or PIVKA (proteins induced by vitamin K antagonists). Nonetheless, this property has been harnessed in the assay of PIVKA in liver disease [17], in dysprothrombinopathies [18, 19] and in the diagnosis of disseminated intravascular coagulation [20]. Snake venom prothrombin-activating enzymes have also been used to prepare meizothrombin [21, 22], non-enzymic forms of thrombin and meizothrombin [14] and in studies of recombinant prothrombin cleavage [23], and prothrombin activation [24].

**Factor V**

Activation of factor V (FV) can be achieved by a serine protease from the venom of Russell’s viper (*Daboia russelli*) (RVV-V) which cleaves the single-chain glycoprotein at Arg1545. The enzyme can be used for the routine assay of FV by virtue of its selective activation of FV [25] and although the use of RVV-V for FV assay is somewhat limited, the reagent remains a useful tool in the study of FV [26] (see further comments below on assay of FV Leiden, PC and APC resistance).
Factors VII and X

Russell’s viper venom also contains a potent activator of factor X (FX) (RVV-X), and the enzyme has become widely used for FX itself [27], for distinguishing between factor VII (FVII) and FX deficiency [28] and lupus anticoagulant assay [29]. The clotting time of plasma using RVV-X is known as the Stypven time™ [30] and a normal Stypven time used in conjunction with a prothrombin time (PT) suggests FVII deficiency whereas a prolonged Stypven time indicates FX deficiency [28]. Measurement of FX can be performed with RVV-X in functional clotting assays [27], with a chromogenic substrate [31] and by a solid-phase enzyme-linked coagulation assay [32]. In the clotting assay, FX is converted to FXa by RVV-X in the presence of calcium ions, FVa and phospholipid. This complex activates prothrombin initiating clot formation, thus clotting time is proportional to FX concentration. The chromogenic assay utilises a specific substrate (Pefachrome FXa™) which is cleaved by the RVV-activated FXa. Not only has RVV-X an established place in FX assay, but is also useful for identifying FX recognition sites [33], determining the effects of deglycosylation on FX activation [34] and for assay of platelet factor 3 [35, 36].

Lupus Anticoagulants

Lupus anticoagulants (LA) are a heterogeneous population of immunoglobulins which interfere with phospholipid-dependent clotting tests, i.e. the activated partial thromboplastin time (APTT), PT and kaolin clotting time (KCT). Because of the molecular heterogeneity associated with LA, no single standalone test can provide a satisfactory screen and in the past, the APTT and KCT have been used for this purpose. Screening for LA has been reviewed elsewhere [37, 38] and snake venom activators are now used including RVV-X, and activators from venom of the taipan, Australian brown snake and saw-scaled viper. The dilute Russell’s viper venom time (dRVVT) is quick, sensitive and inexpensive [29]. It has been found to be less sensitive than the KCT [39], but new formulations of the dRVVT give increased sensitivity [40, 41]. A test has also been developed based on the taipan time combined with a platelet neutralisation procedure [42] and a further snake venom-based confirmatory test for LA has been developed utilising Textarin™ and Ecarin™ [43]. This has proved to be a sensitive and relatively specific test and the T/E ratio has proved a useful confirmatory test for the diagnosis of LA patients with unexplained prolonged APTT [44].

PC and APC Resistance

PC is a vitamin-K-dependent protein which plays a key role in haemostasis, most notably in its active form, APC, as an anticoagulant proteinase inactivating FVa and VIIIa. Functional assay of PC is hampered by incomplete activation using thrombin and by interference of the same thrombin in chromogenic assays. These problems are overcome by the use of a potent, fast-acting PC activator (Protac) from Southern copperhead snake venom, A. c. contortrix [45, 46]. Protac is used in a chromogenic method [47, 48], in functional clotting assays [49] and in a new global clotting assay (ProC Global) [50]. Results from these assays compare favourably with those from immunological methods [51] and the Protac-based method seems preferable to thrombin-based assays since the latter incur a degree of misclassification of both normal and congenitally PC-deficient patients [52].
wise a global test based on the ratio of APTT in the absence and presence of Protac success-
fully identified all patients with defects in the PC anticoagulant pathway [53] and may en-
able recognition of dysfunctional PC by differential results from chromogenic and coagu-
lomeric assays [54]. Use of Protac has greatly simplified the assay of PC, as well as the assay of protein S, the co-factor required for the inactivation of FVα and FVIIIα by APC, the latter being assayed by both amidolytic [55] and clotting methods [56]. In addition, this snake venom enzyme has minimized the associated costs of these assays, permitting the scaling down of sample sizes for use on micro-
titre plates [47].

APC resistance is probably the major cause of thrombophilia and its molecular basis has been determined (FV Leiden) [58]. Functional assays using snake venom activators can accurately detect the genotypic abnormality including the dRVVT (PC Impedance Test, Gradipore), the textarin time (Pentapharm) and STA-Staclot (Diagnostica Stago), using a specific activator from Crotalus viridis helleri venom [59]. The dRVVT offers improved sensitivity and may make DNA analysis unnecessary [60]. In addition, the textarin time may used [61], but with both this method and the ACV (A. contortrix venom) test, conclusive DNA analysis is strongly recommended [62].

Von Willebrand Factor Studies

Botrocetin™ is a platelet-aggregating protein found widely in Bothrops jararaca ven-
on. Botrocetin depends on the presence of von Willebrand factor (VWF) for its effect on platelets, and this property has been utilised in VWF assay [63, 64]. Together with risto-
cetin, the two agonists can be used to differ-
entiate molecular variants of VWF: botro-
cetin partially aggregates platelets from patients suffering from Bernard-Soulier dis-
ease where glycoprotein receptor Ib (GPIb) is absent. However, ristocetin will not induce platelet aggregation in plasma from these patients since it depends on GPIb. The same distinction can be made in type IIa von Willebrand disease (VWD) where high mo-
olecular weight VWF multimers are absent [65]. A solid-phase inhibition assay for VWF-
binding to platelet GPIb has now been pro-
posed using botrocetin [66]. This ristocetin-
botrocetin combination has proved invaluable in the characterization of human plate-
let VWF [67] and in the detection of a miss-
sense mutation in Type B VWD [68]. A second VWF-dependent snake venom plate-
let aggregant, alboaggregin-B isolated from Trimeresurus albolabris venom has been used to quantitate VWF receptors on the GPIb molecule [69].

Platelet Glycoprotein Studies

Snake venoms contain many materials af-
flecting platelet function [70, 71] including the metalloproteinase-disintegrins which inhibit platelet aggregation via interference with surface glycoprotein receptors and the C-type lec-
tins [72]. These compounds offer a unique opportunity for the study of platelet-platelet and platelet-endothelium interactions, al-
though as yet, no routine test of haemostasis utilises a disintegrin.

Conclusions

Snake venoms are widely used in the coag-
ulation laboratory for routine assay of coagu-
lation factors and as research tools. Some applications have been adopted as the pre-
ferred option to conventional coagulation assays and may even be more appropriate than some DNA-based tests. Other compounds like the disintegrins, with powerful in vitro and in vivo actions as anti-platelet aggregants, are useful tools in the study of platelet physiology and potentially, as reagents in the routine coagulation laboratory.

References


