The Potential of *Bothrops moojeni* Venom in the Field of Hemostasis

Established Use and New Insights

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Key Words

Bothrops moojeni · Snake venom analysis · Hemostasis · Prothrombin activator

Abstract

Early studies in the 1930s on the venom of South American Lancehead snakes of the *Bothrops* genus lead to the discovery of compounds active in blood coagulation such as batroxobin and botrocetin. The scope of our investigations is to have a deeper look at the crude venom of *B. moojeni* using state-of-the-art proteomics methods, as well as newly developed bioassays screening for activities in the different fields of application. The proteomics techniques used up to now have included different chromatography methods, mass spectrometry, and bio-computing. The bioassays are focussed on enzymatic and other activities in the field of hemostasis and fibrinolysis. Besides the known activities several new and interesting ones have been found. They still need to be studied and confirmed in more specific supplementary assays.

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Background

Many snakes have highly efficient venoms with pronounced hematological effects in animals and humans. The influence of these venoms on blood coagulation has been investigated by several authors for decades [1–12].

Among the venoms of *Bothrops*, *B. jararaca* and *B. atrox* have been studied most extensively [7]. Studies done by Eagle [13] already in 1937 showed that many snakes of the Crotalidae family coagulated citrated horse plasma. This is due to the direct action of the venom on fibrinogen [6]. Investigations on this thrombin-like activity led to the isolation and purification of a component from the venoms of *B. atrox* and *B. moojeni* snakes, known as batroxobin [14]. This snake venom enzyme, under its trade names Defibrase[®] and Reptilase[®], is commercially available from Pentapharm Ltd. (Basel, Switzerland). It is still widely used in pharmaceutical and diagnostic applications. For collection of high amounts of the venom needed for batroxobin production a snake farm breeding exclusively *B. atrox* and *B. moojeni* has been established in Brasil. Thus, the controlled breeding of the snakes and the pooling of large amounts of venom collected from different animals makes this venom an abundant, stable and homogenous source for further investigations.

Fig. 1. Comparison of the GF profile of the crude venom of *Bothrops moojeni* (b) and the GF profile of the standards (a) chromatogram obtained with 300 mg of crude *B. moojeni* venom.

Aim of the Work

At the present time, most of the *Bothrops moojeni* venom material needed for batroxobin production is not further processed. It contains, however, a lot of active compounds still not fully characterized and described. Therefore, the aim of our project was to develop and use biochemical and proteomic strategies in order to further investigate the crude *B. moojeni* venom, allowing the identification of new bioactive compounds, which could be used for diagnostic or pharmaceutical purposes.

Experiments

Gel filtration (GF) chromatography of the crude *B. moojeni* venom was chosen as a first approach. Pooled and desiccated venom obtained from the snake farm Pentapharm do Brasil was dissolved in deionized water and separated into 18 GF fractions on a Superdex-75 column (see fig. 1). Superdex-75 gel, two columns in-line (XK 26/70, bed height 54 cm and XK 26/100, bed height 87 cm), total CV of 748 ml, running conditions: buffer of 50 mM $\text{CH}_3\text{COONH}_4$, 150 mM NaCl, pH 7.5. *B. moojeni* crude venom (5 g) was separated in 20 individual runs.

The reproducibility of all performed experiments was very good so that pooling into 18 distinct GF fractions collected from run to run was successfully performed.

For preliminary evaluation of the potential of the 18 GF fractions, a high-throughput screening method was used. The test method consisted of 14 screens monitoring different levels and mechanisms of the blood coagulation system. This screening was performed on an automated coagulation test system, Behring Coagulation System (BCS) (Dade Behring, Marburg, Germany), with optical clotting detection principle. It was designed for detection and evaluation of any kind of effect on distinct parts and functions of the blood coagulation system, such as:

- the intrinsic pathway
- the extrinsic pathway
- fibrinolysis
- the APC system
- antithrombin function
- the prothrombinase complex
- blood coagulation activation
- thrombin dynamics in intrinsic or extrinsic pathway.

According to the results of this preliminary screening the most active fractions were selected for further purification and characterization.

Fig. 2. Semi-preparative HPLC profile of GF 12 fraction.

By use of on-line Liquid Chromatography-Electrospray Ionization-Mass Spectrometry more than 100 molecular masses were identified in crude venom. The preliminary results of the high-throughput screening provided some clues about the possible activity of the *B. moojeni* GF fractions. Six GF fractions from the low molecular mass range (GF 9–18) were selected as the most interesting ones for further characterization and purification. Additional separation was necessary to find individual components influencing the blood coagulation system in a specific way. The 6 most active fractions were each (10 mg) further separated by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC); RP-HPLC separation was performed on a Waters Alliance 2690 System (semi-preparative HPLC column: Vydac #218TP510 protein & peptide C₁₈, 10 × 250 mm); see figure 2.

A total of 600 HPLC sub-fractions were obtained from the purifications of 6 GF fractions, and all of them were analyzed further.

The screening, performed on the automated coagulation test system BCS, was used again, giving more precise

results, which could then be confirmed or disproved using other test methods. Moreover coagulation tests were performed on a ball coagulometer with mechanical clotting detection and in Rotem[®] system (thromboelastography), and for further analyses different chromogenic substrates were used.

In addition, 200 of the HPLC sub-fractions were analyzed by Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry using 4-HCCA (α-cyano-4-hydroxycinnamic acid) matrix, resulting in about 1,200 measured molecular masses in the range of 500–10,000 Da. From the large amount of crude venom used as source material for the separation steps minor components could also be detected. The measured molecular masses indicate the highest number of compounds ever detected in raw snake venom. The actual number of different venom components is, however, probably smaller, as the same peptide chain might give several signals if it underwent different modifications.

As expected, several phospholipase A₂ enzymes were found in the low-molecular-weight fractions of *B. moojeni* crude venom. According to the Matrix Assisted Laser

Fig. 3. *B. moojeni* GF fractions 1–8, SDS-PAGE analytical scale, batroxobin is present in GF 5.

Desorption Ionization-Time of Flight-Mass Spectrometry and Liquid Chromatograph-Electrospray Ionization-Mass Spectrometry about 20 molecular masses corresponding to the PLA₂ mass range were identified in the crude venom and the HPLC sub-fractions. Not all of these fractions showed enzymatic activity typical for PLA₂, indicating that some of them probably contain enzymatically inactive types of phospholipases.

According to the preliminary BCS high-throughput screening, three fractions (GF 8–10) showed strong clotting time prolongation, which was later attributed to their very high amounts of phospholipase A₂. Interestingly some of them influenced also the prothrombinase complex, and FXa-binding phospholipases could be isolated and characterized in further separations and more specific assays.

An SDS-PAGE gel (fig. 3) was performed on the high-molecular-mass fractions. It showed that beside the well-known batroxobin found in GF 5, many other proteins are present in these fractions and many unknown activities can still be expected.

It is still unclear whether the determined effects can be attributed to single substances or mixtures and further purifications and characterization steps are necessary. However, a strong prothrombin activator has already been found in the GF 3.

In order to characterize that component better, fibrinogen clotting time measurements were performed. The

Table 1. Comparison of prothrombin-activating properties of ecarin and GF fraction 3

Prothrombin activator	Clotting time, s			
	plasma		plasma + UFH 0.5 U/ml	
Ecarin 5 U/ml	26.5	25.8	26.1	25.8
GF 3; 1:100 diluted	20.8	20.8	21.4	20.8

clot-inducing properties of the compound contained in this fraction were compared with those of ecarin in plasma containing unfractionated heparin. The experiments showed that both substances are insensitive to the presence of unfractionated heparin in plasma and do not need any additional cofactor to be enzymatically active (table 1).

In parallel to the above ‘function to structure’ approach, a ‘structure to function’ approach was used for the identification of possible substances present in the crude venom of *B. moojeni*. That was performed for different HPLC sub-fractions of three GF fractions. After finding the amino acid sequences of the components present in the fractions, the possible activities were assigned to them after comparison with other known substances from different snake venoms. In these experiments, we could identify 12 metalloproteinase fragments, 11 *L*-ami-

no acid oxidase fragments, 1 peptide with homology with the N-terminal part of a small myotoxin, different PLA₂ fragments and small peptides. Also 6 bradykinin-potentiating peptides could be characterized (data not shown).

Conclusion

All GF fractions have been analyzed in a preliminary screening for activities on blood coagulation system. Six of them, further separated by HLPC, have been analyzed

in a broad screen identifying individual and specific effects on blood coagulation. Activities of identified compounds and structures need to be confirmed and their possible use in diagnostic and pharmaceutical applications is currently under investigation. Confirmation of the structures of these proteins is performed using proteomics and biochemical techniques. Other fractions not yet screened in detail need additional separation and purification steps.

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